

Functional diversification of AA17 LPMOs reveals multiple polysaccharide-oxidising activities in plant-pathogenic oomycetes

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Abstract

The AA17 lytic polysaccharide monooxygenase (LPMO) family was recently founded as a novel polygalacturonan targeting enzyme family with members exclusively found in oomycetes. Here, by analysing and classifying over a thousand AA17 sequences across the Oomycota, we report diversification of the family into distinct sequence clusters. By combining phylogenetic analyses and reaction product profiling by mass spectrometry of enzymes representing eight sequence clusters, we demonstrate that family AA17 also harbours enzymes that oxidatively cleave β -1,4-glucans and substituted xylans. Mapping these specificities onto the phylogeny reveals that β -1,4-glucan-active AA17s are widespread in oomycetes, whereas pectin- and xylan-active clades are restricted to plant-pathogenic lineages. X-ray crystallography of a xylan-active AA17 in complex with a xylo-oligosaccharide revealed a unique substrate-binding mode validating the new xylan cleaving activity in this family. Our findings thus disclose a major functional radiation within the AA17 family, suggesting that the LPMO oxidative machinery has evolved in oomycetes to match host cell wall composition, redefining family AA17 as an exceptionally diversified virulence-associated enzyme platform.

Introduction

Lytic polysaccharide monooxygenases (LPMOs) are copper-dependent enzymes that catalyse oxidative cleave of glycosidic bonds in polysaccharides and were first described for their activity on cellulose and chitin^{1,2}. The mechanism is catalysed by a single copper (Cu) atom coordinated by a histidine brace^{2,3}. LPMOs specifically oxidise either the reducing end (C1-oxidising) or the non-reducing end (C4-oxidising) of the polysaccharide, generating a sugar-ketone at the corresponding carbon, and these oxidised products can subsequently undergo spontaneous hydration to form either an aldonic acid (C1) or a gem-diol (C4)⁴⁻⁶. LPMO catalysis requires an electron donor, such as ascorbic acid, to prime the resting state Cu(II) to the active state Cu(I)⁶⁻⁸. The reduced Cu(I) uses either molecular oxygen (O₂) or hydrogen peroxide (H₂O₂) to hydroxylate the target carbon and cleave the glycosidic bond^{6,7}.

LPMOs are classified in the Carbohydrate-Active enZymes (CAZy) database within the Auxiliary Activities (AAs) families^{9,10} and are broadly found across viruses, bacteria, fungi, oomycetes, algae and metazoans. In contrast to bacteria and fungi, oomycete genomes encode a distinctive LPMO repertoire composed of families AA15, AA16 and AA17, while lacking classical LPMO families abundant in fungi (AA9, AA11, AA13, and AA14) and bacteria (AA10)^{11,12}. While genes encoding AA15 family members is only found in animal-infecting oomycetes (*Saprolegniales*)¹¹, members of the AA17 family have origin in both animal-infecting and a wide range of plant-pathogenic oomycetes¹². The latter suggests that these enzymes are central to the unique plant-pathogenic strategies of the Oomycota.

The AA17 family was established in 2021 following the discovery of the first pectin-active (polygalacturonan-active) LPMOs in the oomycete *Phytophthora infestans*¹², which is the causative agent of the potato and tomato late blight responsible for approximately \$1 billion per year in potato crop

losses across Europe alone¹³. These enzymes are highly induced during host colonisation, functioning as essential virulence factors for successful infection¹².

Interestingly, the AA17 genes are highly expanded in the oomycetes. In most *Phytophthora* species, the AA17 family thus comprises more than 20 paralogues making it the largest LPMO repertoire encoded in these genomes^{10,11}. However, despite the extensive expansion of AA17 encoding genes across oomycete genomes, only five members have been biochemically characterised to date, all displaying strict specificity toward homogalacturonan^{12,14}, with the keystone *P. infestans* enzyme, known as PiAA17C, being able to catalyse cleavage of moderately methyl-esterified homogalacturonan as well¹⁵.

Oomycetes are a diverse group of plant and animal pathogens that occupy a wide range of aquatic and terrestrial niches¹⁶. They include historically infamous plant pathogens such as *P. infestans* (the causal agent of the Irish potato famine in the 19th century^{17,18}), which remains a persistent threat to modern agriculture¹⁸, as well as devastating animal pathogens like *Saprolegnia* species, which cause severe mycoses in aquaculture^{19–21}. Indeed, the substantial expansion of AA17 paralogues across oomycete lineages raises the possibility that this family has diversified to adapt to different polysaccharide environments associated with pathogenic lifestyle and host range. In turn, it is tempting to speculate whether a diversification of these enzymes might reflect a broader substrate specialisation, representing an evolutionary adaptation to host cell wall composition.

Here, we combine large-scale phylogenetic analysis of AA17 sequences with biochemical and structural characterisation of representative members spanning major evolutionary clusters across the oomycetes. We demonstrate that AA17 LPMOs are not restricted to pectin but comprise distinct subfamilies with members capable of oxidatively cleaving β -1,4-glucans and substituted xylans. Furthermore, mapping these substrate specificities onto the phylogeny reveals lineage-restricted activities primarily associated with plant-pathogenic species, and X-ray crystallography of a xylan-active AA17 in complex with its substrate reveals a binding mode distinct from previously characterised LPMOs. Together, these findings uncover an extensive functional radiation within the AA17 family, suggesting that oxidative polysaccharide cleavage has evolved as a host-adaptive enzymatic strategy in oomycetes. This expanded functional insight into AA17 enzymes provides a molecular basis for exploring new plant biomass refining routes and new approaches to mitigate oomycete-driven crop disease.

Results

Family AA17 can be divided into 17 Sequence Similarity Network (SSN) clusters

CAZy annotation of oomycete proteomes revealed that their genomes encode LPMOs exclusively from families AA15, AA16, and AA17, while the classical cellulose- and xylan-active LPMO families AA9 and AA10 are absent. AA17 is the most expanded LPMO family in oomycetes, with most plant pathogenic

species encoding over 20 paralogues. In contrast, AA16 members are restricted to the *Pythophthora* and *Phytium* genera, whereas AA15 enzymes are detected only in Saprolegniales. These lineage-specific distributions are consistent with functional specialisation towards cellulose and chitin degradation, respectively¹⁰.

Although AA17 enzymes have been investigated in the context of plant virulence in *P. infestans*¹² and *Phytophthora sojae*¹⁴, the presence of AA17 homologues in non-plant pathogenic oomycetes such as *Saprolegnia* and *Aphanomyces* indicates that this broadly represented enzyme family remains substantially underexplored.

To systematically probe AA17 diversity, we compiled a dataset of 1015 putative AA17 sequences, including the five currently characterised members (Fig. 1). Phylogenetic analysis (Fig. 1a) showed that most AA17 sequences originate from the order Peronosporales (82%), encompassing plant pathogenic genera such as *Phytophthora*, *Peronospora*, and *Phytium*, whereas Saprolegniales (9%) and Pythiales (9%) represent smaller fractions, with a single representative from Albuginales (< 0.01%). Notably, all biochemically characterised AA17s from *P. infestans* and *P. sojae* cluster within a single Peronosporales-specific clade (Fig. 1a). The phylogeny resolves the family into 12–15 distinct clades, with members from Albuginales, Peronosporales, Pythiales, and Saprolegniales distributed across the tree (Fig. 1a, Supplementary Material Fig. S1).

Consistent with their functional similarity and shared activity on polygalacturonic acid^{12,14}, all five characterised AA17s localise within the same clade (Fig. 1a). Pairwise protein sequence identity across the family is extremely low, with the most distant members sharing only 8.7% identity and more than 75% of pairwise comparisons falling below 28% identity, supporting extensive evolutionary divergence and the existence of yet-undiscovered enzymatic activities and/or biological roles (Supplementary Material Table S1).

