On the expansion of biological functions of lytic polysaccharide monooxygenases

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Summary

Lytic polysaccharide monooxygenases (LPMOs) constitute an enigmatic class of enzymes, the discovery of which has opened up a new arena of riveting research. LPMOs can oxidatively cleave the glycosidic bonds found in carbohydrate polymers enabling the depolymerisation of recalcitrant biomasses, such as cellulose or chitin. While most studies have so far mainly explored the role of LPMOs in a (plant) biomass conversion context, alternative roles and paradigms begin to emerge. In the present review, we propose a historical perspective of LPMO research providing a succinct overview of the major achievements of LPMO research over the past decade. This journey through LPMOs landscape leads us to dive into the emerging biological functions of LPMOs and LPMO-like proteins. We notably highlight roles in fungal and oomycete plant pathogenesis (e.g. potato late blight), but also in mutualistic/commensalism symbiosis (e.g. ectomycorrhizae). We further present the potential importance of LPMOs in other microbial pathogenesis including diseases caused by bacteria (e.g. pneumonia), fungi (e.g. human meningitis), oomycetes and viruses (e.g. entomopox), as well as in (micro)organism development (including several plant pests). Our assessment of the literature leads to the formulation of outstanding questions, promising for the coming years exciting research and discoveries on these moonlighting proteins.

I. Introduction

The breakdown of carbohydrates is essential for the global carbon cycle. On Earth, the most abundant sources of carbohydrates are found in the form of cellulose and chitin, used as structural biopolymers in the plant and animal/fungal kingdoms, respectively. These polysaccharides are mainly acted upon by enzymes collectively termed as carbohydrate-active enzymes (CAZymes), which help in their build-up, modification as well as breakdown (Cantarel *et al.*, 2009) (www.cazy.org). In the context of cellulose and chitin conversion, glycoside hydrolases (GHs) act as main players by catalysing the hydrolytic cleavage of polysaccharide chains into smaller oligosaccharides and monosugar building blocks, which can in turn be used as carbon/energy source by

microorganisms. However, polysaccharide chains packed into crystalline lattices are hardly accessible to most canonical enzymes. To circumvent this geometrical and thermodynamic hurdle, Nature has evolved *ad hoc* biocatalytic tools, *viz.* the lytic polysaccharide monooxygenases (LPMOs) (Horn *et al.*, 2012).

Lytic polysaccharide monooxygenases activity was recently discovered in 2010, and they are represented by monocopper enzymes with the unique ability to oxidise the surface of crystalline polysaccharides (Vaaje-Kolstad *et al.*, 2010; Forsberg *et al.*, 2011; Phillips *et al.*, 2011; Quinlan *et al.*, 2011), disrupting thereby the surface topology (Eibinger *et al.*, 2014, 2017; Villares *et al.*, 2017) and creating chain ends tractable by canonical GHs for further depolymerisation. LPMO substrate specificity was later extended to noncrystalline carbohydrate substrates, hinting at a more diverse role and possibly alternative functions of LPMOs (described later). Beyond their biological importance, LPMOs have rapidly proved to be industrially relevant enzymes to boost the efficiency of commercial enzymatic cocktails for plant biomass degradation processes and yield fermentable sugars at competitive cost (e.g. for biofuel production) (Johansen, 2016; Chylenski *et al.*, 2019).

Over the past 10 yr, a significant number of reviews have covered diverse aspects of the LPMO field. We direct the reader to recent comprehensive reviews dealing with mechanistic considerations (Meier *et al.*, 2017; Bissaro *et al.*, 2018b; Wang *et al.*, 2020), applied enzymology (Johansen, 2016; Chylenski *et al.*, 2019), structural aspects (Vaaje-Kolstad *et al.*, 2017; Tandrup *et al.*, 2018) and enzyme secretomes complexity (Berrin *et al.*, 2017; Bissaro *et al.*, 2018b). Recent developments have suggested that LPMOs scope of action would not be restricted to the mere oxidation of structural biopolymers. Here, we aim at drawing a portrait of this expansion of LPMO-related functions. To this end, after succinctly presenting the LPMO field and debated dogmas, we review the emerging biological functions of LPMOs, with a particular emphasis on plant–microorganisms interactions. We then discuss unanswered questions and potential directions that the field may take in the coming years.

II. The bumpy road of LPMO research

Several decades of research have paved the way towards the discovery of LPMOs and the birth of an entire field and community. In Box 1, we provide a historical perspective of this scientific journey.

1. 2010–2020: the race for 'firsts'

The year 2010 marked a turning point in the field of glycan-processing enzymes. First, like the boosting effect reported for CBP21 on chitinase activity (Vaaje-Kolstad *et al.*, 2005a), a study on a fungal GH61 revealed that its addition to a cellulases mixture enhanced cellulose hydrolysis, reinforcing the parallel between GH61s and CBM33s (Harris *et al.*, 2010). At that time, although the boost effect was clear, their enzymatic mechanisms still remained unknown. This same year, Vaaje-Kolstad and colleagues brought a major piece of the jigsaw, the assembly of which started back in the 1950s (Box 1): they demonstrated that the hitherto

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Box 1 A bit of history on pre-LPMOs times

In 1954, in a study entitled *The swelling factor in cellulose hydrolysis*, Reese and Gilligan hypothesised the existence of a nonhydrolytic proteinaceous system ('C_1 factor'), described as targeting and disrupting crystalline parts of cellulose, rendering them more tractable by cellulases (Reese & Gilligan, 1954).

In 1974, Eriksson *et al.* showed the importance of oxygen and oxidative processes in cotton cellulose conversion by the white-rot fungus *Sporotrichum pulverulentum* (anamorph *Phanerochaete chrysosporium*) (Fig. 1) (Eriksson *et al.*, 1974). The authors highlighted the existence of an unknown oxidative activity that could induce swelling of crystalline cellulose.

In the late 1990s, scientists working on soil bacteria (*Streptomyces*) observed the secretion of a mysterious protein with high affinity for chitin (Schnellmann *et al.*, 1994; Kolbe *et al.*, 1998), classified as a chitin-binding protein (CBP). A 21-kDa CBP orthologue (called CBP21) was then found in *Serratia marcescens* (Suzuki *et al.*, 1998). However, due to the lack of experiments substantiating the catalytic activity, these proteins were defined as a new carbohydrate-binding module family, the CBM33 (Sunna *et al.*, 2000). Meanwhile, the characterisation of another unknown protein (later named EGIV), revealed a very weak endoglucanase activity, and became the founding member of the GH61 family (Saloheimo *et al.*, 1997).

In 2005, Vaaje-Kolstad *et al.* (2005a) demonstrated that CBP21 significantly boosted chitin hydrolysis by chitinases. Structural insights revealed that this protein shared little commonalities with canonical CBMs (Vaaje-Kolstad *et al.*, 2005b) (Fig. 1). A few years later, the first GH61 structure (Karkehabadi *et al.*, 2008) revealed features reminiscent of CBP21 while sharing no similarity with other GH structures. Devoid of a conserved active site of most known GHs, no rational molecular mechanism could be proposed to explain the endoglucanase activity of GH61s (Harris *et al.*, 2010). Interestingly, a conserved metal-binding site could be identified in both CBP21 and GH61.

