Cell wall–associated effectors of plant-colonizing fungi

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ABSTRACT

Plant-colonizing fungi secrete a cocktail of effector proteins during colonization. After secretion, some of these effectors are delivered into plant cells to directly dampen the plant immune system or redirect host processes benefitting fungal growth. Other effectors function in the apoplastic space either as released proteins modulating the activity of plant enzymes associated with plant defense or as proteins bound to the fungal cell wall. For such fungal cell wall–bound effectors, we know particularly little about their molecular function. In this review, we describe effectors that are associated with the fungal cell wall and discuss how they contribute to colonization.

INTRODUCTION

Fungal plant pathogens represent important threats to agriculture and have severe consequences for food security (Fisher et al. 2012). Due to the emergence of resistance to commonly used antifungals, the search for new targets needs to be intensified. Secreted effectors, which contribute significantly to disease severity, could become promising candidates for such developments. Besides becoming infected by fungal pathogens, most land plants are colonized by plant–beneficial mycorrhizal and endophytic fungi, which can provide plants with mineral nutrients and protect from stress and pathogens (Bonfante and Genre 2010; Yan et al. 2019; Tedersoo et al. 2020). Also, in these cases are fungal effector molecules central to establishing a functioning symbiosis (Porras-Alfaro and Bayman 2011; Lo Presti et al. 2015; Kothe and Turnau 2018).

Fungal cells are encased by the fungal cell wall, a complex structure of interconnected polysaccharides and proteins. In general, the inner layer of the fungal cell wall is composed of a highly cross-linked chitin–glucan matrix consisting of chains of β-1,3- and β-1,6-glucan followed by an outer layer rich in mannosylated proteins (Fig. 1). In the fungal cell wall, chitin, a polymer of β-1,4-linked N-acetylglucosamine, assembles into microfibrils due to hydrogen bonding between chains and adopts a crystalline structure (Geoghegan et al. 2017; Gow et al. 2017). Upon contact with the host plant, fungal invaders are recognized via conserved microbe-associated molecular patterns (MAMPs). Such fungal patterns can be chitin or β-glucan oligosaccharides, which are either released during synthesis or freed through the action of host chitinases and glucanases, respectively (Sánchez-Vallet et al. 2015; Fesel and Zucaro 2016). Alternatively, plant degradation products (damage-associated molecular patterns, DAMPs) resulting from enzymatic attack by invading fungi, or endogenous peptides, constitutively present or newly synthesized following fungal colonization, are recognized by the immune system via pattern recognition receptors (Boller and Felix 2009). MAMPs and DAMPs elicit pattern-triggered immunity (PTI), which prevents colonization of nonadapted pathogens and provides for basal resistance by restricting infection of adapted pathogens in susceptible hosts (Couto and Zipfel 2016; Saijo et al. 2018). PTI is associated with the production of antimicrobial secondary metabolites, and the synthesis of defense-related proteins or peptides is associated with antimicrobial activity. Additionally, reactive oxygen species (ROS), which have toxic effects on fungi and provide for cell wall cross-linking, are generated, and lignification as well as the synthesis of callose can limit spreading of fungal hyphae in the infected tissue. A programmed local cell death response, referred to as hypersensitive response, can also restrict progression of these pathogens, which require living plant tissue for proliferation (Bigard et al. 2015; Miller et al. 2017; Hou et al. 2019). Successful pathogens overcome PTI by deploying their large arsenal of secreted effectors. In addition, they use effectors to manipulate host processes to support fungal growth. Many fungal effectors are
conventionally secreted via the endoplasmic reticulum (ER)-to-Golgi route and either function in the plant apoplast or are transferred to the plant cytosol via yet unknown mechanisms. Transferred effectors act in discrete cellular compartments and modulate processes such as transcription, protein synthesis, protein secretion, protein stability, the synthesis of secondary metabolites, signaling pathways, and epigenetic regulation (Lo Presti et al. 2015; Toruño et al. 2016).

Due to the presence of chitinases, glucanases, proteases, and other enzymes attacking fungi as well as acidic pH conditions, the apoplast is a hostile environment for fungal growth. To survive in this environment, fungi use apoplastic effectors to inhibit, destroy, or act as decoys for these enzymes.