To complement the phylogenetic analysis, we constructed a sequence similarity network (SSN) (Fig. 1b). At an E-value cutoff of 10^{-77} (Supplementary Material Fig. S2) selected based on the closeness centrality principle²², the SSN resolved 17 clusters plus several minor groups with fewer than 10 members. Additional SSNs based on the closeness centrality score yielded consistent clustering patterns and confirmed the presence of multiple deeply divergent AA17 subfamilies (data not shown). To experimentally interrogate this diversity and maximise coverage of evolutionary space within the AA17 family, we selected 14 representative members spanning eight distinct SSN clusters (Fig. 1a-b). Three of the targets were selected from the cluster containing the previously characterised enzymes, while the remaining 11 were selected from distant clusters hypothesised to possess new activities.

Table 1
Target sequence information

Target	Cluster	Organism	Accession number	MW [kDa]	Amino Acids
AaAA17A	8	<i>Aphanomyces astaci</i>	ETV73941.1	19.4	166
PeAA17A	3	<i>Peronospora effusa</i>	UIZ26907.1	20.3	172
PfAA17A	1	<i>Phytophthora fragariae</i>	KAE8922300.1	20.4	173
PiAA17D	4	<i>Phytophthora infestans</i> T30-4	EEY61639.1	20.7	172
PkAA17A	1	<i>Phytophthora kernoviae</i>	RLN47764.1	20.4	175
PnAA17A	4	<i>Phytophthora nicotianae</i>	ETM55515.1	20.5	170
PnAA17B	7	<i>Phytophthora nicotianae</i>	KUF86759.1	18.9	164
PnAA17C	5	<i>Phytophthora nicotianae</i> P1569	ETI31959.1	19.9	171
PnAA17D	4	<i>Phytophthora nicotianae</i> P1569	ETI56037.1	20.6	171
PnAA17E	4	<i>Phytophthora nicotianae</i> P1569	ETI56084.1	20.4	171
PnAA17F	2	<i>Phytophthora nicotianae</i> P1976	ETO70334.1	20.3	175
PhAA17A	1	<i>Plasmopara halstedii</i>	CEG47543.1	20.1	172
SdAA17A	6	<i>Saprolegnia diclina</i> VS20	EQC34358.1	19.5	168
SpAA17A	6	<i>Saprolegnia parasitica</i> CBS 223.65	KDO26913.1	19.6	168

Oomycete AA17 LPMOs specificities

Fourteen AA17 LPMOs representing eight distinct phylogenetic clusters were recombinantly produced in *E. coli* with an N-terminal pelB leader sequence for periplasmic secretion and a C-terminal Strep-tag as described in Sabbadin *et al.* 2021¹². The purified copper-loaded enzymes (Supplementary Material Fig. S3) were screened for activity against a broad panel of substrates, including: polygalacturonic acid, phosphoric acid swollen cellulose (PASC), mixed linkage β -glucan (β -1,3 and β -1,4 linkages), xyloglucan, arabinoxylan, beechwood xylan, birchwood xylan, chitin, galactomannan, and polyglucuronic acid.

Homogalacturonan

To date, polygalacturonic acid, which forms the backbone of pectin, is the only substrate reported for AA17 LPMOs, and implicated in the *Phytophthora* infection mechanism^{12,14}. Here, purified enzymes were incubated with polygalacturonic acid in the presence of ascorbate and reaction products were analysed with MALDI-TOF MS (Fig. 2a-b). Native and oxidised cleavage products consistent with previously reported AA17 oxidation^{12,14,15} were detected for four AA17, showing C4 oxidative cleavage and subsequent spontaneous decarboxylation and hydration (Fig. 2c) (additional spectra in Supplementary Material Fig. S4). Among the tested enzymes three (*PkAA17A*, *PfAA17A*, and *PhAA17A*) cluster with the Peronosporales-specific cluster 1 (Fig. 1a-b) and share 67.1–80.9% sequence identity with the characterised *PiAA17C*¹², whereas *PnAA17C* shares 55.1% sequence identity and groups in a neighbouring clade and a distinct SSN cluster (cluster 5) (Fig. 1a-b).

For definitive insight into the structural changes upon AA17 treatment of polygalacturonic acid, NMR spectroscopic analysis was conducted for *PkAA17A* activity products, which corroborated the C4 cleavage and spontaneous decarboxylation resulting in a fully hydrated C4 gem-diol in water (Supplementary Material, Fig. S5-7).

β-1,4-linked glucans

The remaining AA17 enzyme targets did not exhibit activity toward polygalacturonic acid. However, six enzymes (*AaAA17A*, *PeAA17A*, *PnAA17B*, *PnAA17F*, *SdAA17A* and *SpAA17A*) displayed previously unreported activity on β-linked glucose polymers, including PASC, mixed-linkage β-glucan (β-1,4 linkages) and xyloglucan, as validated by MALDI-TOF analysis of reaction products (Fig. 3) (additional spectra in Supplementary Material Fig. S8-10).

Five of the six enzymes generated oxidised, hydrated and native oligosaccharide products upon incubation with PASC in presence of ascorbate, consistent with C4 oxidation (Fig. 3a). In contrast, *PnAA17B* produced a distinct product pattern featuring additional sodium adducts corresponding to aldonic acid products, indicating predominant C1 oxidation. Three of the six enzymes also showed lytic activity on mixed linkage β-glucan (Fig. 3b) and three enzymes had lytic activity on xyloglucan (Fig. 3c), with partial overlap between the two groups. Interestingly, *SdAA17A* and *SpAA17A* (both from Saprolegniales) were active on all three β-1,4-linked substrates and group together within cluster 6, whereas *AaAA17A*, *PeAA17A*, *PnAA17B* and *PnAA17F* (active on PASC) are distributed across distinct clusters (clusters 7, 2, 8 and 3, respectively).

Importantly, none of the β-1,4-glucan active enzymes group within the same clades or SSN clusters as the polygalacturonic acid-specific AA17s, supporting functional diversification across the AA17 family. NMR spectroscopy of *SdAA17A* on xyloglucan validated the C4-oxidative mechanism through formation of a quaternary carbon at the C4 position, resulting from full hydration of the C4-keto to a C4 diol in water (Supplementary Material Fig. S11, Fig. S15).

Xylan

The four remaining targets *PiAA17D*, *PnAA17A*, *PnAA17D* and *PnAA17E* grouped in SSN cluster 4 (Fig. 1a-b) and did not exhibit activity towards polygalacturonic acid or any β -1,4-glucan substrates tested. However, these enzymes released native and oxidised xylo-oligosaccharides upon incubation with beechwood xylan (containing 4-O-methylglucuronic acid substitutions) in the presence of ascorbate (Fig. 4a), plus native and oxidised sodium adducts for substituted oligosaccharides, with peak masses consistent with a C4 oxidation mechanism (additional spectra in Supplementary Material Fig. S12). All four enzymes also showed analogous activity on arabinoxylan (Supplementary Material Fig. S13), indicating that cluster 4 members can cleave structurally distinct substituted xylans.

AA17 family clusters display distinct surface features associated with substrate specificity

Collectively, our data identify xylan, alongside β -1,4-glucans, as an additional previously unrecognised substrate class for AA17 LPMOs and substantially expand the functional repertoire of this enzyme family. NMR spectroscopy on xylan products generated by treatment of beechwood xylan with *PnAA17D* confirmed the C-4 specific oxidative cleavage of the xylan backbone followed by hydration to a geminal diol (Supplementary Material Fig. S14-15). Together, these findings reveal that AA17 functional diversification mirrors the ecological and pathogenic specialisation of the oomycete lineages.