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noncatalytic protein CBP21 was able to cleave glycosidic bonds in β-chitin through a redox reaction (Vaaje-Kolstad *et al.*, 2010). Using isotopically labeled dioxygen, the authors demonstrated the introduction of an oxygen atom into the chitin carbohydrate chain in the presence of a reducing agent. This was the rationale to define CBP21, and its subsequently reported orthologues, as monooxygenases. In 2011, several studies reported an analogous oxidative activity on cellulose (Forsberg *et al.*, 2011; Phillips *et al.*, 2011; Quinlan *et al.*, 2011). Quinlan and colleagues clarified the nature of the metal centre: a mono-copper atom coordinated by two histidines, therefore called today a 'histidine brace' (Ciano *et al.*, 2018). In 2012, CBM33s and GH61s were collectively termed 'LPMO' (Horn *et al.*, 2012). These pioneer discoveries spurred intense research efforts, first notably in European laboratories and today worldwide (Fig. 1). LPMOs were rapidly assigned to new auxiliary activity (AA) families in the CAZy database (Levasseur *et al.*, 2013): GH61s were reclassified as AA9s and CBM33s as AA10s. A scientific race was engaged to discover new substrate specificities, structures and mechanistic features. Today, LPMOs are distributed in eight AA families: AA9–AA11 and AA13–AA17 (see later). The substrate specificity of LPMOs varies among these AA families. but is mostly towards cellulose, chitin, xylan, starch or
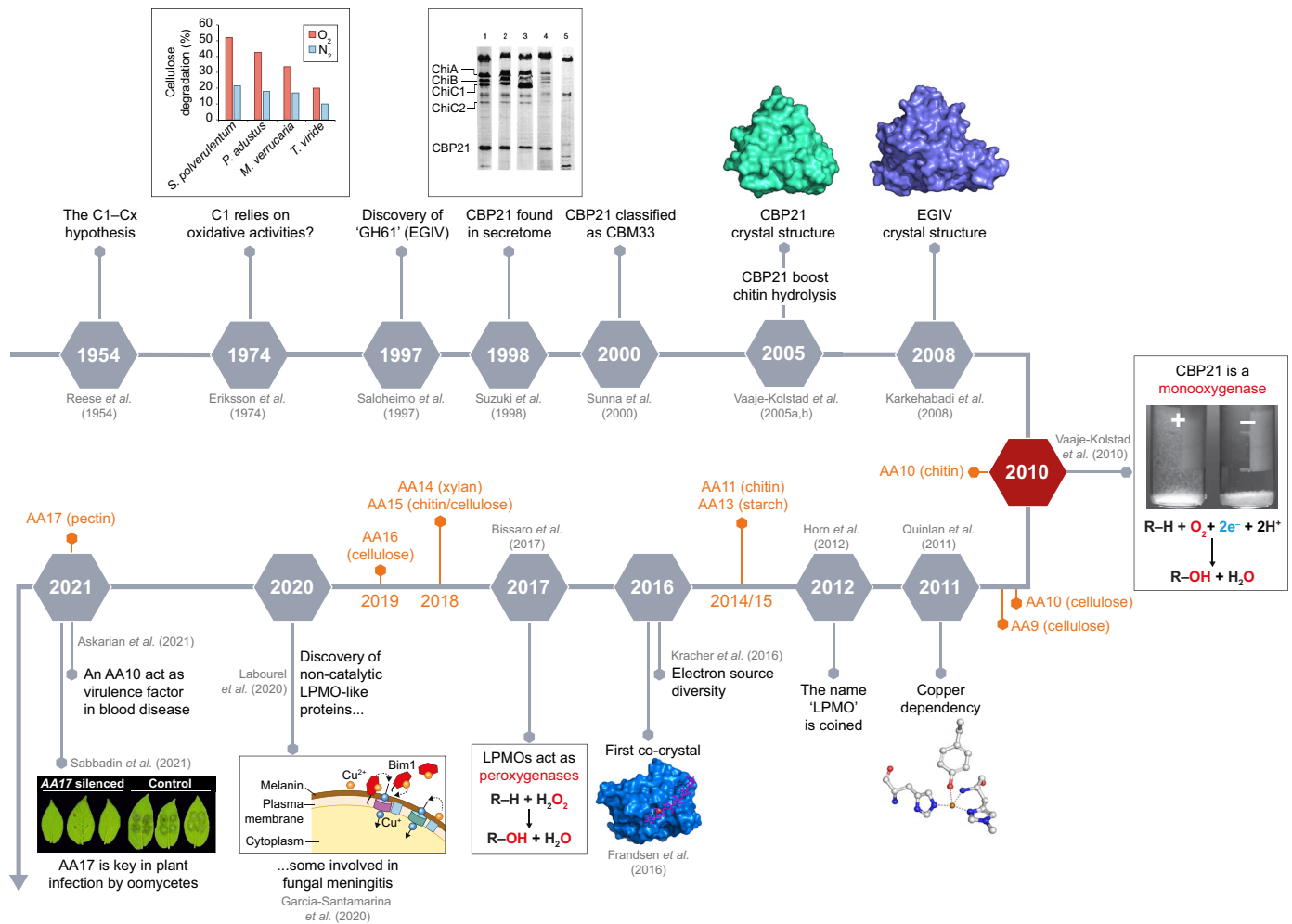
Fig. 1 Lytic polysaccharide monooxygenase (LPMO) research timeline and milestones. Major milestones are associated with the following references, by chronological order: 1954 (Reese & Gilligan, 1954), 1974 (Eriksson *et al.*, 1974), 1997 (Saloheimo *et al.*, 1997), 1998 (Suzuki *et al.*, 1998), 2000 (Sunna *et al.*, 2000), 2005 (Vaaje-Kolstad *et al.*, 2005a,b), 2008 (Karkehabadi *et al.*, 2008), 2010 (Vaaje-Kolstad *et al.*, 2010), 2011 (Quinlan *et al.*, 2011), 2012 (Horn *et al.*, 2012), 2016 (Frandsen *et al.*, 2016; Kracher *et al.*, 2016), 2017 (Bissaro *et al.*, 2017), 2020 (Garcia-Santamarina *et al.*, 2020; Labourel *et al.*, 2020), 2021 (Askarian *et al.*, 2021; Sabbadin *et al.*, 2021b). The discovery of new LPMO families (along with substrate specificity) is displayed in red. The picture on the right-hand side (year 2010) shows the disruptive effect (swelling) of CBP21 on chitin in the presence of oxygen and reductant (vial labelled '+') compared with a reaction devoid of electron source ('−'). The picture on the left-hand side (year 2021) shows the loss of oomycete pathogenicity upon silencing of AA17-encoding gene. AA, auxiliary activity; CBP, chitin-binding protein; EG, endoglucanase.

pectin. They are abundantly present in bacterial and fungal communities but, have also been found in viruses, plants and insects.

(**1**) The AA9 family contains sequences of fungal origin only, and encompasses several substrate specificities, mainly targeting glucose-based polymers such as cellulose, cello-oligosaccharides or even hemicelluloses with a β-1,4-linked glucose backbone (gluco-mannans, xylo-glucans, mixed linked glucans, etc.) (Agger *et al.*, 2014; Bennati-Granier *et al.*, 2015; Frommhagen *et al.*, 2015; Simmons *et al.*, 2017). Of note, the AA9 family also contains nonactive proteins lacking the conserved His-brace motif (Frandsen *et al.*, 2019).

(**2**) The AA10 family is probably the most diverse family in terms of taxonomic coverage, inasmuch as it contains sequences originating from bacteria (97%), viruses (2.9%), archaea (0.1%) and even from some pathogenic fungi of the *Ustilaginomycetes* class

(0.1%). Members of this LPMO family can target either chitin or cellulose, or both. Interestingly, one characterised member of this family from the actinomycete bacteria *Kitasatospora papulosa* seemed to display a broader substrate specificity, cleaving cellulose, chitin and xylan (Corrêa *et al.*, 2019), albeit this result needs to be confirmed.

(**3**) The AA11 family, introduced in 2014, is a rather small, fungal-only family (preponderant in ascomycetes fungi), with only a few characterised members, all active on chitin (Hemsworth *et al.*, 2014; Wang *et al.*, 2018).

(**4**) The AA13 family, first reported in 2014 (Vu *et al.*, 2014), contains fungal-only LPMOs acting on starch substrates (Vu *et al.*, 2014; Leggio *et al.*, 2015; Momeni *et al.*, 2020).

(**5**) The AA14 family, published in 2018, unveiled with the characterisation of two homologues from the white-rot fungus *Pycnoporus coccineus*, an oxidative activity on heteroxylan covering

cellulose fibrils in wood (Couturier *et al.*, 2018). While AA14 LPMOs did not show activity on soluble xylan, synergism with GH30 xylanases was observed on wood fibres (Zerva *et al.*, 2020). (**6**) The AA15 family LPMOs are found in various organisms including viruses (14.5%) and eukaryotes (85.5%) such as algae, oomycetes and complex animals. The two first members were characterised from the insect *Thermobia domestica* and displayed activity on both cellulose and/or chitin (Sabbadin *et al.*, 2018). More recently, two AA15 LPMOs from the termite *Coptotermes gestroi* (Cairo *et al.*, 2020) and one AA15 from the oomycete pathogen *Aphanomyces astaci* ('crayfish plague') (Sabbadin *et al.*, 2021a) showed activity on chitin only.

(**7**) The AA16 family contains members mainly found in fungi and some oomycetes (Filiatrault-Chastel *et al.*, 2019). This family was first identified in the secretome of *Aspergillus japonicus*. Characterisation of several orthologues revealed activity on cellulose (Filiatrault-Chastel *et al.*, 2019). Recent studies have also demonstrated a boosting effect of AA16s on cellulose degradation by a cellulase cocktail and suggested implications of this LPMO family in plant pathogenesis in oomycetes (Jagadeeswaran *et al.*, 2020) (see Section III).

(**8**) The AA17 family is the most recent LPMO family, only found in oomycetes, with a significant expansion of gene copies (up to 65) in hemibiotrophic and necrotrophic pathogens (Sabbadin *et al.*, 2021b). Three AA17s from *Phytophthora infestans* were demonstrated to oxidatively cleave (C4-oxidation) homogalacturonan and oligogalacturonides (degree of polymerisation 10–15), constituents of pectin in plant cell walls (PCW). Prior de-esterification of pectin (by pectin methyl esterases) was required to observe LPMO activity (Sabbadin *et al.*, 2021b).

2. Pitfalls in a recent but established dogma

From the early 2010s, LPMOs have been described as catalysing, via a monooxygenase reaction, the disruption of crystalline regions of polysaccharides (e.g. in chitin or cellulose), otherwise hardly accessible to canonical GHs. Benefiting from the hindsight of a decade of research, it becomes increasingly clear that this dogma is slowly shattering in several respects, and therefore merits to be discussed and nuanced. Before doing so, some facts should be presented.

The peculiar function of LPMOs is intimately related to their 3D structure (Fig. 2). Although some variations across the different families exist, the basal structure of LPMOs is fairly well conserved (Frandsen *et al.*, 2016; Vaaje-Kolstad *et al.*, 2017), consisting of an immunoglobulin-like β-sandwich fold, composed of 8–10 antiparallel β-strands, linked to each other by α-helix loops. At the active site, located on a usually rather flat, solvent-exposed surface (Fig. 2), two universally conserved histidines coordinate a Cu(II) ion through three nitrogen atoms (Ciano *et al.*, 2018). In addition, a conserved tyrosine (or a phenylalanine in chitin-active AA10 and AA15) is also essential for catalysis (Fig. 2). We underscore that in-depth LPMO–substrate interaction studies are only available for a handful of AA9 (Courtade *et al.*, 2016; Frandsen *et al.*, 2016; Simmons *et al.*, 2017) and AA10 (Aachmann *et al.*, 2012; Bissaro *et al.*, 2018a) LPMOs, due to the fact that working with insoluble substrates (e.g. cellulose or chitin) is a true technical challenge.

