Identification of the molecular determinants driving the substrate specificity of fungal lytic polysaccharide monoxygenases (LPMOs)

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Understanding enzymatic breakdown of plant biomass is crucial to develop nature-inspired biotechnological processes. Lytic polysaccharide monoxygenases (LPMOs) are microbial enzymes secreted by fungal saprotrophs involved in carbon recycling. LPMOs modify biomass by oxidatively cleaving polysaccharides, thereby enhancing the efficiency of glycoside hydrolases. Fungal AA9 LPMOs are active on cellulose, but some members also display activity on hemicelluloses and/or oligosaccharides. Although the active site subsites are well defined for a few model LPMOs, the molecular determinants driving broad substrate specificity are still not easily predictable. Based on bioinformatic clustering and sequence alignments, we selected seven fungal AA9 LPMOs that differ in the amino-acid residues constituting their subsites. Investigation of their substrate specificities revealed that all these LPMOs are active on cellulose and cello-oligosaccharides, as well as plant cell wall–derived hemicellulosic polysaccharides, and carry out C4 oxidative cleavage. The product profiles from cello-oligosaccharide degradation suggest that the subtle differences in amino-acid sequence within the substrate-binding loop regions lead to different preferred binding modes. Our functional analyses allowed us to probe the molecular determinants of substrate binding within two AA9 LPMO subclusters. Many wood-degrading fungal species rich in AA9 genes have at least one AA9 enzyme with structural loop features that allow recognition of short β-(1,4)-linked glucan chains. Time-course monitoring of these AA9 LPMOs on cello-oligosaccharides also provides a useful model system for mechanistic studies of LPMO catalysis. These results are valuable for the understanding of LPMO contribution to wood decaying process in nature and for the development of sustainable biorefineries.

Efficient conversion of plant biomass for the production of biofuels and other sustainable bioproducts and materials is considered largely dependent on the key enzymes lytic polysaccharide monoxygenases (LPMOs) (1, 2). LPMOs are copper-dependent oxidoreductases that use molecular oxygen or hydrogen peroxide as cosubstrate to cleave polysaccharides (e.g. cellulose, hemicellulose, chitin, starch) through an oxidative mechanism (3–5). LPMOs exist either as single-domain enzymes or appended to other protein domains, for example, carbohydrate-binding modules (CBMs) (6, 7), directing the enzymes to their target substrate (8–10). To date, LPMOs are grouped into seven families in the CAZy database (11), namely the auxiliary activity (AA) families AA9-AA11 and AA13-AA16 (7), in which members have been found mainly in bacteria and filamentous fungi (12–17), but also recently in arthropod and fern species (18, 19). In all LPMOs studied to date, the active-site redox center is made up of the strictly conserved histidine brace (His-brace) motif, which consists of the N-terminal His and a second His involved in the coordination of one copper atom (20, 21). The AA9 family also contains some members without any LPMO activity, lacking both the His-brace and copper, while features implicated in carbohydrate binding have been maintained (22).