The structural basis underlying the distinct substrate specificities observed across AA17 clusters was inspected by electrostatic surface potential analysis²⁶ and sequence conservation mapping²⁷ (Fig. 5), revealing cluster-specific patterns surrounding the histidine brace (Fig. 5a-d). All clusters showed similar conservation pattern in the multiple sequence alignments, with high conservation for the histidine brace and residues downstream from the His2 at position 123 in the MSA (Fig. 5d). The highly conserved region in the MSA corresponds to the four β -strands structurally located in the core of the protein, which contain up to three disulphide bonds for most of the AA17 clusters (Supplementary Material Fig. S16-24). The structural fold of the AA17 resemble the immunoglobulin-like β -sandwich fold with the N-terminal His-brace of other LPMOs families AA9-11 and AA13-16 (Supplementary Material Fig. S25).

Clusters 1 and 5, which comprise polygalacturonic acid-active enzymes, exhibited small but highly positive electrostatic patches surrounding the histidine brace (Fig. 5a). In contrast, clusters 2, 3 and 7 (β -1,4-glucan-active) showed more negatively charged surfaces in this region. Cluster 4 (xylan-active) and cluster 6 (β -1,4-glucan-active) showed overall neutral to mildly positive surface potentials, while cluster 8 displayed a mixed electrostatic profile.

Several conserved residues surrounding the histidine brace differentiate the polygalacturonic acid-active clusters 1 and 5 (Fig. 5c). There, a conserved glutamine (cluster 1: Q156; cluster 5: Q152) is found adjacent to the secondary histidine of the his-brace, whereas in all other clusters this position is occupied by a histidine that engages in π -stacking with the brace histidine. Additionally, clusters 1 and 5

both have a surface-exposed tryptophan (cluster 1: W21; cluster 5 W19), whereas in other clusters this position is occupied by a tyrosine (clusters 3, 4 and 6), proline (clusters 2 and 7) or phenylalanine (cluster 8). Finally, both clusters 1 and 5 contain a conserved arginine (cluster 1: R95; cluster 5: R91) pointing toward the active site, potentially interacting with the carboxylate group of a galacturonic acid residue upon binding.

Interestingly, β -1,4-glucan-active clusters 2, 3, 6, 7 and 8 predominantly show a classical flat surface topology around the active site corroborating interaction with the flat twofold screw axis of cellulose, exemplified in cluster 7 by three co-planar tyrosine residues - Y17, Y18 and Y37 - aligned with the copper centre and likely involved in neutral polysaccharide binding²⁸. In contrast, cluster 1, 4 and 5 feature uneven surface topology with either a histidine brace buried within a cleft (cluster 1 and 5, polygalacturonic acid-active), or a small groove created by two conserved protruding residues (E95 and Y96 in cluster 4, harbouring the xylan-active targets) (Fig. 5c), capable of interacting with the helical three-fold screw axis of xylose, consistent with divergent substrate specificities within the AA17 family.

Comparative analysis of surface electrostatics, topology and conserved residues across AA17 clusters therefore revealed structural features that correlate with the experimentally observed substrate preferences. Notably, cluster 4 enzymes predicted to target xylan display a distinct surface groove adjacent to the histidine brace and lack the extended aromatic platform characteristic of β -1,4-glucan active LPMOs. These observations suggest that cluster 4 AA17s possess a substrate-binding architecture adapted for recognition of substituted xylans.

Structural analysis of xylan active PnAA17D

To obtain atomic-level insight into this predicted mode of substrate recognition, we determined the crystal structure of the cluster 4 enzyme *PnAA17D* in complex with xylotetraose at 1.6Å resolution (PDB: 31BW, Supplementary Material Table S2). The refined model, displayed with electrostatic surface potential, reveals bound xylotetraose from subsite + 1 to + 4 with the ligand omit electron density shown as a green mesh (Fig. 6a). A close-up view of the active site (Fig. 6b) shows the copper ion in the elongated octahedral coordination geometry, canonical for LPMOs, by the N-terminal amino group, His1, His104, Tyr163 and a water molecule (*). The bound xylotetraose engages through hydrogen-bonding with Asn93, Glu95 and Thr18 at subsite + 2, while residues Lys12, Asn14 and Thr15 contribute to interactions at subsite + 4. Electron density of the sugar moiety proximal to the copper centre (subsite + 1) is weaker for atoms C3-C5 and the associated hydroxyl groups, likely due to insufficient binding at this position, causing movement of the sugar throughout the crystal lattice. However, the C4 of the + 1 subsite shows correct orientation compatible with the canonical LPMO reaction, with the hydrogen pointing towards the copper, available for hydrogen abstraction followed by hydroxylation and glycosidic bond cleavage, as described for chitin-active LPMOs by Bissaro et al.²⁹, and consistent with AA9 LPMO crystal complex structures^{4,30,31}.

Structural comparison by superimposition of *PnAA17D*-Xyl₄ and *LsAA9A*-Xyl₅ (PDB: 5NLO) (Supplementary Material Fig. 25), shows that the binding of the xylo-oligomer follows the same directionality. However, the xylo-oligomers of the two structures are shifted 1.8Å-1.9Å relative to each other and a 90-degree rotation of the subsite + 1 observed in *LsAA9A*-Xyl₅ is not found in *PnAA17D*-Xyl₄, ultimately resulting in a shorter distance from the C4 to the axial water, occupying the position of H₂O₂ in the *LsAA9A* structure. Several interactions are congruent between the two structures, notably the polar interactions: T18, N39, S103, S157 and non-polar interaction by L101 of *PnAA17D*, all excluding S157 which are highly conserved in the AA17 clade 5 (Fig. 5c). Notably, unlike the quite well studied *LsAA9A* enzyme, *PnAA17D* shows no aromatic binding residue at subsite - 3, but displays substantially more polar and non-polar interactions in subsites + 2 to + 4.

Oomycete AA17 repertoire reflects evolutionary specialisation to host polysaccharide composition

All oomycetes possess AA17 with β -1,4 glucan activity, i.e. all *Phytophthora*, *Saprolegnia*, *Aphanomyces*, *Plasmopara*, and *Peronospora* possess AA17 within SSN clusters 2, 3, 6, 7 and 8 linking these enzymes to β -1,4 glucan degradation. This activity may be linked to the oomycete cell wall remodelling during growth and host infection but can also reflect the ability to attack cellulose present in the cell walls of the host plants being invaded by these oomycetes.

The CAZy annotated *Saprolegnia parasitica* encodes 11 such AA17s (and only these), predicted to target β -1,4 glucan bonds similar to the herein characterised AA17 targets on β -1,4 glucan substrates. Since the main habitat of *Saprolegnia parasitica* is freshwater fish, this enzymatic activity is most likely unrelated to the host polysaccharides, but rather evolved to remodel the oomycete cell wall, known to contain significant amounts of β -1,4 glucan³². In contrast *Phytophthora*, *Plasmopara*, and *Peronospora* all invade plant material containing cellulose, which hints that AA17 encoded within these organisms is part of the pathogenicity enzyme toolkit of these oomycetes.

Only oomycetes within the Peronosporales (*Phytophthora* and *Peronospora*) and Pythiales (*Pythium*) have AA17 within the cluster 4 capable of degrading xylan, which concurs with xylan being a major type of hemicellulose component in the plant cell wall. These xylan degrading AA17s are in line with other AA17s suggested to be a part of the plant pathogenic enzyme toolkit of these organisms, particularly since oomycetes contain negligible amounts of xylan within their cell wall³².

So far, only Peronosporales have been shown to possess AA17 with activity on pectin (cluster 1 and 5), which concurs with the aggressive pathogenicity of these oomycetes on plants rich in pectin, including potato, soy and tobacco plants. Indeed, this interpretation is supported by the findings that these specific AA17s are heavily upregulated during plant infection¹².