A first aspect of the LPMO dogma that needs to be discussed is the nature of the substrate: we, and others, have observed that AA9 LPMOs are often more active on amorphous cellulose than on microcrystalline cellulose (Bennati-Granier *et al.*, 2015; Villares *et al.*, 2017). Intriguingly, some LPMOs are also active on oligosaccharides (Isaksen *et al.*, 2014; Bennati-Granier *et al.*, 2015; Frandsen *et al.*, 2017, 2019), and such activity is unlikely to be related to the direct boosting of biomass depolymerisation. Such LPMOs could, for instance, be involved in alleviating the inhibition of cellulases by cello-oligosaccharides upon oxidation of the latter, a role also suggested for cellobiose dehydrogenases (Igarashi *et al.*, 1998). This remains, however, to be demonstrated. Other LPMOs, such as AA13s and AA14s, with more groovy active sites, would better be described as acting on glycosidic bonds encountered in recalcitrant environments – for example in starch (for AA13s; Leggio *et al.*, 2015; Vu *et al.*, 2019), or on xylan coating cellulose fibrils (for AA14s; Couturier *et al.*, 2018; Zerva *et al.*, 2020) – rather than on crystalline substrates. Lately, the crystal structure of an AA17, active on homogalacturonan, has revealed a
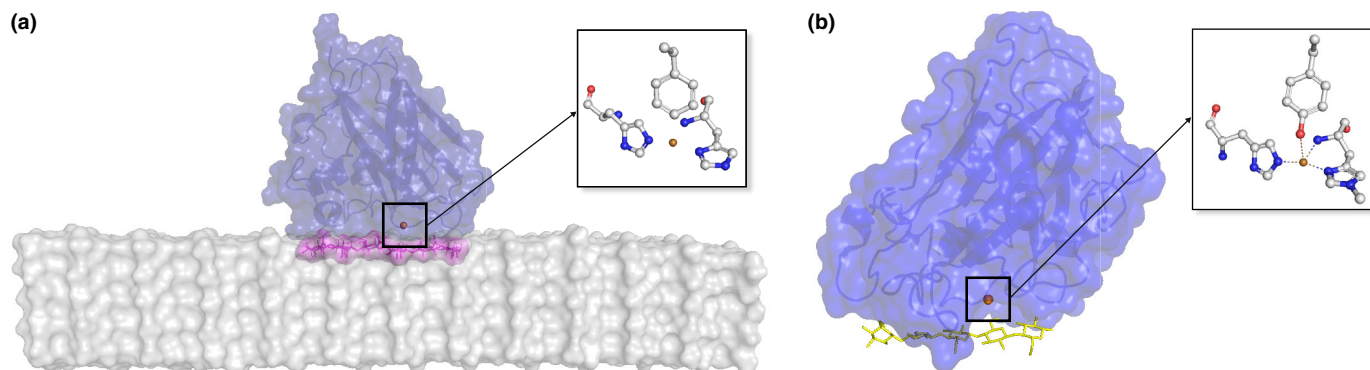
Fig. 2 Lytic polysaccharide monooxygenase (LPMO)–substrate interaction. (a) A model of *Sm*AA10A bound to β-chitin (the chitin crystal is shown in grey and the chitohexaose fragment interacting with the LPMO is highlighted in magenta) (Bissaro *et al.*, 2018a) and (b) *Ls*AA9A-cellopentaose complex (Protein Data Bank (PDB) 5NLS; (Frandsen *et al.*, 2016)). The model in panel (a) was obtained from experiment-guided molecular dynamics (MD) simulations (Bissaro *et al.*, 2018a) and the structure shown in panel (b) was obtained from crystals soaked with oligosaccharides before X-ray diffraction (Frandsen *et al.*, 2016). Inserts show the histidine brace (copper shown as orange spheres).

rather grooved and charged active site, consistent with an adaptation to interact with charged polysaccharides (Sabbadin *et al.*, 2021b).

A second aspect of the LPMO dogma pertains to their catalytic mechanism. Like many other oxidoreductase classes, LPMOs have not been exempt from scientific debates and evolutions when it comes to their mode of action. It is now agreed upon that LPMOs require a priming reduction of the resting Cu(II) state into the active Cu(I) state. It should, however, be underscored that the identity of the reducing agent remains to be clarified for several of the biological contexts involving LPMOs (described later). *In vitro* studies have shown that reduction can occur via reaction with a variety of molecules (Kracher *et al.*, 2016; Frommhagen *et al.*, 2018), including small organic reductants (Vaaje-Kolstad *et al.*, 2010), redox enzyme partners (Phillips *et al.*, 2011; Garajova *et al.*, 2016; Kracher *et al.*, 2016; Várnai *et al.*, 2018; Momeni *et al.*, 2021), lignin and their compounds (Westereng *et al.*, 2015), or even photoactivated molecules (Bissaro *et al.*, 2016, 2020a; Cannella *et al.*, 2016). A more controversial aspect pertains to the nature of the biological cosubstrate bringing the oxygen atom necessary for hydroxylation of either the C1 or C4 carbon of the scissile glycosidic bond (Vaaje-Kolstad *et al.*, 2010; Phillips *et al.*, 2011), and inducing cleavage of the polysaccharide chain (Beeson *et al.*, 2012; Wang *et al.*, 2018). From 2010 onwards, the proposed oxidative mechanism (until its revision in 2016/2017, described later) was described as a monooxygenase reaction, in which O_2 is proposed to serve as an oxygen atom donor. However, in a preprint in 2016 (eventually published in 2017 (Bissaro *et al.*, 2017)), Bissaro and colleagues demonstrated the occurrence of an efficient peroxygenase reaction (Bissaro *et al.*, 2017), that is using H_2O_2 rather than O_2. At this time, multiple kinetics studies have shown that both apparent affinity and catalytic efficiency are several orders of magnitude higher for H_2O_2 (Hangasky *et al.*, 2018; Kuusk *et al.*, 2018, 2019; Bissaro *et al.*, 2020a; Hedison *et al.*, 2020; Jones *et al.*, 2020; Kont *et al.*, 2020; Momeni *et al.*, 2021). This conclusion is further supported by synergistic studies with H_2O_2-producing redox partners (Bissaro *et al.*, 2017; Hegnar *et al.*, 2019; Kracher *et al.*, 2020; Momeni *et al.*, 2021). However, there is the possibility that the actual mechanistic path depends on the biological context and substrate/cosubstrate availability. Furthermore, it should be highlighted that mechanistic studies have mainly been conducted on AA9 and AA10 LPMOs and much remains to be done with the other LPMO families. Clarifying these mechanistic aspects is of utmost importance to then interpret, as correctly as possible, the putative connection between partner enzymes in omics studies, to design relevant *in vivo* localisation studies, and more generally understand the molecular basis underlying the interactions between LPMO-producing (micro)organisms and their host/partner.

Overall, the portrait we draw of the current LPMO field shows that, while there has been dazzling progress within a rather narrow time window, the complexity and subtleties of the biological function(s) of LPMOs clearly escape our understanding. In the next section, we aim at providing an overview of such functions, whether they be supported by sound evidence, or sometimes still at a hypothetical stage.

III. Emerging biological functions

LPMOs are more than mere biomass-degrading enzymes. They have been observed to possess varied biological functions from enabling developmental processes in insects to acting as virulence factors in pathogenic bacteria, oomycetes and fungi, all the while moonlighting as degraders of recalcitrant polysaccharides. This avenue widens the scope of LPMO research while teaching us to look beyond the obvious.

Before diving into specific examples, we carried out a global survey of the number of research articles (over the period 2008–2021) on host–pathogen interactions reporting the expression of an LPMO-encoding gene (studied at transcriptomic level) or the production of an LPMO (at proteomic level, or biochemically characterised) by so-called emitters, whether they be bacteria, oomycetes, fungi, plants or viruses (Fig. 3a). This analysis showed an increasing number of reports, yet modest and mainly focused on bacteria and fungi. Strikingly, this same set of data, represented as an emitter–target map shows that some regions remain empty (Fig. 3b), which could pinpoint territories yet to be explored (e.g. viruses vs animals). To better illustrate the *potential* diversity of LPMOs involved, we have also made an inventory of LPMO and LPMO-like encoding genes found in a selection of bacterial and fungal pathogens discussed here below (Fig. 3c). This inventory clearly illustrates the dichotomy between bacterial (harbouring only a few AA10-encoding genes) and fungal pathogens, which possess mainly AA9s, but also for some plant pathogens – such as *Magnaporthe oryzae* (causing rice blast) or *Leptosphaeria maculans* (causative agent of blackleg disease in Brassica crops, mainly affecting *B. napus*) – a significant number of AA11s and AA16s.

We also underscore that while there are several lines of evidence for the involvement of these LPMOs in pathogenesis processes, crystal-clear proof remains to be provided for many of them (see details later).

