Oxidative plant cell-wall processing enzymes are of great importance in biology and biotechnology. Yet, our insight into the functional interplay amongst such oxidative enzymes remains limited. Here, a phylogenetic analysis of the auxiliary activity 7 family (AA7), currently harbouring oligosaccharide flavo-oxidases, reveals a striking abundance of AA7-genes in phytopathogenic fungi and Oomycetes. Expression of five fungal enzymes, including three from unexplored clades, expands the AA7-substrate range and unveils a cellooligosaccharide dehydrogenase activity, previously unknown within AA7. Sequence and structural analyses identify unique signatures distinguishing the strict dehydrogenase clade from canonical AA7 oxidases. The discovered dehydrogenase directly is able to transfer electrons to an AA9 lytic polysaccharide monooxygenase (LPMO) and fuel cellulose degradation by LPMOs without exogenous reductants. The expansion of redox-profiles and substrate range highlights the functional diversity within AA7 and sets the stage for harnessing AA7 dehydrogenases to fine-tune LPMO activity in biotechnological conversion of plant feedstocks.
The involvement of oxidative processes in polysaccharide degradation by fungi has been proposed by the pioneering work of Eriksson et al. in 1974. This notion has gained strong support by the recent discovery of lytic polysaccharide monoxygenases (LPMOs) that uniquely catalyse the oxidative cleavage of glycosidic bonds in (semi)crystalline polysaccharides such as starch2–4, chitin5, cellulose6–8 and cellulose-bound hemicelluloses, e.g., xylologucan9 and xylan10. Besides LPMOs, filamentous fungi co-secrete an impressing diversity of carbohydrate-specific oxidoreductases11,12. To date, only four fungal oligosaccharide-oxidising enzymes from the auxiliary activity family 7 (AA7) in the Carbohydrate Active enZyme (CAZy) database12, have been characterized. In addition, oligosaccharide oxidases from plants have been reported13,14, but are currently not assigned into AA7. Our insight is, thus, clearly limited regarding the biological roles as well as the diversity of substrates and redox features within this family. Fungal AA7 enzymes catalyse the oxidation of the reducing end C1-OH in e.g., cellobiose oligosaccharides15 and lactose16, xylooligosaccharides17 as well as chitooligosaccharides18 to the corresponding lactones. Electrons derived from oligosaccharide oxidation reduce the FAD cofactor that is subsequently re-oxidised via electron transfer to O2 to generate H2O2 (oxidase activity). Notably, comparable dehydrogenase and oxidase activities have been observed for an AA7 enzyme via electron transfer to artificial electron acceptors instead of O216.

The tertiary structures of hitherto described AA7 enzymes15–17,19 share a common fold comprising an N-terminal FAD-binding domain (F domain) and a C-terminal substrate-binding domain (S domain). This fold is common within the vanillyl alcohol oxidase (VAO, EC. 1.1.3.38) super family20 that harbours AA7. All previously characterised AA7 oxidases are distinguished by a cysteiny1 and histidyl bi-covalently tethered FAD cofactor. By contrast, other VAO family members harbour mostly a monocovalently (or less commonly a non-covalently) bound FAD cofactor20.

Reactive oxygen species (ROS), and especially H2O2, play important roles in lignocellulose degradation by fungi, but the underpinning molecular details of these roles remain poorly understood11. Although O2 has long been considered as the co-substrate of LPMOs57, recent findings suggest that H2O2 is the more favourable co-substrate during polysaccharide oxidative cleavage21–23. LPMO catalysis is mediated by a Cu cofactor6 that must be reduced from Cu(II) to Cu(I) to prime the reaction. Enzymatic priming of LPMOs by the modular pyrroloquinoline-quinone-dependent pyrano dehydrogenase24 (CAZy family AA12 dehydrogenase domain appended to an AA8 cytochrome b haem domain) and FAD-dependant glucose-methanol-choline (GMCh) superfamily of oxidoreductases (AA3), most notably the fungal cellobiose dehydrogenase (CDH) that also possesses a cytochrome b haem domain25–27, has been reported. The activity on cellobiose oligosaccharides and the transcriptional co-regulation as well as the co-secretion with cellulose active AA9 LPMOs28,29, justified extensive studies on CDH as a model for direct electron transfer and priming of LPMOs25,27,29. Not all fungi possess the CDH/LPMO pair, suggesting the presence of additional redox partners and mechanisms for LPMO activation. By analogy, the co-secretion of AA7s with LPMOs upon fungal growth on plant biomass30,31 prompted us to hypothesise a redox interplay between these two enzyme classes.

Here, we report phylogenetic analyses, combined with the selection and characterisation of five fungal AA7s, three of which belong to previously unexplored clades. We demonstrate activity on four saccharides, previously not reported as AA7 substrates. In-depth analysis of a cellobiose oligosaccharide dehydrogenase with a mono histidyl-tethered FAD highlights clade-dependant redox-profiles within AA7. Importantly, we unveil direct activation and potentiation of LPMO activity on cellulose by this newly-discovered dehydrogenase. This study provides biochemical, structural and mechanistic insights into AA7 enzymes as components of the fungal redox network secreted during growth on biomass. Our findings suggest a way to tune LPMO activity for enzymatic degradation of major recalcitrant polysaccharides using AA7 dehydrogenases.

**Results**

Clade-dependant variations in the FAD covalent tethering residues in AA7. To date, the molecular specificity signatures in AA7s have not been unveiled. To explore the sequence diversity in this family, we used the sequence of the previously characterised *Fusarium graminearum* chitooligosaccharase oxidase *FgChito*16 as a query to retrieve sequences comprising 470–570 amino acids (aa) from a BLAST search. The sequences (n = 1927), originating from fungi, eukaryotic microorganisms and plants, were aligned and curated to generate a phylogenetic tree formed by six clades (Fig. 1a). Clade I, which is the largest (34% of all sequences), is dominated by plant and fungal sequences (Fig. 1b). Indeed, this clade contains plant non-carbohydrate active enzymes, e.g., the berberine bridge enzyme from *Eschscholzia californica* EcBBE32 and the monolignol oxidase from *Arabidopsis thaliana* AtBBE-like 1533, as well as the oligo-galacturonide oxidase from *A. thaliana* AtOGOX13, although none of these sequences are currently assigned into AA7.