Discussion

This study reveals that the AA17 family of lytic polysaccharide monooxygenases is far more functionally diverse than previously recognised, encompassing distinct enzyme clades that oxidatively cleave homogalacturonan, β -1,4-glucans and substituted xylans. Until now, all characterised AA17 enzymes had been shown to oxidatively cleave pectin, leading to the view that AA17 represents a pectin-specific LPMO family associated with plant infection. Clearly, the substrate diversity present within family AA17 is indicative of the tailored oomycete CAZyme degradome, specialised to the pathogenic lifestyles and different oomycete invasion strategies with AA17s matching host polysaccharides. The β -1,4 glucan active AA17s, like for example AA9, AA10 and AA11 found in other organisms, may have a dual role in some oomycetes: 1) facilitating host cell wall degradation and penetration and 2) modulating the oomycete cell wall during growth and host invasion, the latter seen for both bacterial³³ and fungal³⁴ LPMOs. The AA17s acting on β -1,4 glucan bonds are of particular relevance as oomycete cell wall remodelling enzymes, as cellulose is the main polysaccharide in the oomycete cell wall³². This activity is spread across all different types of oomycetes including the ones tested here, whereas the pectin and xylan activities are limited to the plant pathogenic oomycetes of the Peronosporales and Pythiales. Previous LPMO activities reported on xylan from AA9 and AA14, have mainly shown activity towards cellulose bound xylan in a two-fold screw axis confirmation^{35,36}. In contrast, the AA17 xylan activity identified here does not seem to require the two-fold screw axis of cellulose bound xylan; rather, it seems that these enzymes interact with the three-fold screw axis of soluble xylan which compares to the activity on soluble polygalacturonic acid first reported for AA17. It is tempting to speculate that AA17 activity on specific plant polysaccharides such as pectin and xylan may have evolved from the more ancestral AA17s attacking β -1,4 glucan that evolved, since most phylogenomic analyses place Saprolegniales as branching earlier, with Peronosporales and Pythiales forming a more recently diversified lineage. This hypothesis is supported by the presence of β -1,4-glucan active AA17s within every taxonomic group of oomycetes, whereas pectin active AA17s so far are only found in plant pathogenic Peronosporales. Clearly the AA17 family is of critical importance for the lifestyle of oomycetes, first; because the AA17 enzymes have only been found in oomycetes, second; because all oomycetes contain a high copy number of AA17 encoding genes, and third; because oomycetes only encode a selected few other LPMOs (AA15 and AA16). The presence of AA15 and AA16 encoding genes in oomycetes correlates with oomycete taxonomy and host biology, with the chitin active AA15 only present in the Saprolegniales order (aquatic animal pathogens) while cellulose active AA16 is only present in the orders Peronosporales and Pythiales (plant pathogens).

So far, pectin LPMO activity is exclusive for the AA17 family, while the activities on β -1,4 glucan and xylan have been shown in other LPMO families, i.e. AA9 (cellulose and xylan), AA10 (cellulose and xylan), AA14 (cellulose and xylan), AA15 (cellulose and chitin), and AA16 (cellulose). Only the two LPMO families AA11 (chitin) and AA13 (starch) do so far not possess members having cellulose activity while all other LPMO families do.

Interestingly, the members of all LPMO families, except for family AA18, show similar tertiary structure with the catalytic domain as a β -sandwich fold containing the N-terminal histidine brace with the

catalytic copper atom positioned for oxidative cleavage of polysaccharides. This structural and catalytic generalisation of the catalytic domain of LPMO families suggest that AA9-11 and AA13-17 could be categorised as a clan in the CAZy database, based on the similar fold and catalytic machinery across the families, as per the original definition of clans introduced for glycosyl hydrolases³⁷, namely “a group of families that are thought to have a common ancestry and are recognized by significant similarities in tertiary structure together with conservation of the catalytic residues and catalytic mechanism”.

Materials and Methods

Materials

Genes were purchased from GenScript (Piscataway, NJ, US) and Twist Bioscience (San Francisco, CA, US). Substrates arabinoxylan (Wheat flour), xylan (Beechwood), xyloglucan (Tamarind Seed) and β -glucan (Barley; low viscosity) were purchased from Megazyme (Bray, Ireland). Avicel® PH-101, polygalacturonic acid (PGA), L-ascorbic acid, d-desthiobiotin, norhamane (β -carboline) and 2,5-Dihydroxybenzoic acid (DHB) from Sigma-Aldrich (St. Louis, MO, US). NZy auto-induction LB medium was purchased from NZYTech (Lisbon, Portugal), Strep-Tactin® 4Flow® high-capacity resin from IBA LifeSciences (Göttingen, Germany). Crystallization screens were from Molecular Dimensions (Newmarket, UK).

Phylogenetic analysis

Phylogenetic analysis of family AA17 was conducted with AA17 sequences from the CAZy database, used as query sequences for blast expansion with blastp³⁸ and top 100 hits for each query sequence were included in the analysis. Fragments were removed and the sequences were N- and C-terminal truncated to the catalytic domain. Redundant sequences were removed with CD-hit²³ at 98% to 1015 sequences. The phylogenetic tree was generated with automatic model selection in IQ-tree²⁵ with 1000 iterations of bootstrapping and visualized in iTOL.

Sequence similarity networks (SSNs) was generated as described in Horning & Terrapon (2023)²² with E-values calculated with blastp³⁸ and using the closeness centrality principle to determine SSN clusters. Consurf²⁷ analysis was made for selected SSN clusters using mafft²⁴ to generate multiple sequence alignments for each cluster which was used as input in the consurf analysis, a representative AlphaFold3³⁹ model or structure was used as basis for the consurf colouring.

Production

Plasmids containing the codon-optimised targets of interest were ordered from Genscript or Twist, in either pET-22b(+) or pET-29b(+) with the addition of a N-terminal pelB leader sequence and C-terminal Strep-tag® II. *E. coli* Rosetta 2 (DE3) pLysS were transformed with pET-22b(+) plasmids and *E. coli* BL21 (DE3) were transformed with pET-29b(+) plasmids. All targets were plated in LB plates with appropriate antibiotics, a single colony was used to inoculate 30 mL LB medium with appropriate antibiotics (100 μ g

mL^{-1} ampicillin and $34 \mu\text{g mL}^{-1}$ chloramphenicol for *E. coli* Rosetta 2 (DE3) pLysS with pET-22b and $50 \mu\text{g mL}^{-1}$ kanamycin for *E. coli* BL21 (DE3) with pET-29b(+)) and grown over night at 180 rpm at 37°C . The preculture was used to inoculate 500 mL of NZy autoinduction media with appropriate antibiotics to a starting OD_{600} of 0.05. The cells were grown at 16°C for 3 days at 140 rpm. The cells were harvested by centrifugation and resuspended in 20 mL lysis buffer (100 mM Tris, 150 mM NaCl, 0.5 mg mL^{-1} lysozyme at pH 8.0) left at 20°C and 120 rpm for 10 min, and then frozen at -20°C . The frozen solutions were thawed and combined with 500 μL DNase solution (1 mg mL^{-1} DNaseI and 1 mM MgCl_2) and left at 20°C at 120 rpm for 10 minutes. After centrifugation the supernatant was collected and loaded on to a Strep-Tactin® 4Flow® high-capacity column. The loaded columns were washed with binding buffer (100 mM Tris, 150 mM NaCl, pH 8.0) and the proteins were eluted with elution buffer (100 mM Tris, 150 mM NaCl, 2.5 mM d-desthiobiotin, pH 8.0). The proteins were copper loaded with equimolar concentration of CuSO_4 and buffer exchanged to 20 mM Tris, pH 7.0 with 10kDa MWCO Vivaspin® 20 Ultrafiltration units. For protein crystallization the eluate was further purified by size exclusion chromatography (SEC) with a HiLoad 16/600 Superdex 200pg (Sigma-Aldrich, St. Louis, MO, US) column connected to a Äkta Pure System, the system was equilibrated with SEC buffer containing 20mM Tris, 100mM NaCl at pH 7.4 and protein was eluted in 2mL fractions, pooled and concentrated using 10kDa MWCO Vivaspin® 20 ultrafiltration (Sartorius) units, by centrifugation at 3500g and 4°C until the protein concentration was $\sim 10 \text{ mg mL}^{-1}$.