1. Role of LPMOs in fungal pathogenesis

The rice blast fungus, *Magnaporthe oryzae*, ranks among the top 10 most devastating plant pathogens worldwide (Dean *et al.*, 2012), causing serious crop yield losses and endangering global food security. This fungus exhibits a hemibiotrophic lifestyle and invades the plant by means of specialised appressoria cells, making use of high turgor pressure to puncture the plant surface, together with various effectors (Wilson & Talbot, 2009). *Mo*Aa91, an AA9 LPMO of *M. oryzae* was identified in the plant apoplast and acted as an inducer of appressorium formation on artificial inductive surfaces (Li *et al.*, 2020). Of note, upon *Mo*Aa91 gene deletion the formation of appressoria cells was delayed. It is even interesting to note that *Mo*Aa91, in association with *Mo*Slp1, an extracellular LysM domain protein anchoring to chitin, helped the fungus in evading the host immune response by preventing fungal-derived sugars (i.e. fungal cell wall carbohydrates) to be recognised by pattern triggered immunity that relied on the host chitin elicitor-binding protein precursor (CEBiP) (Li *et al.*, 2020). However, we must also underscore that *Mo*Aa91 is a multimodular protein composed of an N-terminal AA9 domain and a C-terminal chitin-
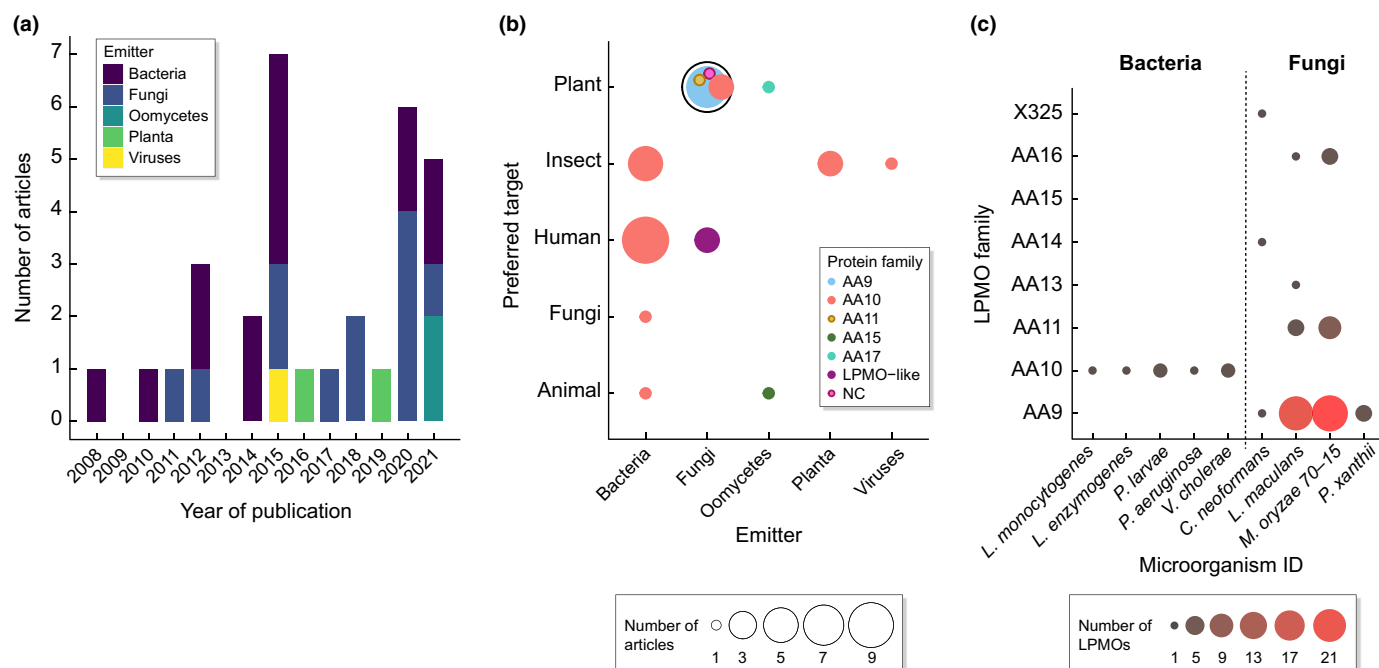
Fig. 3 Literature analysis of the occurrence of lytic polysaccharide monooxygenases (LPMOs) found in various pathosystems. (a, b) Number of research articles reporting an LPMO (or LPMO-like) shown to be relevant by either transcriptomic, proteomic and/or enzyme characterisation in different emitters (Kawada *et al.*, 2008; Chaudhuri *et al.*, 2010; Valente *et al.*, 2011; Stauder *et al.*, 2012; Wong *et al.*, 2012; Yakovlev *et al.*, 2012; Garcia-Gonzalez *et al.*, 2014; Loose *et al.*, 2014; Chiu *et al.*, 2015; Hamre *et al.*, 2015; Huang *et al.*, 2015; Paspaliari *et al.*, 2015; Zhang *et al.*, 2015; Mekasha *et al.*, 2016; Shukla *et al.*, 2016; Liu *et al.*, 2017, 2018, 2020; Hegnar *et al.*, 2019; Yadav *et al.*, 2019; Garcia-Santamarina *et al.*, 2020; Labourel *et al.*, 2020; Li *et al.*, 2020; Munzone *et al.*, 2020; Askarian *et al.*, 2021; Polonio *et al.*, 2021; Sabbadin *et al.*, 2021a,b; Yue *et al.*, 2021), and displayed as (a) a function of the year of publication per emitter and (b) an emitter–target map, in which the circle size indicates the number of articles and the colour indicates the protein family. Note that the 'preferred target' shows the potential target of the emitter, and not necessarily the target of the LPMO, which in several cases remains to be fully demonstrated (see main text). The category 'Animal' refers to nonhuman animals. (c) Analysis of the number of LPMO and LPMO-like encoding genes found in the genomes of several selected microbial pathogens discussed in the present review, including the bacteria *Vibrio cholerae* N16961, *Paenibacillus larvae* subsp. *larvae* ATCC 9545, *Listeria monocytogenes* EGD, *Pseudomonas aeruginosa* UCBPP-PA14, *Lysobacter enzymogenes* CX03; and the fungi *Magnaporthe oryzae* 70-15, *Cryptococcus neoformans* var. *grubii* H99, *Leptosphaeria maculans* JN3 and *Podosphaera xanthii*. NC, not classified (i.e. *Px*LPMO1).

binding domain and that inactivation of the former did not alter strain pathogenicity, therefore supporting a mechanism involving chito-oligosaccharide binding rather than oxidative activity (Li *et al.*, 2020).

Adding to the varied functions of LPMOs, the following discussion is about an LPMO-like protein from *Cryptococcus neoformans* (Garcia-Santamarina *et al.*, 2020), the causative agent of lethal meningitis in humans (Srikanta *et al.*, 2014). This LPMO-like protein is encoded by the gene *BIM1*, and is expressed in large quantities by *C. neoformans* during Cu limitation (Garcia-Santamarina *et al.*, 2020). During pathogenesis and brain colonisation, *C. neoformans* shifts from a Cu detox mode to an actively Cu-acquiring state in Cu-limited conditions. Bim1 binds Cu^{2+} and plays a crucial role in Cu acquisition and successively in cryptococcal meningitis. Garcia-Santamarina and colleagues demonstrated that *BIM1*-knockout mutants are Cu deficient (Garcia-Santamarina *et al.*, 2020). The role of Bim1 in Cu uptake and accumulation depends on Cu(II) coordination and cell-surface association via a glycophosphatidylinositol (GPI) anchor. Overall, this LPMO-like protein is a critical factor for Cu acquisition in fungal meningitis (Garcia-Santamarina *et al.*, 2020). It is worth noting that *C. neoformans* also expresses other LPMOs belonging to AA9, AA11 and AA14 families, the activities of which are yet to be

determined (Garcia-Santamarina *et al.*, 2020). Importantly, Labourel and coworkers have characterised orthologues of BIM1, called X325, found in yeast and filamentous fungi with different lifestyles, including the ectomycorrhizal fungus *Laccaria bicolor* (Labourel *et al.*, 2020) (see Section III.5 on symbiotic interactions for more details). Of note, the authors have shown that these LPMO-like proteins do not display LPMO activity on any of a wide range of tested polysaccharides. Interestingly, while the 3D structure of one fungal X325 member revealed a conserved LPMO fold, it also showed an atypical His brace with an additional Asp ligand to Cu(II). Interestingly, a parallel can be drawn between Bim1/X325 and mammalian ceruloplasmin or bacterial CopC, which are also involved in binding extracellular Cu but are not redox active (Brander *et al.*, 2020). Whether all X325 proteins act as copper transporters, or if some can catalyse hitherto undetected reactions, remains to be unveiled.

Podosphaera xanthii is the causative organism of cucurbit powdery mildew disease. A novel LPMO, called *Px*LPMO1, was discovered and characterised by Polonio and colleagues in 2021 (Polonio *et al.*, 2021). *Px*LPMO1 was seen to possess a predicted protein fold characteristic of LPMOs but showed low sequence similarity with the same. *Px*LPMO1 displayed a putative chitin-binding domain-3 and was demonstrated to catalyse the oxidative

cleavage of chitin and chito-oligosaccharides. Similarly to what was observed for *Mo*Aa91 (mentioned earlier), RNA silencing of *Px*LPMO1 activated chitin-triggered host immunity (chitin receptors) and oxidative burst. Furthermore, *Px*LPMO1 was found to be coexpressed along with host (melon) endochitinases, at early stages during infection, supporting the hypothesis that *Px*LPMO1 would modify chito-oligosaccharides released from the fungal cell wall by plant enzymes, therefore contributing to evade the plant immune system. Several orthologues of this protein have been observed in other fungal pathogens (such as *Rhynchosporium commune*, *Cadophora* sp. and *Phialophora americana*) demonstrating more avenues to look into for LPMO roles in fungal