The sequences in clades Ia, II and IV are mainly from Ascomycota, whereas mostly Basidiomycota sequences populate clades IIb and VI. Remarkably, the majority of the retrieved Ascomycota sequences were from genera known to harbour phytopathogens, e.g., *Fusarium, Magnaporthe, Colletotrichum, Bipolaris, Alternaria* or *Botrytis*. The enrichment of phytopathogen sequences is striking in clade II. Thus, fungus-like eukaryotic plant pathogens from Oomycota clustered in clade Iib (90.5% of sequences), whereas the remaining sequences in clade II originate mainly from Ascomycota phytopathogens. All the four previously described AA7 fungal oligosaccharide oxidases clustered in a branch of clade V (Ya) (Fig. 1a). The scarce insight into AA7 is evident from the lack of characterized members from (sub)clades II, III, IV, Vb and VI.

Since bi-covalent FAD tethering has been the hallmark of all hitherto characterised AA7s, we analysed the conservation of the cysteine and histidine FAD-tethering residues across the phylogenetic tree. Strikingly, clade II harboured exclusively non-canonical sequences with substitution of the FAD-binding cysteine, histidine or both, while these residues were highly conserved in the other clades (Fig. 1c). We selected five AA7 candidates belonging to different clades (Fig. 1a, Supplementary Table 1) for recombinant expression and functional characterization.

**Identification of a strict oligosaccharide dehydrogenase in AA7.** The selected fungal sequences share 25–39% amino acid sequence identity and originate from *Aspergillus nidulans* (AnaA7A, clade I), *Fusarium graminearum* (*FgCelD7H7C*, clade IIa and FgChi7B, clade Va), *Magnaporthe oryzae* (*MoCh7A*, clade Vb) and *Polyporus brunalis* (*PbChi7A*, clade VI) (Supplementary Table 1). The selected AA7 enzymes were successfully expressed in *Pichia pastoris* and highly pure enzymes were obtained from a single affinity purification. The substrate specificity of each recombinant enzyme was assessed against a panel of 40 compounds including saccharides with a degree of polymerization (DP) 1–4 as well as sugar alcohols and aromatic alcohols (Supplementary Table 2) by monitoring both their oxidase activity (i.e. H2O2 production using a peroxidase coupled assay) and dehydrogenase activity. The tertiary structures of hitherto described AA7 enzymes15–17,19 share a common fold comprising an N-terminal FAD-binding domain (F domain) and a C-terminal substrate-binding domain (S domain). This fold is common within the vanillyl alcohol oxidase (VAO, EC. 1.1.3.38) super family20 that harbours AA7. All previously characterised AA7 oxidases are distinguished by a cysteine and histidyl bi-covalently tethered FAD cofactor. By contrast, other VAO family members harbour mostly a monocovalently (or less commonly a non-covalently) bound FAD cofactor20. Reactive oxygen species (ROS), and especially H2O2, play important roles in lignocellulose degradation by fungi, but the underpinning molecular details of these roles remain poorly understood11. Although O2 has long been considered as the co-substrate of LPMOs57, recent findings suggest that H2O2 is the more favourable co-substrate during polysaccharide oxidative cleavage21–23. LPMO catalysis is mediated by a Cu cofactor6 that must be reduced from Cu(II) to Cu(I) to prime the reaction. Enzymatic priming of LPMOs by the modular pyrroloquinoline-quinone-dependent pyrano dehydrogenase24 (CAZy family AA12 dehydrogenase domain appended to an AA8 cytochrome b haem domain) and FAD-dependant glucose-methanol-choline (GMCh) superfamily of oxidoreductases (AA3), most notably the fungal cellobiose dehydrogenase (CDH) that also possesses a cytochrome b haem domain25–27, has been reported. The activity on cellobiose oligosaccharides and the transcriptional co-regulation as well as the co-secretion with cellulose active AA9 LPMOs28,29, justified extensive studies on CDH as a model for direct electron transfer and priming of LPMOs25,27,29. Not all fungi possess the CDH/LPMO pair, suggesting the presence of additional redox partners and mechanisms for LPMO activation. By analogy, the co-secretion of AA7s with LPMOs upon fungal growth on plant biomass30,31 prompted us to hypothesise a redox interplay between these two enzyme classes.

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activity using 2,6-dichlorophenolindophenol (DCIP) as an artificial electron acceptor.

FgChi7B (clade Va), MoChi7A (clade Vb) and PbChi7A (clade VI) displayed the highest normalised rates ($V_o/E$) on chitooligosaccharides, followed by $N$-acetyl glucosamine (GlcNAc) (Supplementary Fig. 1). Importantly, we also discovered oxidase activity against substrates not reported before in AA7. Thus, MoChi7A was active on $N$-acetyl galactosamine (GalNAc), lacto-$N$-biose (LNB) and galacto-$N$-biose (GNB), while PbChi7A oxidised mannoooligosaccharides (Supplementary Table 3, Supplementary Fig. 1). The $K_M$ values of MoChi7A and PbChi7A towards GlcNAc were about 20-fold lower compared to FgChi7B (Table 1), highlighting a marked difference in affinity for monosaccharides between these enzymes. No activity was detected for AnAA7A on any of the tested substrates, suggesting that AA7 targets a wider range of substrates than currently reported. The pH-dependence of activity profiles showed the highest relative activities in the pH range 7.0 – 7.5, except for PbChi7A, which appeared to have a broader profile (Supplementary Fig. 2).