Genes encoding AA9 LPMO enzymes are found in high numbers in fungal saprotrophs. For instance, species of the Lentinus, Phanerochaete, Neurospora, and Podospora genera display between 14 and 33 AA9 genes (https://mycocosm.jgi.doe.gov/mycocosm/home); however, the reason for this gene multiplicity is unknown. Structurally, these AA9 isoforms share a central β-sandwich core, but the surface-exposed structural features around the His-brace vary as reviewed (2, 21, 23). The LPMO structure from Thermoascus aurantiacus (TaAA9A) (20) revealed a Cu-bound His-brace on a flat extended surface near a conserved Tyr, in the C-terminal (LC) loop, putatively involved in substrate binding. AA9 LPMOs were initially assumed to specifically target crystalline regions of polysaccharides, but later, the activity was also demonstrated on a range of hemicellulose polysaccharides such as xyloglucan (XG), xylan, glucomannan (GM), and mixed-linkage β-glucans (MLGs), and also on oligosaccharides (17, 24–31). The AA9 LPMO from...
Neurospora crassa (NcAA9C) is active on both cello-oligosaccharides and hemicellulosic substrates, suggesting that the enzyme recognizes small stretches of β-1,4-linked glucosyl units (24, 25). Borisova et al. (32) determined the structure of NcAA9C and noticed three consecutive Asn residues in the L2 loop and an insertion in the L3 loop of NcAA9C structure compared with other AA9 LPMOs (such as TaAA9A) acting on insoluble cellulose substrates (Fig. 1 and Fig. S1). Around the same time, we found that an AA9 LPMO from Podospora anserina (PaAA9H) with an L3 loop similar to that of NcAA9C showed activity on cello-oligosaccharides as well as XG and GM (29). Shortly after, we determined the structures of two AA9 LPMOs from Lentinus similis (LsAA9A) and Collariella virescens (CvAA9A, previously Chaetomium virescens), which both belong to the phylogenetic cluster C38 (see Results) and display activity on cello-oligosaccharides and on XG, MLG, and GM (28, 33). The L3 loops in LsAA9A and CvAA9A are slightly shorter than those in NcAA9C and PaAA9H; however, their L3 loops are all extended compared with those of TaAA9A, which is not active on oligosaccharides. Interestingly, truncation of the L3 loop in NcAA9C abolishes XG activity (34), and for a closely related (about 60% sequence identity) AA9 LPMO from Chaetomium thermophilum (denoted as CtrPMO1 [35]), a His-to-Ala mutation in the L3 loop (equivalent to His66 in LsAA9A, see below) has been shown to abolish celloheptaose activity.

The first LPMO–carbohydrate complex structures determined were LsAA9A in complex with cello-oligosaccharides (27, 36). Later, LsAA9A was determined in complex with oligosaccharides derived from hemicelluloses (xylan, GM, and MLG) (28). The LsAA9 complex structures confirmed that the oligosaccharides were bound by one Asn in the L2 loop, polar amino-acid residues in the L3 loop, and the conserved Tyr platform (Tyr 203) in the LC loop but also via charged residues in the L8 loop (after notation [37]). Subsites from −4 to +2 were defined based on the LsAA9–cellolohexaose (cel6) complex. The negative subsites (−4 to −1) were formed by the platform Tyro203 in the LC loop as well as Glu148, Asp150, and Arg159 in the L8 loop, whereas the positive subsites (+1 to +2) were formed by Asn28 in the L2 loop and His66 and Asn67 in the L3 loop. Interestingly, the structures also revealed an uncommon lone pair—π aromatic interaction between the pyranose ring O5 and the imidazole of the N-terminal His of the His brace (27, 36). Very recently, crystallographic cello-oligosaccharide complexes were also obtained with CvAA9A and showed broadly the same interactions as LsAA9A (Tandrup et al., submitted; 6YDC, 6YDD, and 6YDE) (33).

Although the substrate-binding determinants and active-site subsites are well defined for LsAA9A, the molecular determinants of substrate specificity are still not easily predictable for other AA9 LPMOs. In this study, we focused on seven LsAA9A-related enzymes from fungal saprotrophs to provide further insights into the main molecular determinants driving LPMO activity toward cello-oligosaccharides and hemicellulosic.
Investigation of seven fungal AA9 LPMOs

Figure 1. Close-up view of LsAA9A active site and sequence alignment of the AA9 LPMOs studied highlighting the residues putatively involved in substrate binding. A, LsAA9A-cell6 complex (PDB 5ACI) highlighting the subsites −4 to +2 and below schematic representation with residues contributing to substrate binding by hydrogen bonding or aromatic stacking or van der Waals interactions in each subsite. These residues are shown in sticks along with the residues involved in copper coordination (including the secondary sphere). The structural loop elements are colored as in panel B. B, multiple sequence alignment of the AA9 LPMOs investigated in this study. Positions involved in cello-oligosaccharide interactions and the His-brace motif are highlighted in green and blue, respectively. The positions of the primary and secondary coordination spheres of the copper are indicated with a blue or a magenta star, respectively. The L2, L3, L8, and LC loop regions are indicated with cyan, green, orange, and yellow bars below the sequences, respectively. LC, C-terminal; LPMOs, lytic polysaccharide monoxygenases.