pathogenesis (Polonio *et al.*, 2021). Strikingly, our analysis of the *Px*LPMO1 sequence (and predicted structure using RoseTTAFold tool (Baek *et al.*, 2021)) indicated that it is an orthologue of BIM1/X325, but belongs to a phylogenetic clade in which the additional copper-coordinating residue specific to X325 (mainly aspartate or histidine) (Labourel *et al.*, 2020) (described earlier), is replaced by an arginine. This major difference may explain why *Px*LPMO1 displayed oxidative activity while X325 did not, and also suggests that *Px*LPMO1 may be the founding member of a new LPMO family containing active LPMO and nonactive LPMO-like members (Fig. 4).
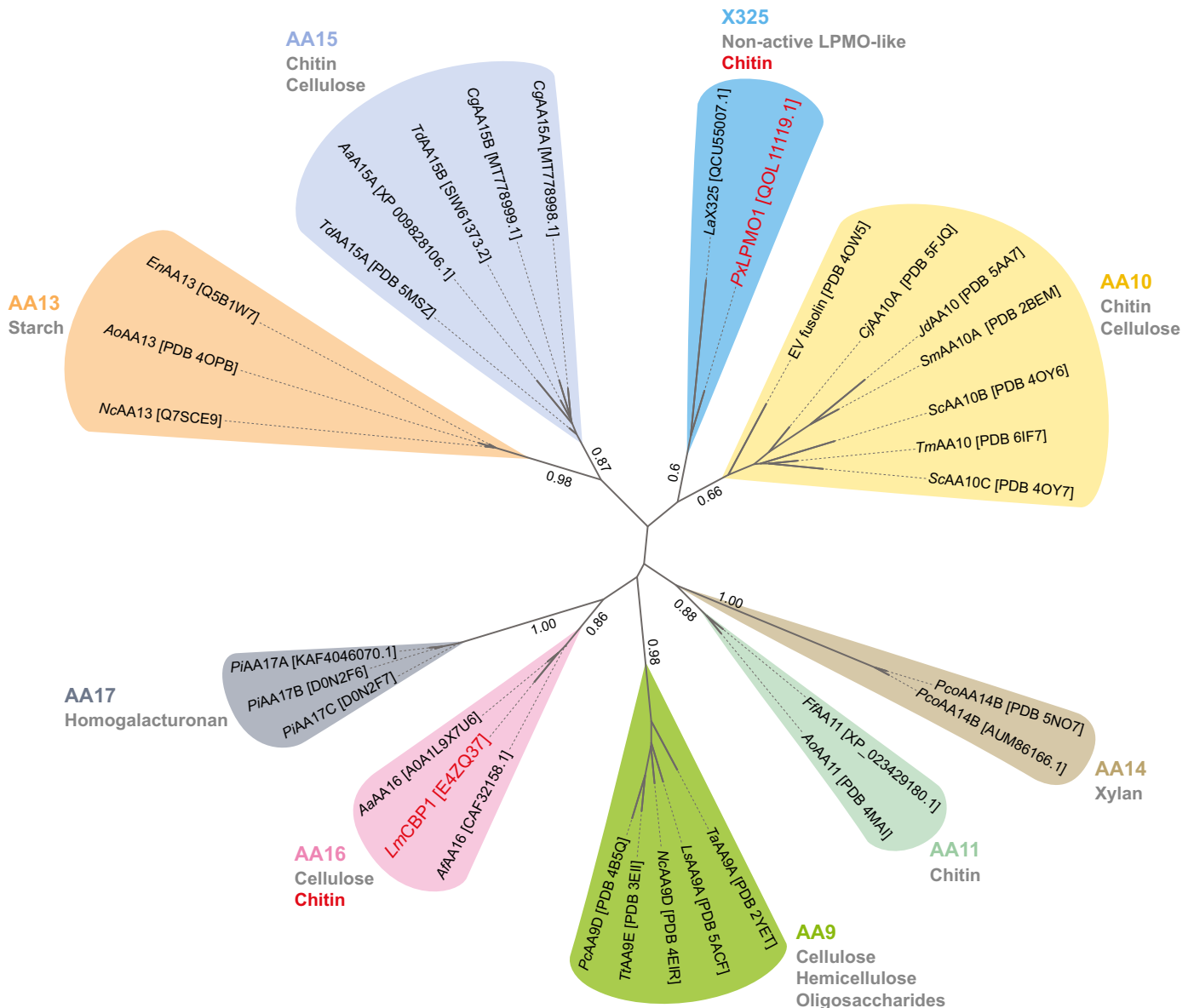

Fig. 4 Phylogenetic tree of lytic polysaccharide monooxygenase (LPMO) families. Sequences of characterised proteins only (accession number or Protein Data Bank (PDB) code provided in the figure) were aligned using T-COFFEE EXPRESSO (Armougom *et al.*, 2006). Phylogeny was inferred using PHYML software (Guindon *et al.*, 2010) and the Whelan and Goldman (WAG) amino acid substitution model (Whelan & Goldman, 2001). Branch support was calculated by 100 bootstrap repetitions (value displayed on tree). The tree was visualised with Interactive Tree Of Life (iTOL) software (Letunic & Bork, 2021). Substrate specificities of each LPMO family are shown in grey (or in red for a few members (printed in red in the tree), in AA16 and X325 families.

Leptosphaeria maculans is a filamentous fungus and the causative agent of blackleg disease in *Brassicaceae* (cabbage, mustard plants). This organism secretes a protein *Lm*CBP1, found by the authors as having an AA10 LPMO domain (our expert analysis contradicts this; described later). *Lm*CBP1 was secreted at high levels during the infection process of *Brassica napus* (Liu *et al.*, 2020), suggesting a role in virulence. Biochemical assays showed that *Lm*CBP1 could bind chitin. Unfortunately, chitinolytic activity was not properly assessed as the reductant was omitted and only chitinase activity was monitored. Our analysis of *Lm*CBP1 catalytic domain sequence indicated that it shares only between 13% and 24% sequence identity with most well characterised AA10 LPMOs. However, structural predictions indicated that it displays a typical fibronectin LPMO fold and possesses the conserved histidine brace. Therefore, there is the possibility that, if cautiously produced and characterised (Eijsink *et al.*, 2019), *Lm*CBP1 could be a functional, chitin-active new LPMO belonging to the AA16 family (see Fig. 4). Additionally, a gene knockout mutant of *Lm*CBP1 demonstrated a decrease in virulence in the host, causing lesser cell death. Furthermore, the LPMO-knockout mutant was more sensitive to H_2O_2 (usually produced by the attacked plant during the so-called oxidative burst) compared with the wild-type strain. This is an interesting observation considering that chitinolytic AA10 LPMOs have been shown to display efficient peroxygenase activity (Bissaro *et al.*, 2017, 2020b; Kuusk *et al.*, 2018). The authors comment that *Lm*CBP1 could be utilised as a means to gain tolerance against H_2O_2, and therefore aid the infection in *Brassicaceae* crops. These results collectively demonstrate LPMOs as agents involved in pathogenesis and, in addition, as potential H_2O_2 scavengers.

In a somehow similar context of pathogen–plant warfare, a recent study by Zarattini *et al.* (2021) used LPMO reaction products to trigger the innate immune response in the model plant *Arabidopsis thaliana*. Precisely, they used the fungal AA9 LPMO from *Thermothielavioides terrestris* to generate a pool of native and (C1/C4) oxidised cello-oligosaccharides (collectively referred to as AA9_COS), which were used to treat plants. Following the treatment with AA9_COS, as well as with cellobionic acid, the plants displayed an increased resistance when exposed to the necrotrophic fungus *Botrytis cinerea*, and were observed to undergo a deep transcriptional reprogramming. In more detail, STRESS INDUCED FACTOR 2 and 4 were seen to play a crucial role and signalled the production of camalexin, an antimicrobial phytoalexin. It was also noticed that the levels of ethylene, jasmonic acid and salicylic acid hormones were increased, along with deposition of callose in the cell wall. This study is unique and displays the possible application of LPMO products (AA9_COS) in triggering the damage triggered immunity (DTI) response in plants (Zarattini *et al.*, 2021). However, more research is needed to pinpoint the exact molecular effector and its related recognition mechanism, including the contribution of the oxidised state of the oligosaccharide (i.e. native vs oxidised oligosaccharides), including its degree of polymerisation and its oxidation site (i.e. C1 vs C4), to making a potent elicitor, all of which currently remain unclear. Furthermore, these results are *seemingly* in contradiction with previously cited studies and recent results on the AA17 LPMO (Sabbadin *et al.*, 2021b) (described later) indicating that LPMO's

action would be aimed at getting through the plant defence barriers. In the Concluding remarks section, we discuss several scenarios that could be envisioned to reconcile these observations. These results allow us to draw parallels to previously described studies demonstrating (native) chitin oligomers as agents that induce plant immune response and to their detailed mechanisms. Also, this study makes us wonder about the evolutionary relationship, if any, between LPMO-derived oxidised oligosaccharides and plant cellular receptors (Liu *et al.*, 2012; Shinya *et al.*, 2015). Interestingly, we underscore that the secretion of chemicals by the plant could also be used as a means to regulate fungal LPMO activity, as a very recent report has shown that some plant extracts (containing cinnamtannin B1) acted as a potent LPMO inhibitor (Tokin *et al.*, 2021).