Apart from FgCelDH7C (vide infra), the dehydrogenase activity for a given substrate was in the same range or higher...
(up to 12-fold) than the corresponding oxidase activity, and the substrate specificity profiles were mostly similar using both assays (Table 1, Supplementary Fig. 1). By contrast, FgCelDH7C acted as a dehydrogenase with a preference for cellobiose/saccharides with \( k_{\text{cat}} \) values of 32–42 s\(^{-1}\) and the highest catalytic efficiency on cellobiose (Table 1, Supplementary Fig. 1h). The enzyme was also active on glucose, α-(1,4)-glucoligos (maltoligosaccharides) and lactose, all sharing a reducing end glucosyl unit. Interestingly, the oxidase activity was estimated to be several orders of magnitude lower (\( V_0/E = 1 - 10^{-11} \text{ s}^{-1} \)) (Supplementary Fig. 1g, h). This unusually higher dehydrogenase/oxidase activity ratio compared to canonical AA7s distinguishes FgCelDH7C as an AA7 dehydrogenase with only trace oxidase activity. Interestingly, FgCelDH7C had a markedly different flavin absorbance spectrum than typical oxidase homologues (Fig. 2a), suggestive of large changes in the FAD chemical environment. The stability of the enzyme was also evaluated to verify the structural integrity (unfolding temperature \( T_{\text{m}} > 55 \text{ °C} \), Supplementary Fig. 2b). Both MoChi7A, which catalyses efficient oxidation of substrates not reported within AA7, and the strict dehydrogenase FgCelDH7C were selected for further in situ NMR spectroscopy to bring insight into their redox chemistry. Initial experiments identified the chemical shift assignments of sub- strates, intermediates and products based on two-dimensional \(^1\text{H},^{13}\text{C}\) HSQC NMR spectra were acquired to follow the time-course conversion of GlcNAC and GalNAC by MoChi7A, showing the initial formation of a 1,5-pyranol (δ) lactone prior to rearrangement to a 1,4-furano (γ) lactone. The carboxylic acid open form was the main product of GlcNAC oxidation (due to lactone hydrolysis), whereas the 1,4-furano (γ) lactone accumulated as the main product of GalNAC oxidation (Supplementary Fig. 4). Similarly, assignments were established for the oxidation of cellobiose by FgCelDH7C (Supplementary Fig. 5). A time series of one-dimensional \(^1\text{H}\) NMR spectra was used for kinetic analysis of the FgCelDH7C catalysed oxidation of cellobiose in the presence of DCIP that displayed exchange line broadening during the reaction (Supplementary Fig. 6a). Line shape analysis showed that oxidised DCIP was reduced in a slow to intermediate exchange regime (exchange rate \( \approx 87 \text{ s}^{-1} \)) (Supplementary Fig. 6b). Interestingly, the sharp signals of reduced DCIP were only observed upon stoichiometric conversion of the oxidized DCIP form and hydrolysis of the lactone to the acid (Supplementary Fig. 6a), most likely due to the irreversible hydrolysis breaking the equilibrium. Notably, the oxidase reaction with O2 as an electron acceptor occurred orders of magnitude slower in the absence of DCIP as electron acceptor (Fig. 2b, c), corroborating the activity data from the coupled peroxidase assay above. Further NMR analyses and activity assays validated the dependence of cellobiosaccharide oxidation on DCIP concentration (Supplementary Fig. 7).

### Table 1: Apparent kinetic parameters of AA7-catalysed oxidation of saccharides.

| Oxidase kinetics | Substrate | Parameter | Enzyme | MoChi7A | PbChi7A | FgChi7B |
|------------------|-----------|-----------|--------|---------|---------|---------|
| GlcNAC | \( k_{\text{cat}} (s^{-1}) \) | 3.7 ± 0.1 | ND | ND |
| | \( K_m (mM) \) | 1.69 ± 0.12 | ND | ND |
| GlcNAc | \( k_{\text{cat}}/K_m (s^{-1} \text{ M}^{-1}) \) | 2.16 × 10^3 | 3.0 ± 0.2 | 6.8 ± 0.3 |
| | \( k_{\text{cat}} (s^{-1}) \) | 3.3 ± 0.2^a | 3.3 ± 0.2^a |
| | \( K_m (mM) \) | 0.80 ± 0.05 | 0.69 ± 0.20 | 15.7 ± 1.13 |
| | \( k_{\text{cat}}/K_m (s^{-1} \text{ M}^{-1}) \) | 4.75 × 10^3 | 4.27 × 10^3 | 4.30 × 10^2 |
| | \( k_{\text{cat}} (s^{-1}) \) | 4.4 ± 0.1 | 3.8 ± 0.04 | 2.3 ± 0.03 |
| Chitobiose | \( K_m (mM) \) | 0.064 ± 0.01 | 0.3 ± 0.02 | 0.27 ± 0.01 |
| | \( k_{\text{cat}}/K_m (s^{-1} \text{ M}^{-1}) \) | 0.33 ± 0.03^a | 1.32 ± 0.07 |
| | \( k_{\text{cat}} (s^{-1}) \) | 6.83 × 10^4 | 1.27 × 10^4 | 8.58 × 10^3 |
| | \( K_m (mM) \) | 5.56 × 10^4 | 10^-1 |
| | \( k_{\text{cat}}/K_m (s^{-1} \text{ M}^{-1}) \) | 1.69 ± 0.12 | ND | ND |
| | \( k_{\text{cat}} (s^{-1}) \) | 3.25 ± 0.10^a | 6.8 ± 0.3 |
| Chitotriose | \( K_m (mM) \) | 0.09 ± 0.01 | 0.28 ± 0.01 | 0.25 ± 0.03 |
| | \( k_{\text{cat}}/K_m (s^{-1} \text{ M}^{-1}) \) | 4.22 × 10^4 | 1.25 × 10^4 | 2.67 × 10^3 |
| | \( k_{\text{cat}} (s^{-1}) \) | 2.0 ± 0.16^a | 0.75 ± 0.13 |

*Apparent kinetic parameters using the dehydrogenase assay. The kinetic parameters are determined from a global fit of the Michaelis-Menten expression to triplicate \( (n = 3) \) independent experiments, except for cellobiose (\( n = 1 \) experiment). The data are shown as means ± standard deviations. For cellobiose the error estimates of the fit to the single data set are shown.

### Table 2: Dehydrogenase kinetic parameters of FgCelDH7C.

| Table 2: Dehydrogenase kinetic parameters of FgCelDH7C. | Substrate | Cellobiose | Cellobiotriose | Cellobiotetraose | Cellopentaose |
|-------------|-----------|------------|----------------|-----------------|--------------|
| \( k_{\text{cat}} (s^{-1}) \) | 30.8 ± 0.81 | 36.1 ± 0.74 | 39.27 ± 0.76 | 45.28 ± 2.03 |
| \( K_m (mM) \) | 5.35 ± 0.39 | 3.94 ± 0.22 | 6.06 ± 0.27 | 11.25 ± 0.94 |
| \( k_{\text{cat}}/K_m (s^{-1} \text{ M}^{-1}) \) | 5757 | 9162 | 6480 | 4025 |

*Structural elements underpinning the dehydrogenase/oxidase activity profiles. We determined the crystal structures of FgChi7B and FgCelDH7C at resolutions of 2.4 and 1.6 Å, respectively (Supplementary Table 4, Supplementary Fig. 8). Both enzymes share the canonical AA7 fold comprising an FAD-binding F domain and a substrate-binding S domain that is formed by a central β-sheet flanked by α-helices (Supplementary Fig. 9a, b). Moreover, the two enzymes share an aromatic cluster comprising: (i) the catalytic base tyrosine (FgCelDH7C Y454), (ii) a tyrosine/phenylalanine (FgCelDH7C F99) that stacks onto and stabilises the catalytic base, and (iii) an aromatic residue that stacks mainly onto the saccharide unit permutate to the reducing end (FgCelDH7C F383) (Fig. 3a). Interestingly, the residues of this aromatic cluster are also highly conserved in most sequences in clades IIa and Va (Fig. 3b), highlighting their importance in catalysis and substrate binding.