Table 1
LsAA9A homologue sequences

| Enzyme name | Organism             | AA9 subcluster | Sequence identity to LsAA9A | Substitutions |
|-------------|----------------------|----------------|----------------------------|--------------|
| LsAA9A      | Lentinus similis     | C38            | -                          | n/a          |
| PchAA9E     | Phanerochaete chrysosporium | C38      | 76%                        | E148Q        |
| PcaAA9A     | Phanerochaete carnosa | C38            | 74%                        | E148Q        |
| BaAA9A      | Bjerkandera adusta   | C38            | 69%                        | E148Q        |
| AgAA9A      | Armillaria gallica   | C38            | 64%                        | N67D, D150Y  |
| ScAA9A      | Schizophyllum commune | C38            | 63%                        | R159K, N67D  |
| AoAA9A      | Aspergillus oryzae   | C29            | 55%                        | Y203W, N67D  |
| AfAA9C      | Aspergillus fumigatus | C29            | 53%                        | Y203W, N67D  |

Subcluster 38 (C38), subcluster 29 (C29), negative subsites (−4, −3, −2, and −1), positive subsites (+1 and +2).

For each AA9 enzyme name, the organism of origin and the cluster they belong to are given. The sequences are listed by their identity to LsAA9A and the substitutions affecting the subsite on either side of the scissile bond are indicated under substitutions.

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Subsites. ScAA9A has an Y203W substitution of the otherwise almost completely conserved platform Tyr involved in stacking interactions with the glycosyl unit in subsite −3. AgAA9A has a D150Y substitution, and because the Asp in LsAA9A interacts with the C6-hydroxyl of the glucosyl unit in subsite −3, it is possible that the Tyr in AgAA9A is able to interact with C6 substitutions on β-(1,4)-linked glucans such as the xylose decorations found in XG. Thus, both Y203W and D150Y likely affect substrate binding in subsite −3 (Fig. 1). AoAA9 and AfAA9 display some differences (D150N, S151R, or R159K) in the L8 loop, which is significantly shorter than the other six AA9s studied and similar to the equivalent loop in AA9 LPMOs acting on insoluble cellulose (such as TaAA9A). Comparison of the TaAA9A, AoAA9A, and AfAA9C sequences indicate that an Arg residue is preserved at subsite −2, either at position 151 or 159.

To investigate the effect of natural variations at these functionally important positions, the catalytic domains of the seven selected AA9 LPMOs together with LsAA9A (control enzyme from the subcluster C38) were produced in Pichia pastoris. After purification, inductively coupled plasma mass spectrometry analyses indicated that the proteins were correctly Cu-loaded, in agreement with the H2O2 production rates observed using the Amplex red assay (Table S1).
The C29 and C38 A9 LPMOs display activity on cellulose

The enzymatic activities of all LsAA9A-related LPMOs were first assayed on two cellulose substrates, amorphous cellulose (phosphoric acid swollen cellulose) and crystalline cellulose (Avicel), with cysteine as a reductant because our previous studies suggest that the use of cysteine eases the interpretation of the chromatograms compared with, for example, ascorbate (27, 28). As expected, we found that all these enzymes displayed some activity on cellulose (Table 2 and Fig. S2). After only 4-h reactions, we detected nonoxidized cello-oligosaccharides (with a degree of polymerization [DP] of up to 4) reactions, we detected nonoxidized cello-oligosaccharides (cell2, cell3, and cell4 in Fig. S2). The results obtained herein with LsAA9A produced in P. pastoris are thus in line with previous reports on the substrate specificity and regioselectivity of the same enzyme recombinantly produced in A. oryzae (27, 28). This indicates that the lack of methylation of His1 in P. pastoris does not affect the product profile or the regioselectivity of the LPMO. Indeed, LsAA9A is a strict C4-oxidizer on cellulose and releases a range of short cello-oligosaccharides dominated by cell2 and cell3, in contrast to the longer products such as cell4, cellopentaose (cell5), and cellohexaose (cell6) released by TapAA9A, which is not active on cello-oligosaccharides (27, 28).