2. Role of LPMOs in pathogenic oomycetes

Oomycetes are a wide group of filamentous, fungal-like microorganisms phylogenetically related to diatoms and brown algae, and causing various severe diseases in agriculture, aquaculture, as well as in natural ecosystems (Derevnina *et al.*, 2016). Oomycete genomes encode three LPMO families (AA15, AA16 and AA17). Sabbadin and colleagues have shown that the array of LPMOs depends on the host preference (Sabbadin *et al.*, 2021a): animal pathogens preponderantly harbour AA15s and AA17s, while plant pathogens mostly have AA16s and AA17s. In 2021, Sabbadin and colleagues reported on the role of an AA15 from the crayfish pathogen *Aphanomyces astaci*. Through the analysis of publicly available transcriptomic data, they showed that one of its AA15 LPMO (called *Aa*AA15A)-encoding genes was differentially expressed in both sporulating and growing mycelia, compared with the zoospore stage (Sabbadin *et al.*, 2021a). *In vitro* biochemical analyses proved the chitinolytic activity of this enzyme. Complete demonstration of *in vivo* function (and substrate) remains, however, to be made. In a second study, still by Sabbadin and coworkers, on plant infection by oomycetes, gene silencing of an AA17 from *P. infestans* (*Pi*AA17C) yielded a significant decrease in lesion sizes on infected potato leaves (Sabbadin *et al.*, 2021b). Combination of biochemical, structural, transcriptomic and *in vivo* experiments unambiguously supported the role of this AA17 in early stages of the *P. infestans* pathogenic cycle, with two putative, nonexclusive functions: (1) contribution to PCW penetration through the oxidation of de-esterified pectin; and/or (2) by evading plant immune system recognition by oxidising pectin-derived oligosaccharides, otherwise recognised as pathogen associated molecular patterns (Benedetti *et al.*, 2018).

3. Viral LPMO as enhancer for microbial insecticide action

Insect pests have been the cause of damage in agriculture since the beginning of the latter, calling therefore for protection measures, including the use of environmentally friendly insecticides. Microbial insecticides, despite being deemed safe for vertebrates, constitute only a small fraction of the total insecticide market (offering mainly chemical products), due to the higher associated costs, narrow spectrum of targeted insects per product, possible

inactivation in the field due to high temperatures and UV radiation and very selective and focused timely application needed for their use (Mitsuhashi, 2018). A suggested mechanism to increase the use of microbial insecticides is the use of synergistic agents that can enhance the infectivity of microbes therefore also reducing the quantity of insecticide used per square area (Mitsuhashi, 2018). Fusolins, produced by insect viruses (entomopox viruses (EVs) and baculoviruses) have been studied for this purpose (a synergistic agent) and have been known to enhance the insecticidal activity of the bacterium *Bacillus thuringiensis* (Bt) (Liu *et al.*, 2011). Spindles are crystallised EV fusolins seen in host cells, and are spindle-shaped microparticles (inclusion bodies) that are extremely stable. Fusolins and spindles both are seen to enhance EV infectivity (Mitsuhashi & Sato, 2000; Mitsuhashi *et al.*, 2007; Takemoto *et al.*, 2008). Strikingly, the globular domain found in fusolin possesses all the features of chitin-active AA10 LPMOs (Chiu *et al.*, 2015), albeit LPMO activity remains to be properly demonstrated by biochemical assays. The mechanism by which these LPMO-containing fusolins enhance microbial infectivity is proposed to be as follows: orally taken fusolins bind to the peritrophic matrix (PM, a chitin rich acellular membrane lining the gut of insects), oxidise its chitin fibrils and alter the PM conformation (Mitsuhashi, 2018). Next, these conformational changes permit the digestive proteases of the gut to further act and digest the PM, which was previously inaccessible. The PM disruption is accelerated jointly by the fusolins and proteases, and this makes any microbes or microbial toxins (such as Bt toxins) easy to pass through the PM and reach the midgut cells. Therefore, fusolins and spindles may be utilised as synergistic agents with microbial insecticide formulations, therefore reducing the use of microbial insecticides sprayed per unit area. Coexpressing fusolins with Bt toxin in crops is another way to increase insect resistance in crops (Liu *et al.*, 2011; Mitsuhashi *et al.*, 2014).

4. Insecticidal activity by a fern LPMO

It has long been noted that ferns and mosses are rarely affected by pathogenic pests or insects compared with flowering plants. Studies using extracts from the ferns *Christella parasitica*, *Pteridium aquilinum* (Eagle fern) and *Hemionitis arifolia* (Heart fern) have demonstrated that, when sprayed on *Arachis hypogaea* (commonly known as peanut plant) they can confer resistance against pests such as the moths *Helicoverpa armigera* (Old World bollworm) and *Spodoptera litura* (tobacco cutworm) (Hendrix, 1980; Sahayaraj & Selvaraj, 2013). Drawing inspiration from this, Shukla *et al.* (2016) screened several species of ferns and identified the protein Tma12 from the edible fern *Tectaria macrodonta*, which displayed insecticidal activity against whitefly, *Bemisia tabaci*. Tma12, when cloned and expressed in transgenic cotton plants (*Gossypium hirsutum*) showed 99% resistance against whitefly. Following this, a later study relying on sequence analysis, X-ray crystallography and H_2O_2 production by Tma12 showed that Tma12 possessed all the hallmarks of an AA10 LPMO (although chitinolytic activity was not assessed), which would make it the first, and so far unique, plant LPMO (Yadav *et al.*, 2019). In a separate study trying to delineate the evolution of land plants and their symbiotic relationships with

cyanobacteria using fern genomes, the authors have observed Tma12 homologues in 1000 other fern transcriptomes and phylogenetic analysis showed Tma12 to be closely related to chitin-binding proteins from bacteria. This raises the question whether these sequences were obtained by horizontal gene transfer (HGT) from bacterial species at some point during evolution as a mechanism to survive pest attack. Although this cannot be denied completely and functional HGT has been observed in other eukaryotes, there is no direct evidence for the same (Li *et al.*, 2018). These studies echo the general consensus that LPMOs could possess a wide range of functions – insecticidal, in this case – in addition to biomass conversion and an avenue to study if any HGT of LPMO genes from bacteria does exist.

5. LPMOs in mutualistic and commensalistic symbiosis

In the previous paragraphs, we have presented several cases of parasitic symbiosis. There are also situations in which the organisms at play can establish a biological interaction with mutual interest, so-called mutualistic symbiosis. The association between fungi and tree roots falls into this lifestyle. While the fungus obtains sugars from the plant, the latter receives trace elements, such as nitrogen and phosphorus, from the former that weaves a web of hyphae in the soil. Of note, most land plants on Earth rely on such mutualistic symbiosis (Martin *et al.*, 2017). In an article by Veneault-Fourrey *et al.* (2014), the authors studied the transcriptome of *Laccaria bicolor*, an ectomycorrhizal fungus – which does not penetrate the plant cells themselves, in contrast with endomycorrhiza – during symbiosis with the California poplar *Populus trichocarpa*. The study revealed that three of the genes coding for AA9 LPMOs were expressed during the period of first contact and in the final period, that is during the formation of the Hartig network. A soft remodelling of the PCW is likely to occur via the concerted 'loosening action' of AA9 LPMOs, GH5 and GH12 endoglucanases (Zhang *et al.*, 2018), together with the modification of pectin in the middle lamella by other CAZymes. Intriguingly, LPMO-like X325 proteins presented above were shown (by immunolocalisation) to be exposed at the interface between fungal hyphae and tree rootlet cells (Labourel *et al.*, 2020). The copper transport function of some X325 orthologues (BIM1 story, described earlier), and the importance of copper trafficking in mycorrhizae, to notably prevent transition metal toxicity, could pinpoint at a role of X325 in establishing a safe symbiotic interface. In agreement with this hypothesis, SymB, an X325 protein from the endophytic fungus *Epichloë festucae* was shown to be a key element of a signalling network impacting hyphal cell fusion and involved in the maintenance of symbiotic interaction between *E. festucae* and the perennial ryegrass *Lolium perenne* (Green *et al.*, 2017).

LPMOs have also been proposed to be involved in a third type of symbiosis, called commensalism, during which (usually) only the host gains benefit from the interaction. For instance, the woodwasp *Sirex noctilio*, a pathogen of pine trees in North America, was reported to be assisted by bacteria belonging to the genus *Streptomyces* that provides the wasp with *ad hoc* tools, such as cellulases, to feed on the tree (Adams *et al.*, 2011). Later, Takasuka

et al. (2013) studied the *Streptomyces* sp. SirexAA-E (ActE) strain associated with the wasp, and found the presence of an AA10 LPMO (called CBM33 in the article) responsible for the degradation of cellulose and hemicellulose. Of note, while the exact mechanism of the symbiosis between *Streptomyces* and *Sirex noctilio* is not explained, the author implied that it may be widespread among insects. Another interesting example is the symbiotic relationship between the bacterium *Teredinibacter turnerae* and the marine caterpillar *Lyrodus pedicellatus*. Like *Streptomyces*, *T. turnerae* produces an AA10 LPMO that is active on cellulose, providing *L. pedicellatus* with wood digestion capacity (Fowler *et al.*, 2019).

6. Lytic polysaccharide monooxygenases in bacterial pathogenesis

Host–pathogen warfare is usually depicted as an arms race in which each antagonist attempts to be smarter than the other. Whether the pathogen plays the false friend card (e.g. biotrophic pathogen) or clear enemy card (e.g. necrotrophic), a very common strategy deployed during early events of bacterial pathogenesis consists of impairing the host defence mechanisms via a series of (enzymatic) reactions (Medie *et al.*, 2012).

Studies on LPMOs from bacteria such as *Vibrio cholerae*, *Paenibacillus larvae*, *Listeria monocytogenes*, *Pseudomonas aeruginosa* and *Lysobacter enzymogenes* have shown this class of enzymes to be involved in pathogenesis and virulence roles (Garcia-Gonzalez *et al.*, 2014; Loose *et al.*, 2014; Paspaliari *et al.*, 2015; Askarian *et al.*, 2021; Yue *et al.*, 2021). As virulence factors, LPMOs may be studied as potential targets for synthesis of inhibitors against the pathogen.