By contrast, the structure of FgCelDH7C revealed a unique active site architecture, as compared to all hitherto available AA7 structures. The first notable overall structural difference is that a loop (residues Q335-F346) that flanks the active site in FgCelDH7C appears shorter due to a preceding helical segment that folds away from the active site, which provides a less occluded and more solvent-exposed FAD-cofactor compared to FgChi7B and all hitherto reported AA7 structures (Supplementary Fig. 9).

Strikingly, the mode of FAD-anchoring represents a second unique characteristic distinguishing FgCelDH7C from the other AA7 structures. Thus, the isoalloxazine ring of the FAD cofactor in FgChi7B is bi-covalently anchored at the C6 atom to C162 SY (6-S-cysteinyl) and the 8a-methyl group to H102 N61 (8a-N1-histidyl), akin to all previously determined AA7 structures (Supplementary Fig. 9a). By contrast, FgCelDH7C is mono-covalently histidyl-tethered due to the substitution of the FAD-tethering cysteine to a glycine (Fig. 3c). Notably, this cysteine is conserved in the majority of AA7 except in clade II sequences that possess a glycine, an alanine or a serine at this position (Fig. 3b, d, Supplementary Fig. 10). A third structural signature of FgCelDH7C, compared to all available AA7 structures, is the substitution of a histidine residue facing the isoalloxazine ring in the FAD-binding domain to a serine (S165) (Fig. 3c).

A fourth different motif in FgCelDH7C entails four arginine residues that protrude into the substrate-binding pocket (Fig. 3c, d), providing a distinctively positive charged milieu compared to other structurally characterised AA7s (Supplementary Fig. 11).
Fig. 2 Spectral properties and time-course NMR analysis of the AA7 cellooligosaccharide dehydrogenase FgCelDH7C. a Spectral comparison of the flavin absorbance of FgCelDH7C and the oxidases FgChi7A as well as MoChi7A, all at 20 µM. The data are means ± standard deviations (n = 3 independent experiments). b and c Show the time resolved in situ 1H NMR analyses of the oxidation of cellobiase by FgCelDH7C in the presence (b) or absence (c) of 1.3 mM 2,6-dichlorophenolindophenol (DCIP) as an electron acceptor, respectively. The presented spectra in panels b and c are from a single experiment (n = 1). Source data are provided as a Source Data file.

The cellooligosaccharide dehydrogenase FgCelDH7C primes and fuels LPMO activity. To investigate if AA7 enzymes can trigger the activity of LPMOs, we set up AA7-LPMO mediated cellulose degradation assays to monitor the release of native (unmodified) and oxidised cellooligosaccharides from cellulose. The two cellulose-active LPMOs from Podospora anserina PaLPMO9E and PaLPMO9H were used in these assays. The interplay between FgCelDH7C and the C1-oxidising PaLPMO9E was evident from the marked increase in native and C1-oxidised cellooligosaccharides released from phosphoric acid swollen cellulose (PASC) relative to the control reaction, which was fuelled by electrons from ascorbate (Supplementary Fig. 12a). Since FgCelDH7C is inactive on cellulose, the release of oligosaccharides in this assay is solely attributed to the PaLPMO9E activity. However, the generation of C1-oxidised cellooligosaccharides, by both FgCelDH7C and PaLPMO9E, precluded determining the contribution of each enzyme to the total amount of oxidized species. Therefore, a similar assay was also performed with the C4-oxidising PaLPMO9H (instead of PaLPMO9E) as it allowed attributing both C4 and C1/C4 oxidised cellooligosaccharides (identification based on the previous work34) exclusively to the LPMO activity. This assay with PaLPMO9H resulted in a significant increase in single- and double-oxidized oligosaccharides relative to the ascorbate control (Supplementary Fig. 12b). We performed similar assays on Avicel (higher crystallinity cellulose than PASC), which revealed that the addition of the preferred FgCelDH7C substrate cellotetraose (DP4) results in a considerable increase in the level of released cellooligosaccharides in a dose-dependent manner (Supplementary Fig. 13). A similar fuelling of LPMO activity was also observed when the same assay was performed using the previously characterised C4-oxidising AA9 LPMO from Lentinus similis (LsAA9A)35,36 (Supplementary Fig. 14). These findings indicate that the ability of FgCelDH7C to fuel cellulose active LPMOs is not LPMO-specific.

We further used the CDH from P. anserina to benchmark the FgCelDH7C-PaLPMO9H system, as CDHs are recognised as key reduct partners to AA9 LPMOs20,27,34. The observed release of cello-oligomers was markedly higher when the LPMO reaction was fuelled by a 3-fold lower FgCelDH7C concentration compared to PaCDHB (Fig. 4, Supplementary Fig. 15). Indeed, the FgCelDH7C-PaLPMO9H pair released 1.5- and 2-fold higher C1-C4 double-oxidised and native cellooligosaccharides, respectively, as compared to the PaCDHB-fuelled reaction. To evaluate if this interplay was specific for FgCelDH7C, we used the promiscuous oxidase MoChi7A in a similar cellulase degradation assay. Interestingly, the high level of AA7-fuelled LPMO activity appeared specific to FgCelDH7C based on the low amounts of C1- and C1-C4 oxidised species observed in assays where FgCelDH7C was replaced with MoChi7A that displays low cellooligosaccharides oxidase side-activity (Supplementary Fig. 16).

Mechanistic insights into the LPMO-AA7 interplay. To investigate the mechanism of the AA7-LPMO interplay, we analysed the effect of H2O2 or the superoxide species (O2−•) by performing the reaction in the presence of either horseradish peroxidase (HRP) that converts H2O2 to H2O241 or superoxide dismutase...
Fig. 3 Active site signatures of AA7 oligosaccharide oxidases versus dehydrogenases. The active sites of the clade IIa discovered dehydrogenase FgCelDH7C (PDB: 6YJI) and the canonical clade Va cellooligosaccharide oxidase SsGOOX (in complex with 5-amino-5-deoxy-cellobiono-1,5-lactam ABL, PDB: 2AXR) are shown. a The FAD-tethering histidine in addition to an aromatic cluster comprising the tyrosine base catalyst, a phenylalanine/tyrosine and the substrate-stacking aromatic residue are conserved features in fungal oligosaccharide dehydrogenases and oxidases. b Sequence logos of patches spanning the structurally similar active site residues shown in (a). c Active site differences between clades IIa and Va. d Sequence logos of patches spanning the structurally divergent active site residues shown in (c) from clades IIa and Va. The amino acid numbering of the deposited protein sequences is used in the figure.