The C29 and C38 A9 LPMOs display activity on cello-oligosaccharides

We assayed the activity of the selected AA9 LPMOs on cello-oligosaccharides (cell3-cell6) in the presence of cysteine. All AA9 LPMOs tested were active on cell6 (Table 2), generating both cell3 and cell4 as the main nonoxidized products (Table 3 and Fig. 3), with constant rates over a 2-h reaction. We clearly found that no C1-oxidized products were formed and the chromatograms did indicate peaks corresponding to C4-oxidized species eluting around 25 and 40 min. Although the latter were not easily observable because of low sensitivity of species with late elution times, it strongly suggests that these A9 LPMOs display a C4 regioselectivity in agreement with previous data on LsAA9A.

The majority of the AA9s tested produced cell4 as the major nonoxidized product followed by cell3, except for ScAA9A, which displayed a different profile because cell3 was the major product pro...
This interesting observation indicates that the majority of the AA9 enzymes can bind cell6 preferably from subsite $-4$ to $+2$ in agreement with previous biochemical and structural reporting on \textit{Ls}AA9A (e.g. in supplementary figures 1 and 4 of [27]). However, based on our observations, we believe that the preferred binding mode of \textit{Sc}AA9A is from subsite $-3$ to $+3$. The rates of substrate conversion (and product formation although not shown) were determined and under our experimental conditions, we found that the majority of AA9 LPMOs converted cell6 with relatively similar constant rates of around 1 min$^{-1}$ (Table 3).

When investigating LPMO activity on cellobiose (Fig. 2, Tables 2 and 3). This interesting observation indicates that the majority of the AA9 enzymes can bind cell6 preferentially from subsite $-4$ to $+2$ in agreement with previous biochemical and structural reporting on \textit{Ls}AA9A (e.g. in supplementary figures 1 and 4 of [27]). However, based on our observations, we believe that the preferred binding mode of \textit{Sc}AA9A is from subsite $-3$ to $+3$. The rates of substrate conversion (and product formation although not shown) were determined and under our experimental conditions, we found that the majority of AA9 LPMOs converted cell6 with relatively similar constant rates of around 1 min$^{-1}$ (Table 3).

![Figure 2. Time-course reaction of cellohexaose degradation.](image_url)

**Figure 2. Time-course reaction of cellohexaose degradation.** Comparison of the preferred binding mode between the following: \textit{A}, \textit{Phanerochaete carnosa} AA9 (\textit{Pca}AA9A) and \textit{B}, \textit{Schizophyllum commune} (\textit{Sc}AA9A). Schematic representations below the chromatograms indicate amino-acid substitution compared with \textit{Ls}AA9A and subsite organization.

![Figure 3. Time-course reaction of cellopentaose conversion exemplified by PchAA9E.](image_url)

**Figure 3. Time-course reaction of cellopentaose conversion exemplified by PchAA9E.** Reactions with cellopentaose (cell5) analyzed after 20, 40, 60, 90, and 120 min. Cleavage of cell5 by PchAA9E produced cell3 (ca. 13-min retention time) and C4-oxidized cellobiose (ca. 25 min retention time) indicating that these enzymes are C4 oxidizers binding cell5 in a single binding mode. cell3, cellotriose.

weak activity was observed for AgAA9A. PchAA9E and PcaAA9A, the AA9 LPMOs most closely related to \textit{Ls}AA9A, showed no activity with cell3 as substrate unlike \textit{Ls}AA9A (27). With cell5 as substrate, we found that PchAA9E, PcaAA9A, AoAA9A, and AfAA9C (exemplified by PchAA9E, Fig. 3) generated cell3 as the major nonoxidized product and C4-oxidized DP2 products (and also a minor peak corresponding to glucose emerged during 2-h reaction). This indicates that the preferred binding mode of cell5 is from subsites $-3$ to $+2$ similarly to \textit{Ls}AA9A. We monitored the conversion of cell5 and the corresponding formation of cell3, which occurred at a constant rate over a 2-h time course (Table 3 and Fig. 3). Again, we found that the four enzymes appeared to convert the cell5 substrate at similar rates of around 1 min$^{-1}$ (Table 3). The small formation of glucose could arise from some scarce
cell3 degradation that occurred under these experimental conditions, although we did not observe activity when running experiments with only cell3 as a substrate.