The causative agent of cholera, *V. cholerae*, has been studied widely and it is known that its survival in the intestine is largely dependent on its ability to adhere to and colonise the intestinal epithelial cell surfaces (Yoon & Waters, 2019). *Vibrio cholerae* secretes, via a type-2 secretion system, a tetra modular protein called GlcNAc binding protein A (GbpA), composed of an N-terminal AA10 domain, two domains involved in bacterial cell-surface binding, thereby enabling microcolony formation, and a C-terminal CBM5/12 domain capable of binding to crystalline chitin that permits the bacteria to attach to and colonise a wide range of crustaceans (Wong *et al.*, 2012; Yoon & Waters, 2019). In addition to this, GbpA was also found to oxidatively degrade various forms of chitin. GbpA-like proteins were also seen to occur in several other pathogenic bacteria, such as *Listeria monocytogenes*, *Bacillus cereus* and *Yersinia pestis*, further supporting their role in virulence (Loose *et al.*, 2014).

Following this, another interesting study was performed in *Paenibacillus larvae*, the causative agent of the epizootic American Foulbrood affecting honeybees, with a worldwide dramatic ecological impact (Genersch, 2008). This infection that kills honey bee larvae has been widely investigated and Garcia-Gonzalez and colleagues have presented evidence of an AA10 LPMO domain-containing protein, *Pl*CBP49, to be a key virulence factor of *P. larvae* (Garcia-Gonzalez *et al.*, 2014). To invade the host, the bacterium needs to weaken or degrade the PM, therefore enabling the direct access of bacterial toxins to the gut epithelial cells of the

honeybee larvae. *Pl*CBP49 was demonstrated to have chitinolytic activity on crystalline chitin and colloidal chitin and was inhibited by copper chelators (e.g. EDTA or cyanide). Using Δ*Pl*CBP49 mutants, the authors could show that the bacterial virulence was significantly reduced (95% survival rate of larvae), and the PM was virtually unaltered, therefore cementing *Pl*CBP49 as a key virulence factor of *P. larvae* (Garcia-Gonzalez & Genersch, 2013; Garcia-Gonzalez *et al.*, 2014).

Listeria monocytogenes, causing the deadly foodborne disease listeriosis, is the next bacterial pathogen under discussion. This organism possesses a complete chitinolytic machinery comprising two GH18 chitinases, ChiA and ChiB, and a multimodular *Lm*LPMO10 (Paspaliari *et al.*, 2015). *Lm*LPMO10 is a 'Gbp' type protein with the ability to bind to α- and β-chitin and cellulose, but active only on α- and β-chitin yielding C1-oxidised products. Unlike chitinases, *Lm*LPMO10 secretion does not increase in a chitin-containing medium, suggesting that its role is more than biomass degradation, most likely in the realm of pathogenesis and virulence, by mainly enabling bacterial adhesion to the host cell (DebRoy *et al.*, 2006; Kawada *et al.*, 2008; Tran *et al.*, 2011; Frederiksen *et al.*, 2013). Single gene deletion mutants of ChiA, ChiB and *Lm*LPMO10 of *L. monocytogenes* showed significant reduction in their ability to colonise murine liver and spleen compared with the wild-type strain (Chaudhuri *et al.*, 2010). This further supports the role of these enzymes as virulence factors. Experimental data unravelling the detailed mechanism of action remains to be uncovered.

In a very recent study conducted by Askarian and colleagues on *Pseudomonas aeruginosa*, a multidrug-resistant pathogen causing various infections (e.g. pneumonia or blood infection), the chitinolytic AA10 LPMO, CbpD, was found to promote the survival of the pathogen in host blood, acting therefore as a virulence factor. Deletion mutants of CbpD rendered the pathogen less lethal. Also, Δ*CbpD* mutants were more prone to cell lysis by the host 'membrane attack complex' (MAC) and it has been proposed that bacterial CbpD would impair the assembly of a functional host MAC. While the exact molecular mechanism of this phenomenon is yet to be established, these data once again extend the functions of LPMOs from biomass-degrading catalysts to key pathogenicity players (Askarian *et al.*, 2021).

Lysobacter enzymogenes is an interesting bacterium with the ability to produce bioactive metabolites against a wide spectrum of fungal pathogens of plants and humans. A chitinolytic AA10 LPMO secreted by *L. enzymogenes* (*Le*LPMO10A) was seen to induce the production of antimicrobial compounds called 'heat stable antifungal factor' and its analogues (Yue *et al.*, 2021). The authors further suggested a mechanism of action, yet to be fully demonstrated, for *Le*LPMO10A: the LPMO would cleave the recalcitrant chitin present in the fungal pathogen cell wall, enabling thereby the delivery of the antifungal factors via outer membrane vesicles.

7. Role of LPMOs in development and cell wall remodelling

Insects mainly feed on plant substances and the efficient digestion of the latter is therefore critical for their development. This

degradation process is achieved by their intestinal enzymes and/or the symbiotic microbes therein.

The first characterised LPMO from insects was from *Thermobia domestica* (firebrat) and became the founding member of the CAZy AA15 family (Sabbadin *et al.*, 2018). Members of this family were also observed in the genomes of several protists, crustaceans, mollusks, chelicerates, algae and oomycetes (Sabbadin *et al.*, 2018). Expressed as transcripts per kilobase million (TPM) at medium (10 < TPM < 100), high (100 < TPM < 1000) or very high (TPM > 1000) levels, these LPMO-encoding genes were seen to be mostly present in the midgut region. As *T. domestica* is devoid of any gut microbiome with polysaccharide degrading capacities, AA15s were suggested to play a crucial role in this process. Biochemical characterisation of two paralogues, *Td*AA15A and *Td*AA15B, showed that the former could oxidise both microcrystalline cellulose and β-chitin, while the latter was active on β-chitin only. Furthermore, *Td*AA15A, the most abundant (expression-wise) AA15 LPMO from *T. domestica*, was seen to synergise with cellulases (GH1, GH6, GH7, GH9) and chitinases (GH18), increasing therefore the yield of oligosaccharides released from cellulose or chitin, respectively (Sabbadin *et al.*, 2018).

The AA15 class of LPMOs is conserved from protists to oomycetes to insects, and were suggested to have a pre-Cambrian origin and therefore were proposed to furnish a possible explanation for the thriving of insects during the Carboniferous period (heavily colonised by plants) with the help of their gut endogenous lignocellulolytic enzymes (Sabbadin *et al.*, 2018). In addition to shotgun proteomics analysis, the analysis of public transcriptomic databases revealed that AA15s from *Drosophila melanogaster*, *Dm*AA15A and *Dm*AA15B, were expressed at high levels during different stages of development and during metamorphosis, and were seen to be coexpressed with genes encoding chitinolytic activities, indicating their involvement in chitin remodelling within the respiratory and digestive systems. Significantly, important phenotypic changes were observed in several *Drosophila* when AA15-encoding genes were silenced or knocked down, including defects in respiration, changes in adult morphology and lethality during pupation (Mummery-Widmer *et al.*, 2009; Hosono *et al.*, 2015). Sabbadin and colleagues proposed that the chitinolytic activities of the early insects of the order *Zygentoma,* initially used for developmental reasons, were later evolved towards cellulolytic functions (Sabbadin *et al.*, 2018; Cairo *et al.*, 2020). Here, one may draw an intriguing parallel with AA10s, in which strict chitinolytic (Vaaje-Kolstad *et al.*, 2010), strict cellulolytic (Forsberg *et al.*, 2011) and hybrid activities have been reported (Forsberg *et al.*, 2014). Indeed, an *in silico* analysis of mutation rate within the AA10 family suggested that chitinolytic AA10s would have preceded the others, which would be in the process of evolving towards different substrate specificities (Book *et al.*, 2014). The biological role of AA10s is, however, still not entirely clear, and may be context dependent.

Cell wall remodelling is a crucial aspect of microorganism life cycle, notably of fungi, which can form different cell types (spores, yeast cells and hyphae) to thrive and explore their environment. Studying the fungus *Neurospora crassa*, Gonçalves and colleagues have shown that a gene, called *cwr-1* (for cell wall remodelling),

together with another gene (*cwr-2*), was involved in a so-called cell fusion checkpoint upon cell-cell contact: that is, regulation of cell wall dissolution, blocking cell fusion upon contact between cells with nonidentical genetic backgrounds. Such regulation is important for reducing the risks of transmission of mycoviruses, senescence plasmids or defective organelles (Gonçalves *et al.*, 2019). *In vivo* inactivation of *cwr-1* altered this regulation and allowed cell fusion between formerly incompatible strains. Strikingly, sequence analysis of *cwr-1* indicated that it was most likely to be an LPMO, belonging to the chitin-active AA11 family (Gonçalves *et al.*, 2019) as it shared 44% sequence identity with its founding member, the AA11 from *Aspergillus oryzae* (Hemsworth *et al.*, 2014).

IV. Concluding remarks and future perspectives

Through this review, we have attempted to highlight how diverse can be the biological functions of LPMOs (Fig. 5). It is truly fascinating how such small, and rather structurally simple proteins have been co-opted by a wide array of living organisms (and viruses) to target nearly all major carbohydrate polymers on Earth. From the C1–Cx factors of Reese & Gilligan (1954) to the discovery of several LPMO families, the research in this area has come a long way. Whereas a decade of LPMO-dedicated research has been necessary to unveil the actual biotechnological potential, biological scope and mode of action of these copper-enzymes secreted by saprotrophic microorganisms (plant biomass-decaying and chitinivorous fungi and bacteria), new facets of LPMO research, including symbiotic relationships (parasitism, mutualism and commensalism), viral infections and developmental biology start to unfold. Excitingly, in regard to this plethora of arenas, much remains to be investigated and understood. While the LPMOs from viruses, bacteria and fungi showcase themselves to be crucial virulence factors, the LPMOs acting as insecticidal agents and those aiding insect development present them to be dextrous proteins with wide-ranging abilities.