Fig. 4 Analysis of AA7-LPMO interplay in cellulose degradation. Reactions were performed on Avicel using FgCelDH7C and PaLPMO9H with subsequent ionic chromatography (HPAEC-PAD) analysis. a A representative chromatogram part showing C1-C4 double-oxidised species in the cellulose degradation assay including combinations of Avicel (5 mg mL⁻¹), FgCelDH7C (0.4 µM), PaLPMO9H (4 µM), PaCDHB (1.2 µM), celletetraose (DP4, 0.8 mM) and ascorbate (Asc., 1 mM) as indicated in the figure. b Comparison of the cellulose degradation assay based on the cumulative area under the peaks of native (DP3, DP5 and DP6), C1 oxidised (except DP2 and DP4 which were added as substrates for the CDH and AA7, respectively) and C1-C4 double-oxidised cellooligosaccharides from the reactions in (a). The data in (a) and (b) (n = 3 independent reactions) were generated in NaOAc/NaOH buffer (50 mM, pH 5.2) at 35 °C. The bar plot in (b) shows the means of total peak area (n = 3 independent reactions, each shown as a white circle) with standard deviations. Source data are provided as a Source Data file.
(SOD) that converts superoxide ions\textsuperscript{37} to \(\text{H}_2\text{O}_2\) and \(\text{O}_2\), respectively. The addition of increasing concentrations (5–38 nM) of HRP, reduced the AA7-potentiated LPMO activity (Fig. 5a). These results are consistent with \(\text{H}_2\text{O}_2\), which is supplied by \(\text{FgCelDH7C}\) and by the side-activity of primed LPMOs, being a preferred LPMO co-substrate. In the presence of increasing concentrations (0.07 – 0.5 µM) of SOD\textsuperscript{37}, we observed a similar inhibitory trend (Fig. 5b). These findings suggest that \(\text{O}_2\)^-- plays a specific role in the \(\text{FgCelDH7C}\)-fuelled LPMO reaction (Fig. 5b). Indeed, the priming reduction of LPMOs with the superoxide ion has been previously proposed\textsuperscript{38}. In addition to \(\text{O}_2\)^---mediated priming, we also investigated the possibility of direct priming electron transfer between the AA7 and the LPMO using Electron Paramagnetic Resonance (EPR) spectroscopy. The extent of LPMO-Cu(II) reduction to Cu(I) was monitored as a decrease of the Cu(II) EPR signal due to the silence of LPMO-Cu(I) species in EPR\textsuperscript{25}. The addition of \(\text{FgCelDH7C}\) to \(\text{PaLPMO9H}\) pre-incubated with cellotriose (DP3) under anaerobic conditions led to a decrease (35–40%) in \(\text{PaLPMO9H}\)-Cu(II) signal (Fig. 5c), whereas DP3 alone failed to induce a change in the Cu(II) EPR signal intensity, consistent with the involvement of \(\text{FgCelDH7C}\) in the observed priming of \(\text{PaLPMO9H}\) (Fig. 5d). The fact that only partial reduction of the Cu(II) center was observed could be due to kinetic or thermodynamic barriers. We have previously determined the redox potentials of the LPMOs \(\text{PaAA9E}\) and \(\text{PaAA9H}\) at pH 5 to +155 mV and +326 mV, respectively\textsuperscript{25}. The redox potentials of canonical AA7 oxidases with a bi-covalently anchored-FAD is about +130 mV, whereas mutants that abolish the cysteinyl-FAD bond exhibited redox potentials in the 60 mV range\textsuperscript{39,40}. Accordingly, the reduction of LPMOs by \(\text{FgCelDH7C}\) is likely to be thermodynamically feasible assuming a redox potential in the same range as the abolished cysteinyl-FAD bond mutants. To test the possibility of a direct electron transfer between \(\text{FgCelDH7C}\) and \(\text{PaLPMO9H}\), we used a strategy...
previously developed to analyse electron transfer between redox partner proteins31. We first established that PaLPMO9H-Cu(II) could be efficiently reduced by the chemical reductant dithionite (Fig. 5d). Then, equimolar solutions of FgCelDH7C and PaLPMO9H were prepared anaerobically. The FgCelDH7C solution was pre-reduced with sub-stoichiometric (80%) amounts of dithionite to avoid excess dithionite in the medium. Identical volumes of the two protein solutions were then mixed which resulted in about 50% reduction of the LPMO active site from Cu (II) to the Cu(I) state (Fig. 5d). Altogether, our data are indicative of a molecular oxygen-independent, direct electron transfer from the AA7-bound FAD to the active site of the LPMO.

**Discussion**

This study sheds light on an enigmatic family of flavo-enzymes mainly occurring in fungi and fungi-like eukaryotic microorganisms as well as in plants. Our phylogenetic analysis mapped the four hitherto described AA7 fungal oligosaccharide oxidases together with a large set of mostly uncharacterised orthologues into six clades. The majority of clade I sequences share a highly conserved motif involving the catalytic tyrosine base and a stacking tyrosine (or phenylalanine) that is likely to rigidify the position of the catalytic base (Fig. 3a, Supplementary Fig. 10). Curiously, a few atypical enzymes lack the catalytic tyrosine, e.g., EcBBE from clade I possess a histidine and an adjacent glutamic acid at the equivalent position in the structure. This is in agreement with an alternative mechanism, involving acid/base catalysis previously proposed for EcBBE42. Nonetheless, the conservation of the catalytic motif in most sequences supports the conservation of the canonical mechanism across all AA7 clades. These findings expand significantly the sequence inventory that can be considered as AA7.

The substrate aromatic-stacking platform (Phe, Tyr or Trp) (Fig. 3a, Supplementary Fig. 10), is conserved in all previously and presently described oligosaccharides-active AA7s, in addition to a clade I enzyme that is specific for aromatic alcohols33, indicative of shared substrate-stacking function of the above aromatic platform. By contrast, sequences that lack the aromatic-stacking residue are unlikely to be active on oligosaccharides, e.g., EcBBE, which features in alkaloid biosynthesis32. In conclusion, the above described aromatic-stacking platform offers a signature for the identification of putative carbohydrate-active AA7 members, which are likely to have more polar substrate-binding pockets than counterparts active on aromatic substrates. An active site arginine (FgCelDH7C R273) (Fig. 3c, d) has been previously proposed as a specificity signature of carbohydrate-active AA7 members18. This arginine correlates with activity on substrates that lack an N-acetyl C2-substituent, e.g., celluligosaccharides and xylo-oligosaccharides. A glutamine or smaller amino acids (Fig. 3d) at this position allow the accommodation of N-acetylated substrates, e.g., chito-oligosaccharides consistent with our structural analysis of FgChit7B and the previously characterised FgChitO18,19 (Supplementary Fig. 17).