With a cell4 substrate, we found that the four enzymes produced mainly cell2, confirming the binding mode from the −2 to +2 subsites, as previously reported for LsAA9A (27, 28). In contrast, AgAA9A appeared to equally produce both cell2 and glucose, although the activity was low, indicating binding from both −2 to +2 and −3 to +1 (Table 2). Incubating any of the substrates in the absence of enzyme or replacing the enzyme with equimolar Cu-acetate resulted in no substrate conversion or product formation.

The C29 and C38 AA9 LPMOs display activity on hemicellulose-derived substrates

Next, we tested the ability of the enzymes to degrade an XG polysaccharide from tamarind seeds. The reaction products were analyzed with chromatography using XXXG, XXLG, and XLLG oligosaccharides as standards (nomenclature after (41) where G and X denote β-(1,4)-linked glucosyl units either nonsubstituted or with xylosyl substitutions at the C6 position, respectively). For the majority of AA9s, we observed significant activity except for BaAA9A and ScAA9A where activity could not be detected even after overnight incubation (Fig. S4A). Among the products from 1-h reactions (Fig. S4B), three peaks with retention times around 15 min could correspond to short nonoxidized oligosaccharides (such as XXXG) and peaks with retention times 20 to 25 min, which could correspond to longer native or C4-oxidized oligosaccharides (after comparison with [24]). After overnight reactions (ca. 22 h, Fig. S4A), these peaks were significantly smaller, indicating a possible activity of these enzymes on XG oligosaccharides. Therefore, we tested the ability of these AA9 LPMOs to degrade an XG XXXGXXXG tetradecasaccharide. Reactions with XXXGXXXG showed that all AA9 (including BaAA9A and ScAA9A) cleaved XXXGXXXG, which lead to a small peak corresponding to XXXG and a larger peak corresponding likely to an XXX hexasaccharide (Fig. 4). This suggests that these AA9 LPMOs preferentially bind the XXXGXXXG nonreducing end in subsite −3 and with a nonsubstituted glucosyl unit in the +1 subsite, which would generate XXX and C4-oxidized XXXG species, as also reported by Agger et al. (24). They can also accommodate a xylose-substituted glucosyl unit in subsite +1 and bind the XXXGXXXG nonreducing end in subsite −4 producing a nonoxidized and a C4-oxidized XXXG species. We also speculated that the D150Y substitution near subsite −3 in AgAA9A (Fig. 5) could enhance binding of a C6-substituted glucosyl unit, and thus, AgAA9A would have the strongest preference for binding XXXGXXXG. Reactions with XXXGXXXG and AgAA9A or LsAA9A were run in parallel for 20 min, 2 h, and 24 h (Fig S5); however, the results of the time-course reaction with XXXGXXXG did not reveal any striking difference between AgAA9A and LsAA9A.

Finally, we tested the ability of the AA9 enzymes to cleave other hemicellulose substrates such as MLG, GM, and xylan. For many of the AA9s, products following enzymatic cleavage were detectable from reactions with MLG and GM. However, for ScAA9A, we only found weak activity on MLG and for BaAA9A, no clear activity on either of MLG or GM (Table 2). No clear activity on xylan or xylohexaose was observed for any of the LsAA9A-related LPMOs.
Homology models of the studied LPMOs

To better interpret and discuss the biochemical results, homology modeling was carried out using LsAA9A as the template. All models were highly reliable and with reasonable geometric quality (Table S2) according to Swiss-Model scores (42). Although still reliable, the AoAA9A model had the lowest quality as expected, given its lowest sequence identity (~55%) to the template (Table 1 and Fig. S6A). The LC loops bearing the Tyr/Trp residue expected to interact with the substrate (see Discussion) are the regions of the homology models that are structurally most different from the LsAA9A template. However, some caution must be applied in interpretation because these regions are also less reliably modeled (Fig S6).