Mass spectrometry

Reactions with 2 μM enzyme, 4 mM Ascorbic acid, 5 g L^{-1} substrate in 50 mM NaOAc, pH 6.0 buffer were incubated at 30°C with 1000rpm in thermomixer for 20 hours. Precipitation of 50 μL sample was conducted with 150 μL acetonitrile (ACN) and the pellet was resuspended in water and 1 μL spotted on a 384 MALDI-TOF MS target plate with addition of 1 μL 2,5-Dihydroxybenzoic acid (DHB) matrix (9 mg mL^{-1} in 70% ACN). For charged polysaccharide polygalacturonic acid, the resuspended pellet was incubated with Dowex 50 resin for 30 minutes and 1 μL of sample was spotted the 384 MALDI-TOF MS target plate with addition of 1 μL norhamane (10 mg mL^{-1} in 70% ACN) matrix. MALDI-TOF MS spectra were recorded with Bruker Daltonics autoflex™ speed in linear mode using the Bruker flexcontrol software.

Structural

PnAA17D was crystallised using the sitting drop vapour diffusion method. The protein sample was concentrated to 10 mg mL^{-1} in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl. This sample was crystallised against the commercial LMB⁴⁰ screen using a Mosquito® Xtal36 automated liquid handling system (SPT Labtech, Melbourn, Hertfordshire, UK) into MRC3 96-well crystallisation plates (SWISSCI, High Wycombe, UK), with a reservoir volume of 40 μL , and drop volumes of 0.3 μL . Crystal rods appeared after one week in a drop consisting of 0.15 μL protein and 0.15 μL reservoir (24% w/v PEG3350, 50 mM HEPES pH 7.5 and 150 mM NaCl). Powdered xylohexaose (Megazyme) was dissolved in each 0.5 μL reservoir and added to the drop containing the crystal. After 1 hour of soaking at RT, the crystal was mounted onto a MicroLoop (MiTeGen) and transferred through a cryoprotecting solution consisting of reservoir conditions

containing dissolved xylohexaose powder and 20% w/v PEG400, before flash freezing in liquid nitrogen. Data for structure determination was collected at 100 K at the BioMAX beamline at MAX IV (Lund, Sweden)⁴¹, using MXCube3⁴² data collection software and IspyB/Exi⁴³ monitoring and management software. The data was processed and scaled using XDS/XSCALE⁴⁴ (Version Jan 19, 2025) (see Supplementary Material Table S2 for data collection statistics). Phases for structure solution were obtained using molecular replacement using PHASER (1.21_5207), using the alphafold3^{39,45} model of PnAA17D. Subsequent refinement was done by alternating restrained refinement in phenix.refine and manual rebuilding in Coot (0.9.8.92)⁴⁶. Further validation of protein and sugar geometry was performed using MolProbity (4.5.2) in the phenix suite, phenix.polder for generation of the omit map for xyloetraose, and additional analyses were performed in PyMOL (v3.1.6.1, Schrödinger).

NMR

For NMR spectroscopic analysis of enzyme function, the samples of substrate and of a post-reaction mixture treated with AA-17 were lyophilised and redissolved in 500 mL D₂O. All NMR spectra were acquired using a Bruker Avance III HD 800 MHz spectrometer equipped with a TCI cryoprobe and an Ascend Magnet (18.7 T) at 40°C. The acquired NMR spectra for the post-reaction mixture included homonuclear 2D ¹H-¹H spectra, where ¹H-¹H TOCSY was acquired as a data matrix of 2048×256 complex data points that sampled 320 ms and 40 ms in the direct and indirect dimension of the 2D spectrum, respectively, while a multiple quantum filtered ¹H-¹H COSY was acquired as 2048×512 complex data points that sampled 212 ms and 53 ms in the direct and indirect dimensions, respectively. Heteronuclear ¹H-¹³C HMBC using a three-fold low-pass J-filter to suppress one-bond correlations (2048×256 complex data points sampling 197 ms and 5.8 ms, respectively, and employing non-uniform sampling of 30% of the data points in the ¹³C dimension), ¹H-¹³C HSQC using multiplicity editing (1024×256 complex data points sampling 80 ms and 13 ms, respectively, and employing non-uniform sampling of 50% of the data points in the ¹³C dimension), ¹H-¹³C H2BC (1024×128 complex data points sampling 128 ms and 5 ms, respectively, and employing non-uniform sampling of 50% of the data points in the ¹³C dimension), and ¹H-¹³C HSQC-TOCSY (1024×256 complex data points sampling 98 ms and 13 ms, respectively, while employing non-uniform sampling of 30% of the data points in the ¹³C dimension) were recorded. For the starting material, ¹H-¹³C HSQC and ¹H-¹³C HMBC spectra were acquired with the same acquisition parameters. All NMR spectra were processed with zero-filling to at least double the number of the acquired data points, baseline corrected and analysed using Bruker Topspin 4.1.3 software.

Declarations

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Author contributions

Conceptualisation: SF, ASM. Methodology: SF, FS, TT, JH, SM. Data analysis: SF, FS, TT, JH, SM, BH, ASM. Writing: SF, FS, TT, ASM. Funding acquisition: ASM.

Competing interests

The authors declare no competing interests

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Figures

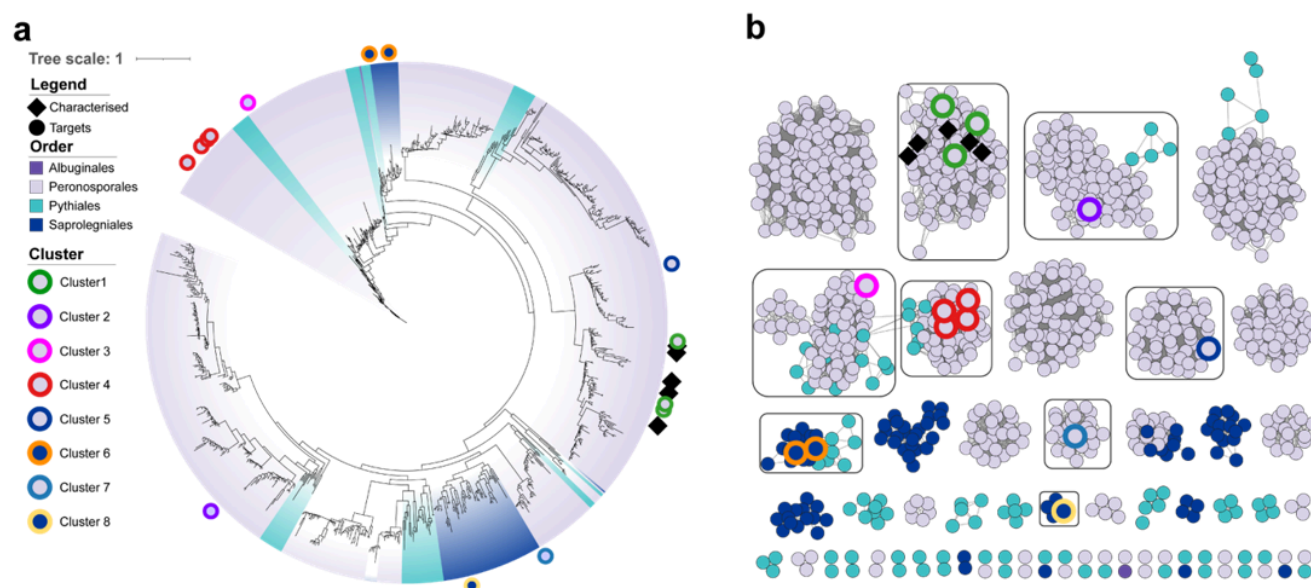


Figure 1

Phylogenetic analysis and sequence-based clustering of oomycete AA17 catalytic domains. A total of 1015 putative AA17 protein sequences were retrieved based on curated CAZy AA17 entries and expanded with BLASTp searches. Sequences were trimmed to the predicted catalytic domain, and redundant sequences were removed using CD-hit²³. **a** A multiple sequence alignment was generated with MAFFT²⁴, and a phylogenetic tree was created with IQ-tree with 1000 bootstraps²⁵ and visualised in iTOL (Tree with bootstrap values in Supplementary Material Fig. S2). **b** Sequence similarity networks (SSNs) were generated from the full curated dataset, and closeness-centrality was used to evaluate group partitioning to select an E-value cutoff 10^{-77} . Tree clades and SSN nodes are coloured according to

oomycete order. Previously characterised AA17 enzymes are shown as diamonds while targets selected for experimental characterisation are shown as circles with coloured borders by clade.