Probably the most widespread class of apoplastic effectors are LysM (lysine motif) proteins. LysM effectors contain up to seven LysM motifs, a carbohydrate-binding module family 50 (CBM50) domain. The LysM domain consists of approximately 40 amino acids and forms a small globular structure that binds to N-acetylgalcosamine-containing carbohydrates, including chitin (Buist et al. 2008; Mesnage et al. 2014). The major function of LysM effectors in the apoplastic space is to sequester chitin fragments released during a fungal infection to circumvent the perception of chitin fragments as MAMPs by plant immune receptors (de Jonge and Thomma 2009; Sánchez-Vallet et al. 2015). Ecp6 from the tomato leaf mold pathogen Cladosporium fulvum (Bolton et al. 2008; de Jonge et al. 2010; Sánchez-Vallet et al. 2013), Slp1 from the rice blast pathogen Pyricularia oryzae (= Magnaporthe oryzae) (Mentlak et al. 2012), and Mg3LysM from the wheat pathogen Zymoseptoria tritici (= Mycosphaerella graminicola = Septoria tritici) (Marshall et al. 2011) are prominent and extensively studied LysM effectors. Similarly, a catalytically inactive form of the glycoside hydrolase family 18 (GH18) chitinase in Moniliformis perniciosa can also sequester chitin fragments (Fiorin et al. 2018).

The release of chitin oligosaccharides from the fungal cell wall can also be dampened by fungal chitinase inhibitors or degradation of chitinases by secreted fungal-syn metallo- or serine protease effectors (Jashni et al. 2015; Ökmen and Doehlemann 2016; Rocafor et al. 2020). Other classes of apoplastic fungal effectors are inhibitors of plant serine proteases and papain-like cysteine proteases, which can destroy fungal proteins with critical roles for virulence (Jashni et al. 2015; Rocafor et al. 2020). Apoplastic effectors that inhibit plant peroxidases can negatively impact ROS production (Hemetsberger et al. 2012), and plant-derived compounds, including signaling or antifungal molecules, can be degraded by secreted fungal enzyme effectors (Ökmen et al. 2013; Nizam et al. 2019). The repertoire of plant defense compounds also includes antifungal proteins such as kiwelines that are secreted to the apoplast after infection and which can be inhibited by apoplastic fungal effectors (Han et al. 2019; Altegoer et al. 2020). Rapid alkalinization factor secreted by Fusarium oxysporum facilitates apoplastic alkalinization, with beneficial consequences for virulence (Masachis et al. 2016; Thynne et al. 2017). In line with the ongoing battle between fungi and their hosts in the apoplast (Jashni et al. 2015), the bioinformatics tool ApoplastP predicts that plant-colonizing fungi encode highly variable numbers of apoplastic effectors, ranging from 28 in Ustilago hordei to 435 in Melampsora lariis-populina (Saperschneider et al. 2018). Whether this is connected to differences in plant colonization strategies is currently unknown.

Since the fungal cell wall is contacting the host plant first, it is no surprise that fungal invaders of plants have also developed strategies to alter their cell wall to avoid being recognized by the plant immune system. One of these is to alter the composition of the cell wall itself by converting chitin to chitosan with the help of specific chitin deacetylases. Chitosan is a relatively poor substrate for chitinases, leading to diminished release of...
chitin oligomers, which can trigger defenses (Hadwiger 2013). In several biotrophic fungi, it has been observed that in planta hyphae are covered by a layer of chitosan (El Gueddari et al. 2002; Geoghegan et al. 2017). However, it is not yet known whether such layers have a shielding function. In Verticillium and Fusarium species, virulence is reduced when genes encoding specific secreted chitin deacetylases are deleted, and it is hypothesized that these enzymes deacetylate chitin oligomers in the apoplast, which reduces their ability to trigger defense responses (Gao et al. 2019). In several fungal plant pathogens, α-1,3-glucan biosynthetic genes are up-regulated during host colonization. Because α-1,3-glucan is refractory to plant-produced enzymes, α-1,3-glucan in the outer layers of the cell wall can act as a shield molecule providing protection from enzymatic degradation (Fujikawa et al. 2009; Yoshimi et al. 2017). In P. oryzae, the α-1,3-glucan synthase gene, MgAGS1, is essential for virulence (Fujikawa et al. 2012; Otaka et al. 2016). β-Glucan synthesis, on the other hand, is strongly down-regulated in biotrophic hyphae of Colletotrichum graminicola, allowing evasion of β-glucan-triggered immunity (Oliveira-Garcia and Deising 2013, 2016a).