Discussion

Here, we have investigated substrate specificities of seven fungal AA9 LPMOs from two related phylogenetic subclusters (C29 and C38) and shown activity on both cello-oligosaccharide- and hemicellulose-derived substrates. Our results support the notion that activity on short cello-oligosaccharides correlate with the activity on plant cell wall β-(1,4)-linked hexose chains. All of the LPMOs behaved relatively similarly to the canonical LsAA9A in terms of regioselectivity, ability to degrade cello-oligosaccharides and hemicelluloses containing β-(1,4)-linked D-glucose in contrast to AA9 from the other groups, which are mostly only active on cellulose. Our results show that all of the investigated LPMOs could bind cell6 from both subsite −4 to +2 and −3 to +3, indicating that the LPMOs in fact recognize five β-(1,4)-linked glucosyl units as in cell5. Indeed, we found a single binding mode for cell5 (from subsites −3 to +2), and both cell5 and cell6 are degraded with similar rates (indicating that subsites −4 and +3 are not strong binding sites). The very similar and low rates measured could be an indication of H2O2 being a limiting factor. It would be interesting to further investigate the activity of these LPMOs using H2O2 as cosubstrate under more controlled conditions. Based on the set of AA9 LPMOs investigated in this study, we conclude that in general, an extended L3 and an L8 loop both with polar/charged amino acid residues are determinants of cello-oligosaccharide activity in AA9 LPMOs. These features are present in AA9 LPMOs from C29 and C38 and small sequence differences within the L3, L8, and LC loops do not significantly impact the activity.

Although the AA9 LPMOs investigated here are overall similar, in-depth biochemical analyses revealed some subtle differences between the enzymes studied in terms of activity and substrate binding. For instance, LsAA9A is able to cleave cell3 (bound by Glu148 and Asn67 in subsites −1 to +2, respectively), unlike the other AA9 LPMOs investigated here. We found that PchAA9E and PcaAA9A (most closely related to LsAA9A) cleave cell4 as the smallest oligosaccharide substrate (from subsites −2 to +2). Structural modeling shows that Gln148 of PchAA9E and PcaAA9A align well with the LsAA9A Glu148. It is possible that E148Q alter the H-bond, which weakens cell3 binding, which could explain the absence of cell3 activity (Fig. 5).
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It also appears that N67D and R159K lead to loss of cell6 activity unless compensated by other substitutions in the L8 loop such as D150N and S151R found in AuAA9A and AfAA9C (Fig. 5 and Fig. S6A). Our results also suggest that the double substitution N67D/E148Q negatively affects cello-oligosaccharide binding because the BaAA9A enzyme only showed activity on cell6 and not on cello-oligosaccharides < DP6 (Table 2).

The ScAA9A enzyme displayed a different product profile by binding cell6 from −3 to +3. A weakened −4 subsite or a strengthened +3 subsite could explain this observation. Indeed, ScAA9A displays a Trp in the LC loop instead of the otherwise conserved substrate-interacting platform Tyr. Based on the ScAA9A model, these aromatic side chains likely make a similar interaction with cello-oligosaccharides, although two Pro residues (Pro204 and Pro205, not found in the other investigated enzymes) appear to affect the conformation of the LC loop (Fig. 1B, Fig. 5, and Fig. S6C). We believe that these subtle differences could alter the accommodation of the substrates in the −4 subsite and that the Trp preferably interacts with the terminal glucosyl unit at the nonreducing end in subsite −3. The ScAA9A 3D model also indicate that the T68Y and N27T substitutions (in loops L3 and L2, respectively), which are the neighboring residues of those making up subsite +2, and the L3R could play a role in binding of the terminal glucosyl unit at the reducing end in subsite +3 (Fig. 5, bottom right inset). Both of these explanations would lead to preferential binding of cell6 from −3 to +3.

A recently determined structure of CvAA9A (which shares 45% sequence identity with LsAA9A and also belongs to subcluster C38) with cell6 bound between subsite −3 and +3 (PDB code 6YDE) shows that the CvAA9A Arg3 is involved in a water-mediated hydrogen-bonding network in contact with the glucosyl unit at subsite +3 (33). In addition, both biochemical and structural analyses show that CvAA9A binds cell4 from −3 to +1 (PDB code 6YDC) unlike the AA9 LPMOs investigated here.