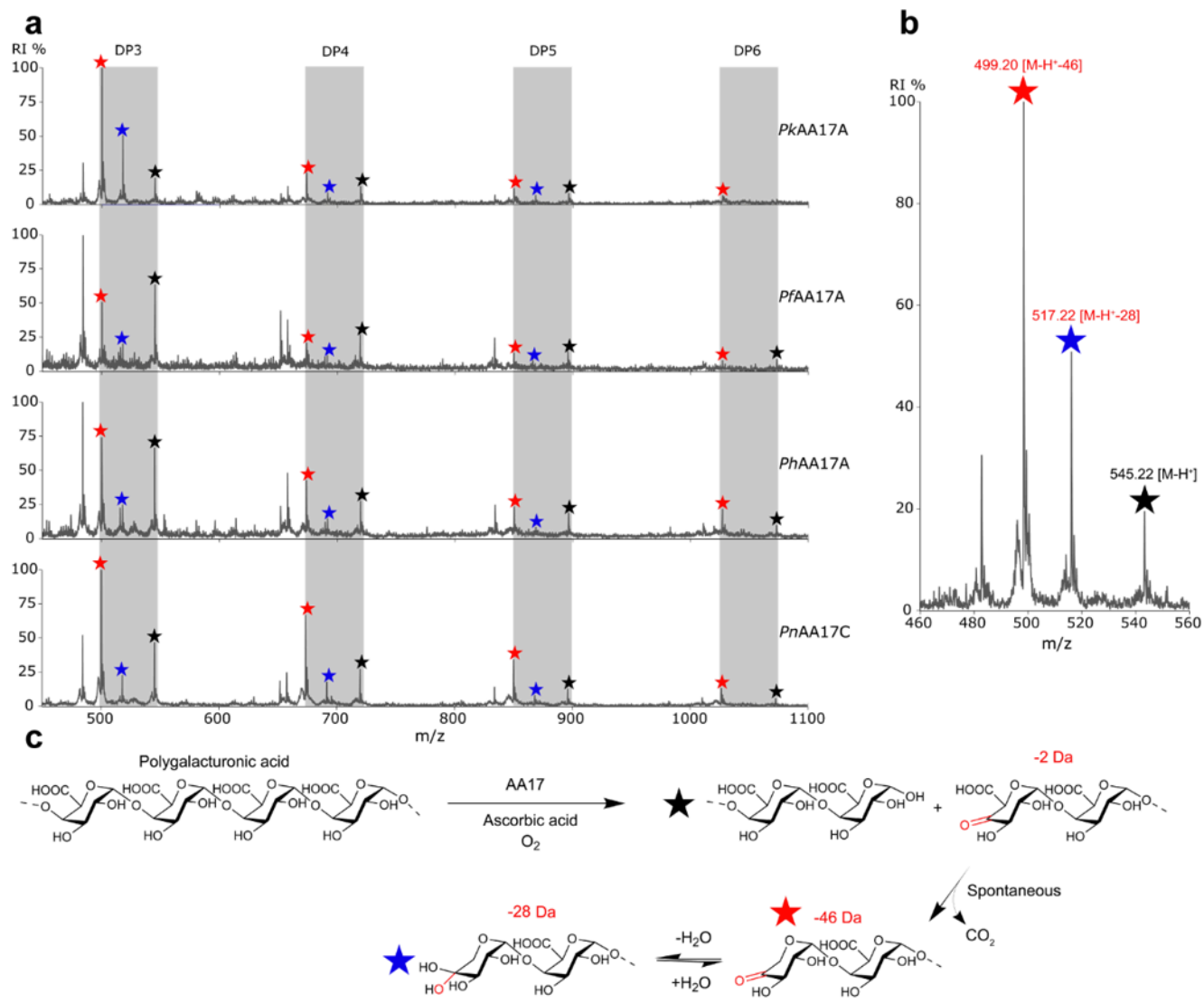


Figure 2

MALDI-TOF Spectrum (negative mode) on polygalacturonic acid. Reaction with 1 μM enzyme, 1 mM Ascorbic acid and 3 $\text{g}\cdot\text{L}^{-1}$ polygalacturonic acid in 50mM sodium acetate pH 6.0 after incubation for 24 hours. **a** Mass spectra in 450-1100 m/z for PkAA17A, PfAA17A, PhAA17A, PnAA17A and polygalacturonic acid control. Species are indicated by colour: native oligosaccharide indicated by black star, oxidized species indicated by red star, and hydrated product by blue star. DP ranges are shown in grey and all spectra are shown as relative intensity. **b** Detailed mass spectrum for DP3 of PkAA17A with oxidised product 499.20 m/z hydrated product 517.22 m/z and native oligosaccharide 545.22 m/z **c** Proposed reaction mechanism of polygalacturonic acid with mass differences related to the native oligosaccharide (black). Native [M-H⁺] (black), oxidised [M-H⁺-46] (red), and hydrated [M-H⁺-28] (blue) are

represented as coloured stars, indicating mass spectrum relation, while mass differences in Da are indicated in red.