Interestingly, FgCelDH7C offers structural and functional insight into the previously unknown clade II (Fig. 3b, d), which is exclusively populated with atypical sequences of enzymes with potentially mono-histidyl (clade Ila), mono-cysteinyl or non-covalently bound FAD (clade IIb). The lower (<10,000-fold) oxidative activity of FgCelDH7C compared to canonical AA7s correlated with conspicuous changes in its active site architecture. The loss of cysteinyl-FAD is likely to elicit a large decrease in the midpoint redox potential, as observed in EcBBE39 (clade I) and StGOOX40 (clade Va) when the cysteinyl bond is abolished39. In both cases, the redox potentials decreased from about 130 mV to 53–61 mV accompanied by about 20-fold decrease in oxidase activity. The changes in the flavin absorbance spectrum due to the loss of the cysteinyl-bond in both enzymes were similar to the FgCelDH7C spectrum (Fig. 2a), which provides a possible specroscopic feature for lower redox-potential AA7 enzymes.

Oxygen gating and activation at the re-side of the isoalloxazine ring in the FAD-domain are also important for the oxidative activity. The entry of O2 to the active site in flavo-enzymes is favoured through hydrophobic tunnels rather than the solvent-filled substrate-binding pocket33. These tunnels converge to a cavity sterically gated by apolar residues33,44, where the O2 is positioned and activated for hydride transfer. Strikingly, we unveiled that the milieu of the FAD, particularly on the re-side, is consideredly different in clade II as compared to canonical AA7s. An eye-catching substitution of a histidine, which is highly conserved in all other clades, to a serine, leucine or valine in clade II is observed (Fig. 6a, b, Supplementary Fig. 10, sequence patch 3). Mutation of a histidine at the equivalent position in the FAD-dependent aryl alcohol oxidase from Pleurotus eryngii is shown to reduce the rate of the oxidative half-reaction by an order of magnitude45. The histidine was proposed to contribute to the positioning and polarisation of O245, thereby reducing its activation-free energy barrier. The substitution of this histidine in clade II enzymes is accompanied with other marked changes in the O2 binding cavity (Fig. 6a, b). These steric and chemical changes are likely to increase the energy barrier for hydride transfer to O243,44, thereby severely reducing the oxidative activity. On the other hand, the uniquely open and positively charged active site of FgCelDH7C, compared to structural homologues, may favour the accommodation of organic electron acceptors to promote the dehydrogenase oxidative half reaction. Taken together, these findings give insights into the structural elements associated with the distinctively high dehydrogenase/oxidase activity profile of FgCelDH7C and possibly AA7 homologues in clade II.

Clade I plant enzymes catalyse diverse reactions, e.g., in alkaloid synthesis32 and lignin building block synthesis demonstrated for the monolignol oxidase from Arabidopsis33. By contrast, the biological roles of oligosaccharide-specific oxidases have remained an enigma. Both canonical AA7 oligosaccharide-specific oxidases and clade II dehydrogenases are ubiquitous in phytopathogenic fungi and Oomycota plant parasites, responsible for some of the most devastating plant diseases in agriculture. Recently, the activity of oligogalacturonide oxidases (clade I) from Arabidopsis has been reported to reduce the elicitor activity of oligogalacturonides that trigger plant immune-response13. Interestingly, plants overexpressing the oligogalacturonide oxidase AOGOX1 were more resistant to infection by the phytopathogen Botrytis cinerea, supporting a role in plant immunity13. Fungal LPMOs are another category of oxidoreductases that have been hypothesized to play a role in plant pathogenesis46, although this is yet to be demonstrated despite the abundance of LPMO genes in most fungal phytopathogens.

Interestingly, the co-secretion of fungal LPMOs and AA7 enzymes has been observed30,31, which inspired us to investigate the interplay between these two enzyme families. A balanced supply of ROS, e.g., H2O2 has been shown to be crucial for sustained LPMO activity, due to the affinity (low µM apparent K_M value)32 of LPMOs to H2O2 and their high sensitivity to oxidative damage21,47. We have shown that FgCelDH7C potentiates LPMO-catalysed cellulose degradation in the absence of any exogenous reductant. The quenching of LPMO activity with either an alternative artificial acceptor for the AA7 (Supplementary Fig. 12) or an enzyme that scavenges H2O2 (Fig. 4a) is consistent with the proposed peroxygenase LPMO mechanism21. In addition, O2− species that are generated by flavo-enzymes in the presence of O248, may contribute to LPMO priming.
consistent with previous findings\textsuperscript{21,38}. To evaluate the specificity of the observed AA7-mediated activation of LPMOs, MoChi7A that possesses a low oxidase activity on cellooligosaccharides was used instead of \textit{Fg}CelDH7C at equimolar concentrations in a similar assay. Under these conditions, the release of oligomers from cellulose was not observed to the same extent, indicating that the priming and/or the balanced fuelling of LPMO activity is more readily achieved with the AA7 dehydrogenase (Supplementary Fig. 15). Remarkably, the performance of \textit{Fg}CelDH7C was comparable with CDH that has been shown to mediate direct transfer of priming electrons from cellooligosaccharide oxidation to LPMOs via the cytochrome \textit{b} domain\textsuperscript{49}. Our EPR experiments were consistent with direct electron transfer to the LPMO when the FAD-cofactor in \textit{Fg}CelDH7C was reduced either with dithionite or cellotriose in the absence of O\textsubscript{2} (Fig. 5c, d). This electron transfer and the interaction with the LPMO is likely to occur at the substrate-binding face (si-side) of the FAD, which is consistent with the rather open active site topology of \textit{Fg}CelDH7C (Supplementary Fig. 9). By comparison, the electron transfer from the larger (about 800 aa) and bi-modal CDH to the LPMO requires inter-domain electron transfer from the dehydrogenase to the cytochrome domain and a subsequent large domain movement to the open CDH form\textsuperscript{49,50}. The deletion of the cytochrome domain impairs the priming of the LPMO, but the LPMO fuelling remains efficient\textsuperscript{29}, likely via the oxidase side-activity of CDH. Similarly, the deletion of the cytochrome domain in the pyrroloquinoline-quinone-dependent pyranose dehydrogenase impaired the fuelling of LPMO activity \textsuperscript{24}.