We also found some differences when investigating the conversion of hemicellulose substrates. In the AgAA9A 3D model, a Tyr (D150Y) is positioned next to the Tyr platform near subsite −3 (Fig. 5 and Fig. S6B) and could play a role in XG binding through interaction with xylose substitutions at the C6-hydroxyl of the glucosyl unit. However, the XG activity of AgAA9A was not markedly higher than that of other AA9 LPMOs characterized here (Fig. S4). Somewhat puzzling, however, we found that BaAA9 and ScAA9A cleaved an XXXGXXXXG oligosaccharide but did not show any activity on the XG polysaccharide substrate, unlike the rest of the characterized AA9 LPMOs. Finally, although LsAA9A is able to cleave xylan and xylohexaose (albeit much less efficient than cellulose substrates), we did not find any significant activity on these substrates with any of the other C29 or C38 AA9s investigated here. Thus, LsAA9A remains the only AA9 LPMO with reported activity on isolated xylan (as found here and previously [28]). The AA9 LPMOs from subclusters C29 and C38 (modular AA9-CBM1 proteins or single-domain AA9 enzymes, respectively) characterized in this study all originate from fungal species commonly associated with saprotrophic and/or plant parasitic lifestyles. Many of these are Agaricomycetes fungal species that display multiple AA9 genes, but on average only one or two putative cello-oligosaccharide-active AA9 LPMOs. During this study, we have also noted that some AA9 sequences, grouped in the neighboring subcluster C27, have similar putative cello-oligosaccharide binding features and are appended to a module of unknown function. These sequences originate mostly from plant pathogen species of the Dothideomycetes class, and it would be interesting to see if there is a correlation between cello-oligosaccharide specificity and pathogenicity.

We and others have previously observed a correlation between LPMO activity on hemicelluloses containing β-(1,4)–linked D-glucose (24, 25, 29, 35) and cello-oligosaccharides for some AA9s (e.g. NcAA9C, PaAA9H, and CtpMO1) from other subclusters (C21 and C23, which contain many Sordariomycetes species and are not closely related to C38 or C29) (Fig. S1). These AA9 LPMOs behave similarly to the AA9 LPMOs investigated in this study. This shows that the LPMO activity on both cello-oligosaccharides and hemicelluloses is not limited to AA9s in a few closely related phylogenetic subclusters but is found more widely in a number of fungal phylogenetic classes in species with different lifestyles. Interestingly, a recent transcriptomic study on P. carnosa showed that the gene encoding PcaAA9A (studied herein) was expressed during several days on growth on white spruce (Picea glauca) and aspen (Populus tremuloides) (43). In another study on Aspergillus nidulans, the gene encoding AN1602 (44) (an AA9-CBM1 protein displaying 65% sequence identity with AoAA9 and AfAA9) was highly transcribed during growth on cellulose and xylan, and the enzyme was shown to be active on both cell6 and XG (44, 45). Recently, XG and cell6 activities have also been reported for an AA9-CBM1 LPMO from Aspergillus tamarii (AtAA9A) for which the catalytic AA9 domain shares about 80% sequence identity with AoAA9A and AfAA9C (46).

It seems that many wood-decaying fungal species use an arsenal of AA9 LPMOs with at least one AA9 member that is able to recognize a short β-(1,4)–linked glucan motif conferring activity on cello-oligosaccharides and hemicellulosic substrates. The functional features of the fungal AA9 LPMOs studied herein do not fit with the common belief that LPMOs are specifically targeting crystalline regions of cellulose fibrils. Rather, C29 and C38 LPMOs seem to target a variety of complex motifs within the plant cell wall. Whatever is their biological function, we have shown that it is possible, from their sequence, to predict their LPMO activity toward cello-oligosaccharides and hemicellulose substrates, although their precise substrate specificity remains difficult to predict. In addition, the AA9 LPMOs displaying a single oligosaccharide-binding mode could be useful model systems for further mechanistic investigations.