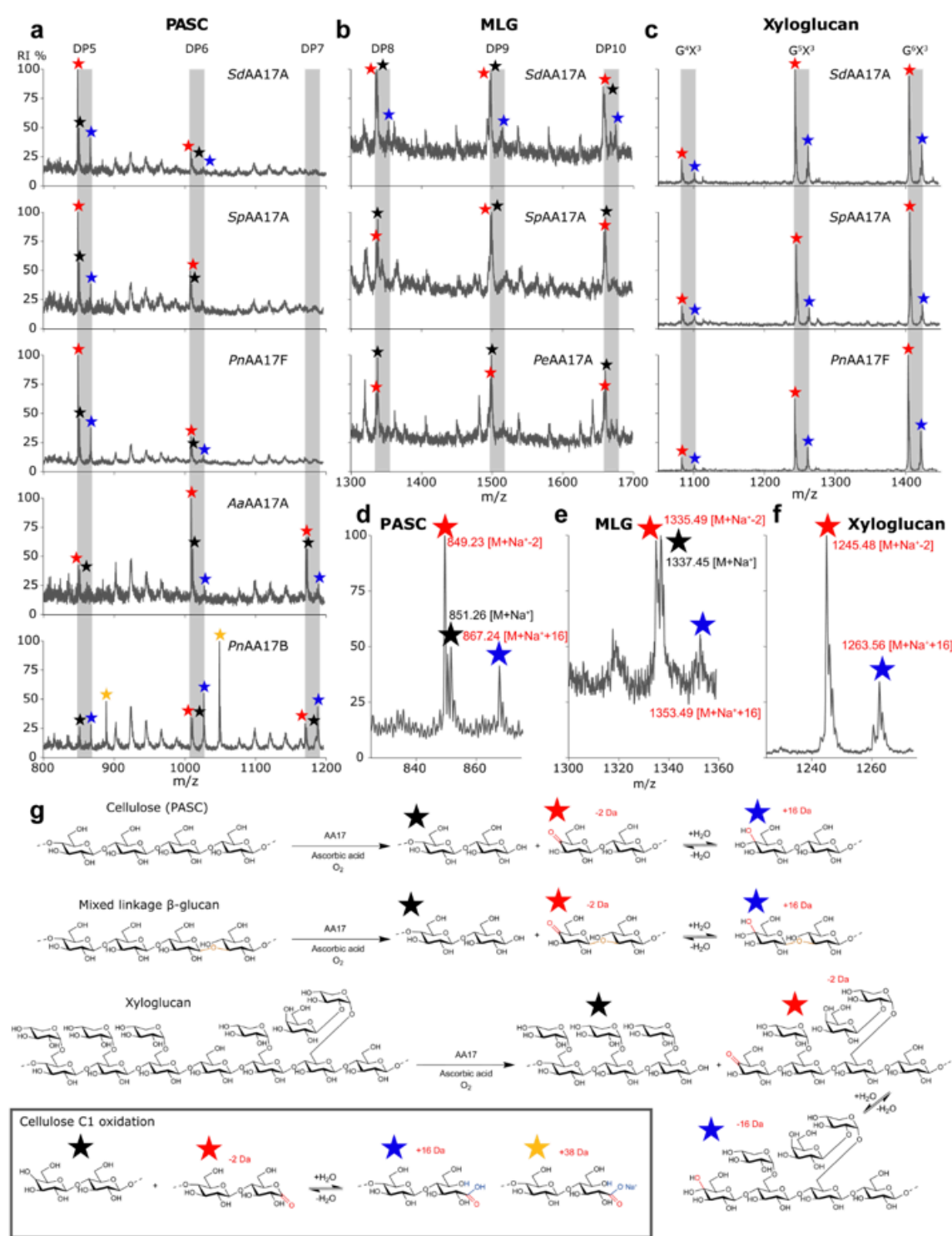


Figure 3

MALDI-TOF Spectrum (positive mode) on β-linked glucans with SdAA17A. Reaction with 2 μM SdAA17A, 4 mM Ascorbic acid and 5 g·L⁻¹ PASC, xyloglucan or β-glucan in 50mM sodium acetate pH 6

at 30°C after 24 hour incubation. **a** Mass spectra of enzymes incubated with PASC in range 800-1200 m/z. **b** Mass spectra for reactions on mixed linkage β -glucan in the range 1300-1700 m/z. **c** Mass spectra for reactions on xyloglucan in the range 1050-1450 m/z, DP denoted with G=Glucose/Galactose and X=xylose with number of units in superscript. **d** Mass spectrum for the reaction product formed by SdAA17A acting on PASC DP5 with masses: 849.23 m/z (oxidised), 851.26 m/z (native), and 867.24 m/z (hydrated). **e** Mass spectrum for the reaction product formed by SdAA17A acting on mixed linkage β -glucan DP8 with masses: 1345.49 m/z (oxidised) , 1337.45 m/z (native), and 1353.49 m/z (hydrated). **f** Mass spectrum for the reaction product formed by SdAA17A acting on xyloglucan G⁵X³ showing the masses: 1245.48 m/z (oxidised) and 1263.56 m/z (hydrated). **g** Proposed reaction mechanisms for cellulose, mixed linkage β -glucan and xyloglucan with adducts indicated by coloured stars: Native [M+Na⁺] (black), oxidised [M+Na⁺-2] (red), hydrated [M+Na⁺+16] (blue), aldonic acid sodium adduct [M+Na⁺+38] (yellow).

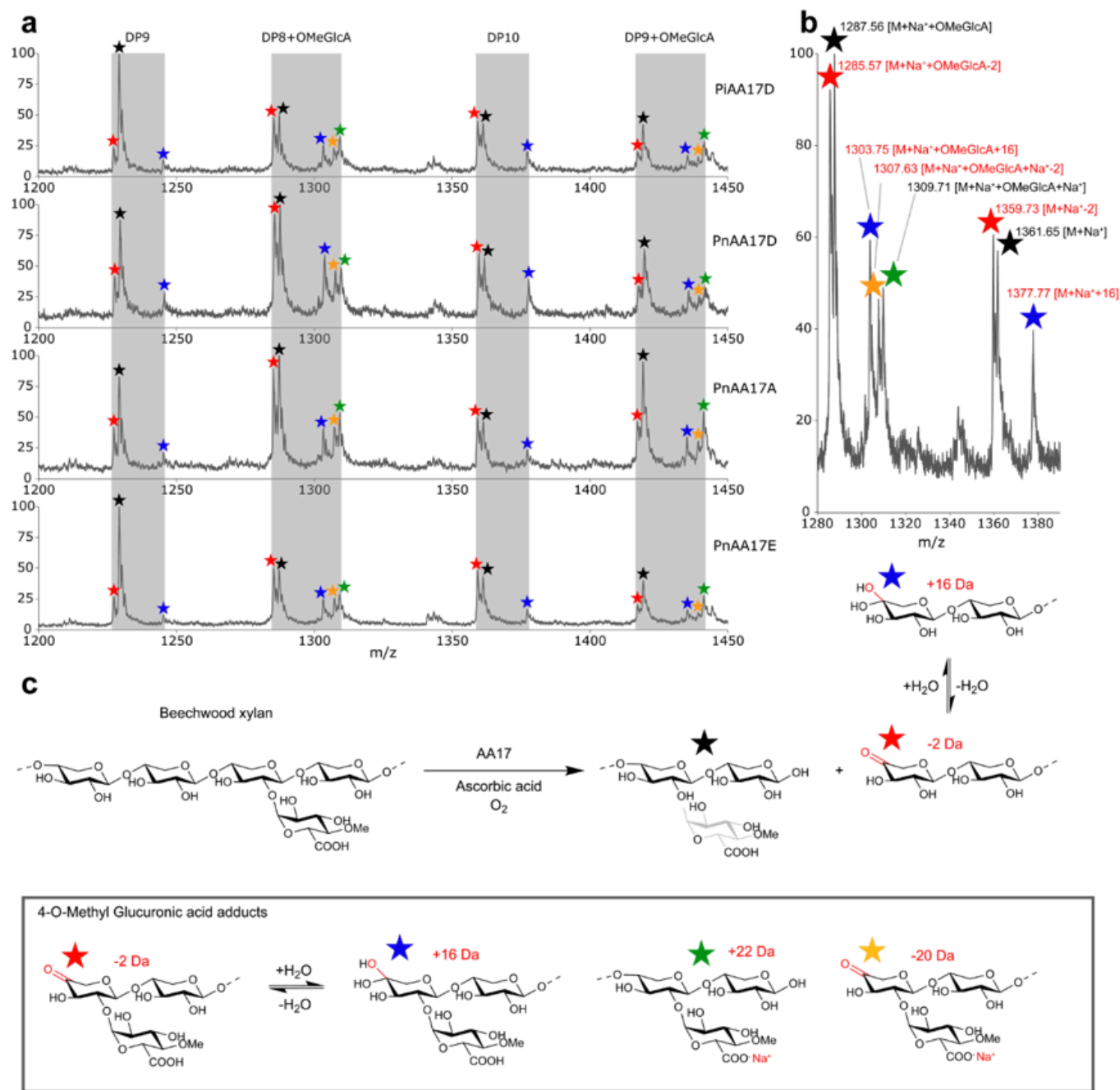


Figure 4

MALDI-TOF Spectrum (positive mode) on beechwood. Reaction with 2 μ M enzyme, 4 mM ascorbic acid and 5 g·L⁻¹ beechwood xylan in 50mM sodium acetate pH 6 at 30°C after incubation for 24 hours. **a** Mass spectra of enzyme reactions incubated with beechwood xylan in range 1200-1450 m/z. **b** Zoom of PnAA17D in the range 1280-1390 m/z. **c** Proposed reaction mechanism for cleavage of beechwood xylan. 4-O-Methyl glucuronic acid adducts indicated by colored stars: native [M+Na⁺] (black), oxidised [M+Na⁺-2] (red), hydrated [M+Na⁺+16] (blue), native sodium adducts [M+Na⁺+22] (green), oxidised sodium adduct [M+Na⁺+20] (yellow).