In summary, the one-module AA7 dehydrogenase confers direct priming of LPMOs in contrast to other known LPMO redox partners that require either small organic molecules or an additional cytochrome haem domain to mediate LPMO priming. The expansion of the substrate range of fungal oligosaccharide-oxidising flavo-enzymes belonging to the AA7 family and the potentiation of LPMO activity, highlight the functional diversity within AA7. These findings set the stage for harnessing other AA7 dehydrogenases to fine-tune the activity of LPMOs that lack known redox partners. The demonstration of a functional and
efficient AA7-LPMO system offers attractive avenues for the development of commercial enzyme blends targeting recalcitrant biomass.

Methods
Bioinformatics and phylogenetic analysis. The sequences of 2200 putative AA7 sequences were retrieved by a BlastP search against the non-redundant protein database interfaced the NCBI server (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using the FgChiO sequence (Genbank accession: XP_011325572) as a query and default settings. The retrieved sequences were filtered by excluding non-eukaryotic proteins and those displaying <25% amino acid (aa) identity and <90% coverage to the query. Two additional AA7-like sequences, which were differentially upregulated in the secretome of Aspergillus nidulans grown on starch31, and a longer sequence possessing an N-terminal extension of about 250 amino acid residues (MoChi7A) were also included in the sequence inventory. The sequences comprising 470–570 aa (n = 1927) were aligned using MAFFT32 with default parameters, and curated using GBload932. The alignment was used to construct a phylogenetic tree using NCBI32,34 and rendered by iTOL32. The visualization of amino acid conservation was made using WebLogo35.

Production of selected AA7 enzymes. Based on the phylogenetic analysis above, five selected AA7 gene fragments encoding mature peptides without signal peptides (Supplementary Table 1) were codon optimised for P. pastoris and purchased from GENEWIZ (NJ, USA). The gene fragments were cloned within the XbaI and XhoI restriction sites of the pPICZzA vector (Invitrogen, Carlsbad, CA, USA) in frame with the Saccharomyces cerevisiae a-mating factor secretion signal and fused to a C-terminal (His6) tag. The synthetic gene harboring plasmids were propagated in Escherichia coli DH5α, linearised with PmeI and thereafter transformed into competent P. pastoris X33 by electro-poration following the protocols from the Easy Select Expression System (Invitrogen). Six transformants per construct were screened for production in deep well plates for three days with MeOH addition 3% (v/v) every 24 h. The best-secreting transformants based on SDS-PAGE gel electrophoresis, from the constructs of the selected enzymes (Supplementary Table 1) were chosen for larger-scale production. These clones were grown in shake flasks containing 2 L of BMGY (Pichia expression manual, Invitrogen) containing 1 mL L−1 of Pichia PM1 trace element solution (2 g L−1 CuSO4.5H2O, 3 g L−1 MnSO4.H2O, 0.2 g L−1 Na2MoO4.2H2O, 0.02 g L−1 H3BO3, 0.5 g L−1 CaSO4.2H2O, 0.5 g L−1 CoCl3, 12.5 g L−1 ZnSO4.7H2O, 22 g L−1 FeSO4.7H2O, H2SO4 1 mL L−1) and biotin 0.2 g L−1 to an OD600 of 2–6, for 16 h at 30 °C using an orbital shaker (200 rpm). The cells from each construct were harvested by centrifugation (4000 × g, 10 min, 4 °C) and the expression was induced by re-suspending the cells into 400 mL of BMMY medium at 20 °C and the culture was continued for 3 days with methanol supplementation to 3% (v/v) every 24 h. The cells were harvested by centrifugation (5000 × g, 10 min, 4 °C) and the pH of each of the supernatants was adjusted to 7.8 using NaOH (2 M), followed by sterile filtration using 0.22 μm filters (Millipore, Burlington, MA, USA). The filtered supernatants were adjusted to 7.8 using NaOH (2 M), followed by sterile filtration using 0.22 μm filters (Millipore, Burlington, MA, USA). The filtered supernatants were harvested onto 5 mL His Trap HP columns (GE Healthcare, Uppsala, Sweden) connected to an Akta purifier 100 (GE Healthcare) at 5 mL min−1 50 mM NaCl equilibrated with buffer A (Tris-HCl 50 mM pH 7.8, NaCl 150 mM, imidazole 10 mM). Non-bound proteins were washed by 10 column volumes (CVs). The bound proteins were eluted with a 34% buffer B (Tris-HCl 50 mM pH 7.8, NaCl 150 mM, hexamethylene urea 0.2 g L−1, 505 nm at 35 °C for 20 min with 30 s intervals). The assay was carried out at eight pH values in the range 5.1–9.0 by exchanging the buffer in the screening assay to the universal Britton-Robinson buffer (acetate, phosphoric acid, and boric acid, 50 mM of each). The dehydrogenase assay was used to allow for comparison of all three enzymes in the same assay and the initial rate of oxidation at each pH was determined from the slopes of the progress curves as above. Differential scanning calorimetry was employed to examine the conformational stability of FgCelDH7C (1 mg mL−1) at pH values of 5.2 and 7.2 in 50 mM (in acetate and phosphate buffers, respectively) in the temperature range of 15–90 °C at a scanning rate of 1 °C min−1 using a NanoDSC instrument (TA instruments, New Castle, DE, USA). The temperature and pH optimum of Pch7A (15 mM) were determined using the HRP assay described above against chitobiose (2 mM). For determination of the pH optimum, a Britton Robinson buffer (100 mM each) was adjusted to pH 4–11 and the oxidation activity was measured after 1 h incubation at 4 °C. The temperature stability of Pch7A (15 mM) was measured at oxidation of 2 mM chitobiose within 1 h at different temperatures (25°C–95°C).