Experimental procedures

Cloning and expression of AA9 LPMOs

All proteins were produced using the in-house 3PE Platform (Pichia Pastoris Protein Express; www.platform3pe.com/) (47). Genes encoding LsAA9A (GenBank ALN96977.1), AfAA9C
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Reactions with AA9 enzymes (at 4-μM concentration) were carried out at 50 °C at 850 rpm in 20-mM Na-acetate, pH 5.5, 100-mM NaCl in the presence of 1 mM cysteine, chosen in preference to other reducing agents for easier interpretation of the chromatograms. For polysaccharides, incubation times were either 1 h or overnight (approximately 20 h) and reaction mixtures were diluted ten times before injection. XG from tamarind seeds (CAS No. 37294-28-3) was purchased from Megazyme, and XXXGXXXG was kindly provided by H. Brumer (UBC, Vancouver, Canada). It was prepared as described in (50). For cello-oligosaccharides (initial concentration of 1 mM), time-course reactions were carried out with time points at 20, 40, 60, 90, and 120 min, and the reaction mixtures injected to the system were x100 dilutions. For the C29 AA9s, the 2 h time-course reactions with cell5 were run in triplicates. For time-course experiments with cell5, the peak areas of the different reaction time points were plotted against time and the slope was converted to molar rates. Control reactions with LPMO and a cello-oligosaccharide in the absence of an electron donor showed no product formation. For all substrates, negative controls were run both without enzyme and in the presence of Cu-acetate in equimolar concentrations to the enzymes (i.e. 4 μM). None of the negative controls with Cu-acetate produced peaks above the background.

Analysis of carbohydrate products

Analysis of carbohydrate products (both oxidized and nonoxidized) after incubation of polysaccharide and oligosaccharide substrates with AA9 LPMOs was performed using high-performance anion-exchange chromatography coupled with pulsed amperometric detection as described in (51). By the use of standards (Megazyme), peak areas were converted to molar concentration. A peak corresponding to the electron donating redox partner cysteine was observed with retention time of 26 to 27 min. Peaks at retention times corresponding to oxidized products have been previously reported (29).

Homology modeling

Swiss-Model (https://swissmodel.expasy.org/) (42) was chosen because we found this method to better handle the methylated His and the position of the Tyr platform (Tyr203 in LsAA9A) than other available methods (e.g. the Phyre2 server (http://www.sbg.bio.ic.ac.uk/) [52]). Swiss-Model runs through the server at https://swissmodel.expasy.org/ with template PDB 5ACH (the closest available for all investigated enzymes) (42). GQME and QMEAN Z-scores used to assess model quality are summarized in (Table S2). The lowest scores in terms of reliability and geometry were GQME of 0.77 and QMEAN Z-score of −2.09, which are still very reasonable for a
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useful model. MolProbity overall scores are similar for models and template, with models generally having slightly worse Ramachandran statistics.

**Data availability**

All data generated or analyzed during this study are contained within this article.

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**Author contributions**—K. E. H. F. and J. G. B. designed the study. K. E. H. F. carried out most of the experimental work. K. E. H. F. and B. H. carried out the bioinformatics analysis. K. E. H. F. and M. H. expressed and purified recombinant lytic polysaccharide mono-oxygenases. K. E. H. F. carried out enzyme assays. K. E. H. F. and M. H. analyzed enzymatic products with high-performance anion-exchange chromatography coupled with pulsed amperometric detection. L. L. L. made homology models. K. E. H. F. and J. G. B. wrote the manuscript with contribution from the coauthors. The manuscript was approved by all coauthors before submission.

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**Abbreviations**—The abbreviations used are: CBM, carbohydrate-binding module; Cell1, glucose; Cell2, cellobiose; Cell3, celiotriose; Cell4, cellotetraose; Cell5, cellopentaose; Cell6, cellohexaose; DP, degree of polymerization; GM, glucosannan; His-brace, Histidine brace; Hindbrace, Hindistane brace; ICP-MS, inductively coupled plasma mass spectrometry; LC, C-terminal; LPMO, lytic polysaccharide mono-oxygenase; MLGs, mixed-linkage β-glucans; XG, xyloglucan.

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