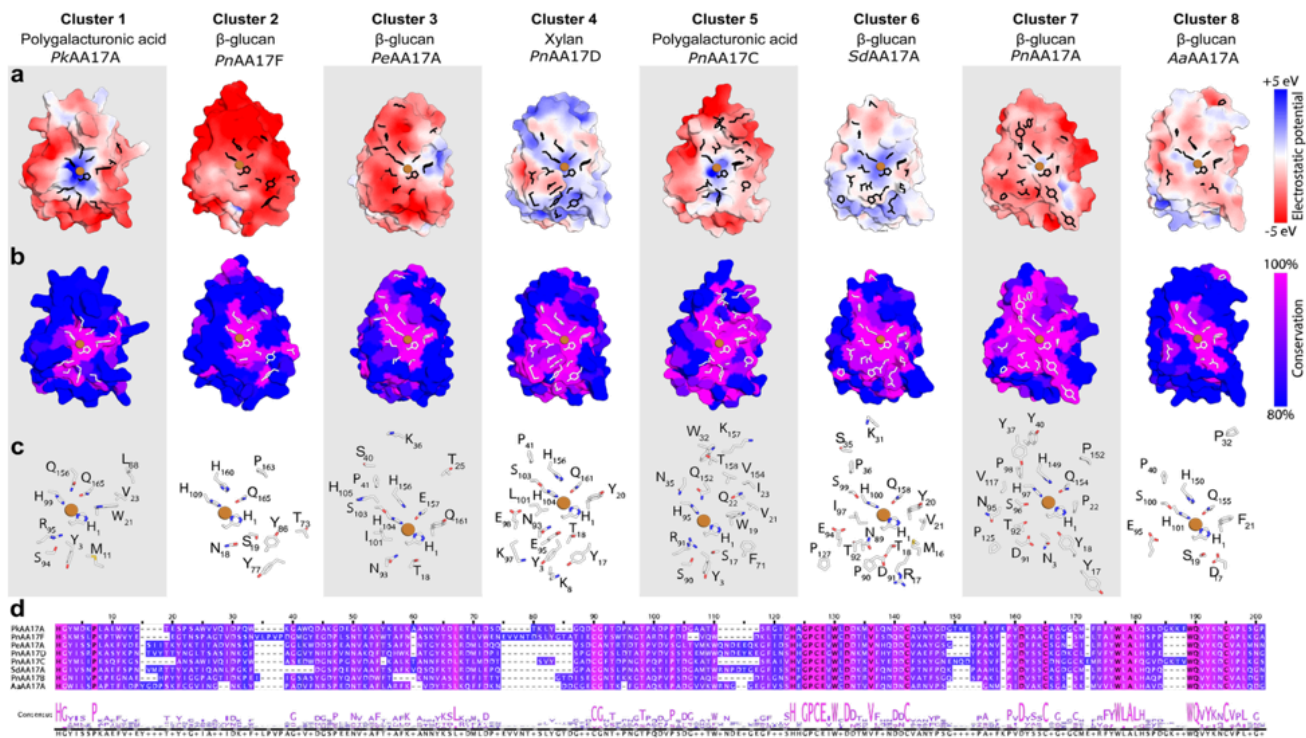


Figure 5

Structural comparison of the eight selected clusters in the AA17 family. For each cluster in the SSN, the members were used to generate cluster-wide multiple sequence alignments (Supplementary Material Fig. S16-24) to investigate the structural features of the eight different clusters. **a** Electrostatic surface potential of the clusters coloured from negative (red) to positive (blue). **b** Surface residue conservation analysis of the eight clusters. Surface area patches are coloured by conservation from 80-100% within their respective clades from blue to magenta. **c** Highlighted binding surface residues >95% conserved. Orange sphere is the Cu^{2+} centre. **d** Multiple sequence alignment of the eight representative LPMOs from each cluster.

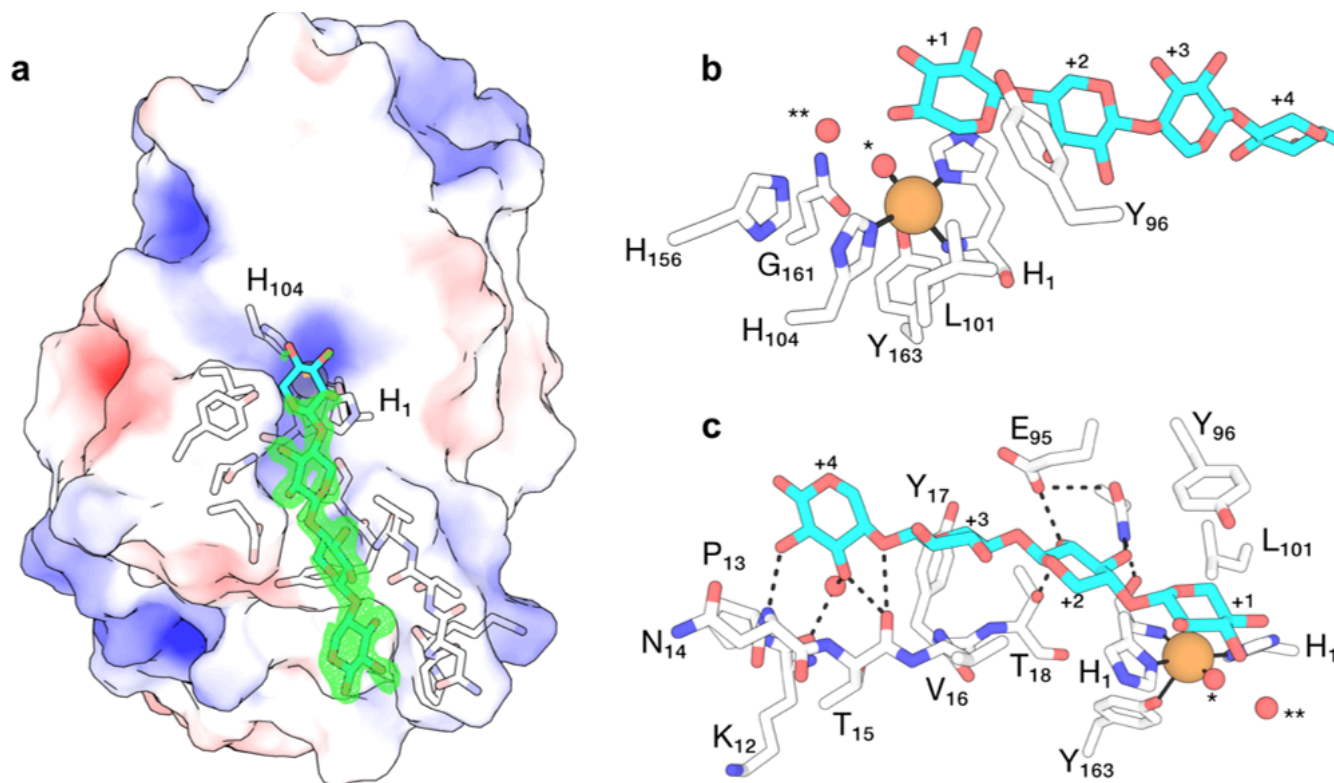


Figure 6

Structural analysis of PnAA17D in complex with xylan. X-ray crystallography structure of PnAA17D in complex xylan introduced by soaking. **a** Electrostatic surface potential of PnAA17D with ligand xylotetraose in cyan and mF_o-DF_c omit electron density map in green mesh at 3.0 σ , generated by omitting the displayed sugars from the model. Residues in proximity to the ligand (4Å) are shown through the surface. **b** Closeup of the active site, showing residues and subsites around the His-brace. Two distinct water molecules (labelled * and **) are found in the axial position of the Cu, with distance Cu–*, and between Cu and other coordinating ligands indicative of a Cu²⁺ binding site³⁸. **c** Polar interactions between the bound xylotetraose and PnAA17D.

Supplementary Files

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