NMR Spectroscopy of FgCelDH7C, FgChi7B and MoChi7A. Initially, the analysis of post oxidation reactions was conducted on a reaction mixture containing FgChi7B or MoChi7A (1 µM of each), 25 mM GalNAc or GlcNAc as substrates, buffered with 50 mM NaOAc buffer pH 5.2 (500 mM, 25 °C, 30 min). The reaction mixture was pre-cooled and the dehydrogenase activity at pH 7.2 was measured by using an 800 MHz Bruker Avance II spectrometer equipped with an Oxford 18.7 T magnet and a TCI cryocube by transferring 500 μL of the post-reaction mixtures to NMR sample tubes (5 mm) and performing H-1, H-1 TOCSY, H-1,C COSY, H-1,C-13C HMBC NMR and multiplicity-edited H-1,C-13C HSQC NMR spectroscopy analyses. H-1 TOCSY spectra were acquired as data matrices of 1024 × 256 complex data points sampling 128 and 64 ms in the direct and indirect dimensions, respectively. H-1,C COSY spectra were acquired as data matrices of 2048 × 128 complex data points sampling 320 and 20 ms in the direct and indirect dimensions, respectively, while H-1,C-13C HMBC NMR spectra were acquired as data matrices of 2048 × 256 complex data points sampling 212 and 5.8 ms in the direct and indirect dimensions, respectively. Multiplicity-edited 1H-1,C-13C HSQC spectra were collected by sampling the NMR spectra for 106 and 15.9 ms in the direct (H) and indirect (13C) dimensions, respectively. Once intermediate and product identification was established (Supplementary Fig. 3), a series of H-1,C-13C HSQC spectra was acquired at 15 °C to follow the conversion of GalNAc and GalNAc by MoChi7A (1 µM) in 50 mM NaOAc buffer pH 5.2 after 5, 15, 60 and 180 min incubation time. The H-1,C-13C HSQC spectra were collected by sampling the NMR spectra for 159 and 8.3 ms in the direct (H) and indirect (13C) dimensions, respectively, with experiments of 19 min duration to yield the time course shown in Supplementary Fig. 4 upon integration of the CH2H2 NMR cross peaks. For cellulose, a time series of H-1,C-13C HSQC spectra for the enzymatic conversion of Chi7B (0.56 mM) was yield assignation of cellulobionolactone and of cellulobionic acid signals as displayed in Supplementary Figure 5. Time-resolved 1H NMR spectra sampling 16384 complex data points of the FID for 1.27 s were subsequently used to track the reaction kinetics for cellulose (2 mM) conversion by FgCelDH7C (0.56 μM) at 25 °C in the presence or in the absence of 1.3 mM DCPD (5 mM) using the same equipment as above. The 1H singlet signal for DCIP as well as signals for H5 of cellulobionolactone and H2 of cellulobionic acid were integrated alongside the H2 and
H3 signals for the reducing end of cellulose. Signal areas were normalized to the number of contributing hydrogen atoms. The same buffer, enzyme concentration and sample setup were subsequently used to track the depolymerization of cellulose oxidation (6 mM cellobiose) by FgCelD7H7 in the presence of 0.025, 0.05, 0.1, 0.2, 0.5 or 1 mM DCIP. All NMR spectra were acquired, processed and integrated in Topspin 3.5 p6 with amine zero filling in all dimensions.

Crystallization and structure Determination of FgCelD7H7 and FgChI7B. Crystallization screens PACT+/+ (Jena Bioscience, Jena, Germany), C5CG+ and Morphos screens (Molecular Dimensions, Sheffield, UK) and optimisation plates of FgCelD7H7 (90 mg mL−1, deglycosylated with EndoH, New England Biolab, Ipswich, MA, USA) and FgChI7B (10.5 mg mL−1), both in 20 mM NaOAc pH 5.5, were performed with a Crystal Gymplion liquid handling robot (Art Robbins Instruments, Sunnyvale, USA). Drops containing equal volumes of protein and reservoir solutions were mixed. Crystals of FgCelD7H7 appeared in the F7 condition (NaSCN, 20% (w/v) PEG 3350), which was optimised by varying PEG content (12.5–25% w/v). The best crystals formed within 4 days at 20 °C, using 150 nL of reservoir and protein solutions (1:1 ratio) in 50 µL reservoir solution. These crystals were cryo-protected with PEG400 in reservoir solution and cryo-cooled in a nylon loop. Crystals of FgChI7B were initially observed in PACT+÷ condition E10 (PEG 3350, 22% w/v, 200 mM sodium potassium phosphate using a 1:1 protein:reservoir ratio. Crystals were optimised by micro-seeding using the above condition at 22 and 24% PEG (w/v). Drops contained 1:0.5:2 of protein:reservoir: seed stock (100 fold diluted seed stock) ratio, respectively. The crystals were cryo-cooled using a mixture of reservoir and PEG400 (0.7:0.3 ratio) added to the drop prior freezing in liquid nitrogen. Diffraction data for FgCelD7H7 and FgChI7B crystals were collected in BioMax beamline at MAXIV (Lund, Sweden) using MXCuBe v3 and id30A3 (MASSIF-3) beamline at ESRF (Grenoble, France) using to 1.64 Å and 2.38 Å resolution, respectively. Both structures were solved using molecular replacement in Phaser59 with model coordinates of Tixyli40 and initial automated model building and refinement using PHENIX.autobuild57 and PHENIX.refine respectively. Manual model rebuilding and map inspection were performed in Coot60 and analysed using MolProbity60. The final validated structural models of FgCelD7H7 and FgChI7B were deposited to the Protein Data Bank under the entry codes 6Y7I and 6Y7O, respectively (Data collection and refinement statistics are in Supplementary Table 4).

Cellulose degradation assays based on the interplay between LPMOs and AA7 enzymes. The assays were performed using suspensions of either 1% PASC51 and/or 0.5% Avicel (Honeywell Fluka, Morris Plains, NJ, USA) (both w/v) in 250 μL of same buffer in the absence or presence of 40 µM dithionite and incubated at 25 °C for 15 min to achieve the full reduction of the catalytic Cu(I) center of PaLPMO9H into Cu(II) by dithionite. Next, the following solutions were prepared and incubated anaerobically for 5 min at 25 °C: (i) FgCelD7H7 (20 µM) with sub-stoichiometric amount of sodium dithionite (16 µM) to reduce 80% of the flavo-enzyme, while avoiding excess dithionite in the solution, (ii) PaLPMO9H (20 µM) without adding in the same buffer. Equal volumes of the two solutions were then pooled into a 160 µL EPR sample and incubated for few minutes prior and EPR analysis.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The atomic coordinates of FgCelD7H7 and FgChI7B have been deposited in the Protein Data Bank (https://www.ribscb.org) under the PDB accessions 6Y7I and 6Y7O, respectively (see also Supplementary Table 4). The GenPept accession IDs of the enzymes characterised in the study are given in Supplementary Table 1. All the data are available from the corresponding authors upon request. Source data are provided with this paper.

Received: 26 October 2020; Accepted: 9 March 2021; Published online: 09 April 2021

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