A major challenge, and still a lacuna, in LPMO research is the paucity of work on substrates in their natural state (which often remain to be clearly identified). In general, the LPMO community usually assesses LPMOs on a range of (commercially) available substrates, which in many cases may fail to catch the right LPMO–substrate couple. Also, the recent X325/Bim1 story (Garcia-Santamarina *et al.*, 2020; Labourel *et al.*, 2020) teaches us that we should look beyond the only prism of oxidative activity, as other functions, such as in this case copper transport, may have evolved.

Among several outstanding questions (Box 2), an intriguing observation is the high number of LPMO gene copies in fungal and oomycetes genomes. While it could suggest a crucial importance of these proteins for these microorganisms, the exact biological role/significance of this gene multiplicity is still not clear. By contrast, the seemingly virtual absence of LPMOs in plants (excepted in ferns) is another area that has not been tapped into. This question leads us also to reflect on the warfare between plants and pathogens (fungi, oomycetes or insects), and the role of LPMOs in the latter. To breach the PCW, pathogens will secrete PCW polymer-active LPMOs and hydrolases, releasing thereby (oxidised) oligosaccharides. Depending on the evolutionarily advancement in the arms race
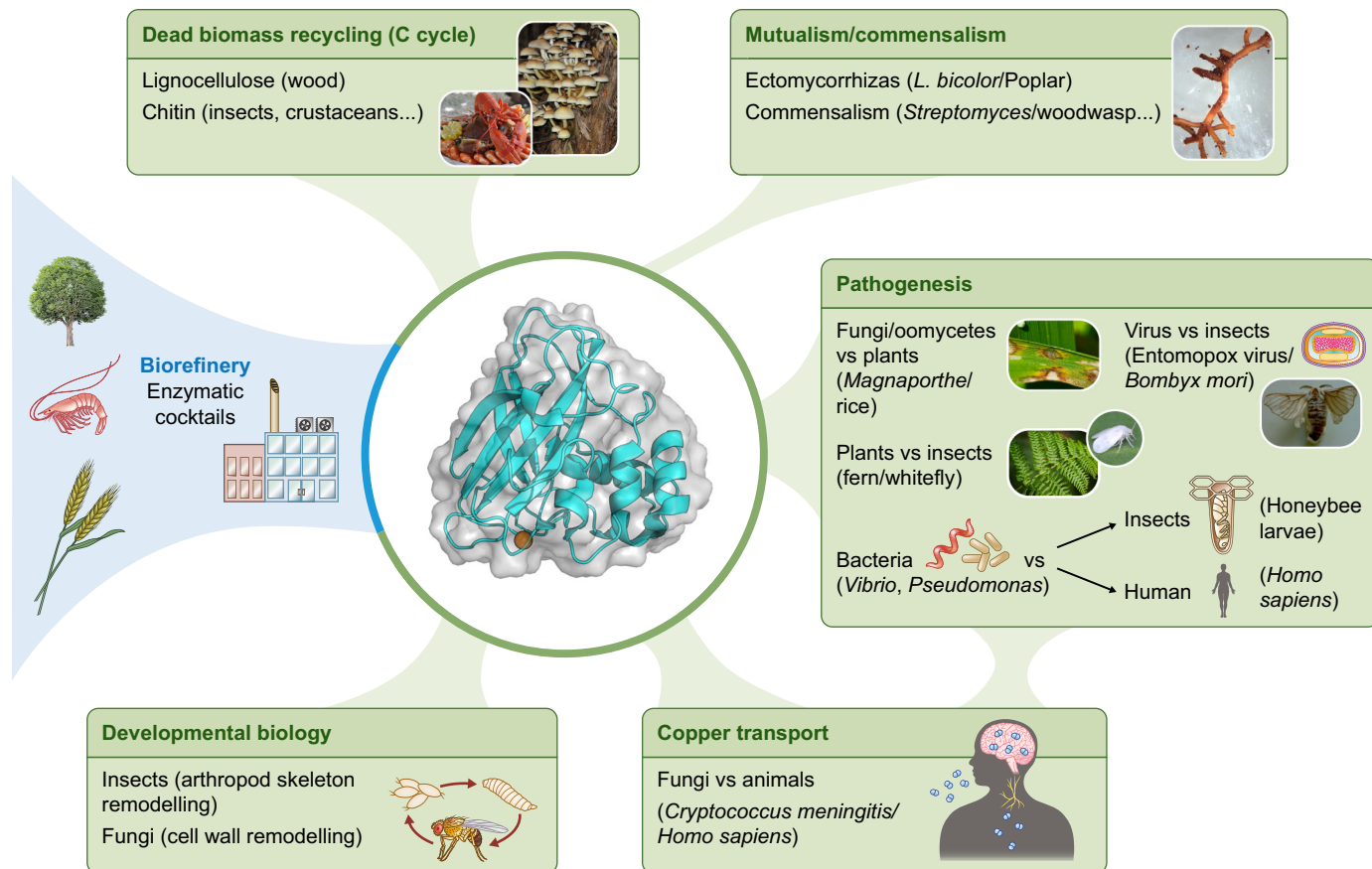
Fig. 5 Overview of the diversity of lytic polysaccharide monooxygenase (LPMO) biological functions. Historically, LPMOs have mainly been harnessed for their boost effect in biorefinery processes (blue zone) and have therefore primarily been studied in natural systems related to biomass degradation and carbon recycling. Lytic polysaccharide monooxygenases have more recently been observed to play crucial roles in pathogenesis in plants, insects and humans, while also enabling some mutualistic and commensalistic behaviours. Another emerging role is their suspected contribution cell wall remodelling during the developmental stages of arthropods and fungi.

Box 2 Outstanding questions

• Why do some microorganisms, especially plant pathogens (e.g. fungi, oomycetes), have dozens of LPMO gene copies?

• What about plant LPMOs? Why are (canonical) LPMOs absent from most plant genomes? Why, like ferns, did other plants not acquire chitin-active LPMOs to defend from fungi/insects? Did they evolve more efficient strategies?

• Does the LPMO oxidative regioselectivity (C1 vs C4) play an important role in the plant–microorganism interaction? Does it have any other biological relevance?

• On substrate specificity of LPMOs: have we already discovered all substrate specificities? What is the evolutionary relationship between *and* within the different families? As proposed for AA10s, could chitinolytic LPMOs be the overall ancestral members?

• On cosubstrate specificity: do LPMOs use H_2O_2 or O_2 in a biological context? What is their respective bioavailability? Do they use it to the same extent? How critical is the cosubstrate nature in the plant–pathogen warfare (oxidative burst)?

(e.g. if the plant is equipped with the right receptors, etc.), these molecular clues of pathogen action can be recognised by the plant, triggering therefore its immune response. Oxidised cello-oligosaccharides have been proposed to induce such response (Zarattini *et al.*, 2021) but it appears rather puzzling, in as much as LPMOs have also been proposed to be used by pathogens to hide their presence by oxidising oligosaccharides. To reconcile these seemingly contradictory observations, we hypothesise that LPMO oxidative regioselectivity (C1 vs C4) and substrate specificity (oligosaccharides vs polysaccharides) needs to be carefully considered. We underscore that C4-oxidising LPMOs are often active on oligosaccharides. Of note, the converse statement is not true as, in a very recent report, Rieder and coworkers showed that an LPMO from *Aspergillus fumigatus* (*Af*AA11B) was active on chito-oligosaccharides (GlcNAc)$_4$ (and hardly active on crystalline chitin substrates), while displaying a C1 oxidative regiospecificity (Rieder *et al.*, 2021). Nonetheless, we think that, in the vast majority of cases, C1-oxidised products can be seen as the mere consequence of the combined action of fungal polymer-active LPMOs and hydrolases, possibly used as hints to inform the plant that its cell wall is under

attack, By contrast, the oligosaccharide C4-oxidising LPMOs may be instrumental as *ad hoc* tools to 'mask' oligosaccharides derived from the action of fungal hydrolases on PCW. This hypothesis would require that C1- and C4-oxidised LPMO products are not recognised in a similar way by the plant and hints at possible new biological implications of LPMOs beyond the oxidation of polysaccharide substrates. This remains, however, to be evaluated.

Another crucial aspect pertains to the role of H_2O_2, a double-edged sword, yet central molecule. Conversely, it has been shown that H_2O_2 can efficiently fuel LPMO oxidative activity, while it can also inactivate them if used at too high a dosage (Bissaro *et al.*, 2017). In addition, H_2O_2 and other ROS are known to be secreted in abundance (oxidative burst) by plants when attacked, to an extent that depends on the host–pathogen couple (Gupta *et al.*, 2021). We believe the study of ROS fluxes, and associated redox enzymes, in the plant–microorganism interaction is indubitably a research topic of utmost importance.

Beyond their instrumental role in dead biomass decomposition, it is also suggested that LPMOs are involved at the other end of the carbon cycle, *viz.* in the establishment of the dialogue between living trees and mycorrhizal fungi, possibly in copper homeostasis and/or cell wall remodelling (Veneault-Fourrey *et al.*, 2014; Labourel *et al.*, 2020; Lebreton *et al.*, 2021). We anticipate that more research should be carried out in this direction to better seize the actual scope of action of LPMOs in carbon cycling in terrestrial ecosystems.

Overall, we have seen that trans-disciplinarity is the key to understanding the function of these different LPMO families and to obtain insights into their (co)substrate specificities, biological relevance and, in the future, unveil their biotechnological power. Following what has been achieved in the field of biorefinery, in which LPMOs have been harnessed to boost saccharification processes, we hope that our review will contribute to assessing the promising biotechnological potential that LPMOs still have to offer, notably to address some major issues in the health and agriculture sectors.

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