Besides structural alterations of the fungal cell wall during host colonization, fungi also secrete protein effectors, which bind to the fungal cell wall and provide direct protection in their cell wall–bound form from antimicrobial plant enzymes, antifungal proteins, as well as phenolic compounds. In this review, we will focus on cell wall modifications attributed to fungal effector proteins and how binding of such effectors provides stealth or changes certain cell wall properties essential for colonization.

**CHITIN-BINDING PROTEINS ASSOCIATED WITH THE FUNGAL CELL WALL**

The cell wall–protective role of fungal effectors is commonly demonstrated by localization studies using either fluorescently labeled proteins or immune-electron microscopy (immuno-EM) and by functional assays in which it is demonstrated that the effector protects hyphae from enzymatic attack. Fungal cell wall–bound effectors can carry different chitin-binding domains (FIG. 1). Carbohydrate-binding module family 14 (CBM14) is a chitin-binding domain that contains six conserved cysteine residues, suggesting the formation of disulfide bridges. An example is the virulence-promoting CfAvr4 effector in C. fulvum that protects the fungal cell wall from chitinases secreted by the tomato host (FIG. 2A; TABLE 1) (van den Burg et al. 2004, 2006). Functional orthologs of CfAvr4 are also identified in other fungal species (TABLE 1) (Stergiopoulos et al. 2010; Kohler et al. 2016). Recent structural analysis of CfAvr4 with ligands revealed that two molecules of CfAvr4 form a sandwich structure that holds two chitohexaose molecules (Hurlburt et al. 2018). Mutational analyses demonstrated that the chitohexaose-binding domain extends through the entire length of CfAvr4 and revealed that its integrity is critical for hyphal protection from chitinases (Hurlburt et al. 2018). SnTox1 from the necrotrophic fungus Parastagonospora nodorum promotes virulence by inducing necrosis on wheat lines carrying the Snl1 susceptibility gene (TABLE 1) (Liu et al. 2012). SnTox1 possesses a chitin-binding-like domain at the C-terminus that resembles the CBM14 domain in other chitin-binding fungal effectors (Liu et al. 2012). SnTox1 protein binds to hyphae and protects them against chitinases (FIG. 2A). It is speculated that apoplastic SnTox1 might activate a membrane-bound plant receptor to induce cell death to allow the fungus access to nutrients, but cell wall–bound SnTox1 might have a second role in protecting hyphae against chitinases (Liu et al. 2016). So far, the proposed two functions have not been separated.

The plant xylem–invading fungus Verticillium non-alalfaef secrete VnaChtBP, an effector carrying six tandemly repeated carbohydrate-binding module family 18 (CBM18) domains (TABLE 1). This domain is unrelated to the CBM50/LysM and CBM14 domains and is characterized so far by homology modeling only. VnaChtBP is highly up-regulated during colonization, its protein product binds chitin oligomers and protects fungal hyphae from chitinases (FIG. 2A), although knockdown of VnaChtBP does not affect virulence. This has been interpreted to reflect redundancy and convergent evolution of this CBM18 effector (Volk et al. 2019).

Although many LysM effectors reside in the apoplastic space to sequester chitin fragments in that compartment (Rocafort et al. 2020), a second class of LysM effectors are bound to the fungal cell wall. The genome of wheat pathogen Z. tritici encodes two such LysM effectors: Mg1LysM as well as Mg3LysM (TABLE 1) (Marshall et al. 2011). Mg1LysM and Mg3LysM effectors protect hyphae from degradation by plant chitinases (FIG. 2A) (Marshall et al. 2011). Although a direct link between binding and protection has not yet been established, a recent structural study demonstrates that the single-LysM-domain-containing effector Mg1LysM forms chitin-dependent as well as chitin-independent dimers. Chitin-induced oligomerization of Mg1LysM ligand–independent homodimers leads to formation of a contiguous supramolecular structure that is anchored to chitin in the fungal cell wall and prevents hydrolysis by host chitinases (Sánchez-Vallet et al. 2020). Structure-guided mutation of Mg1LysM protein
Figure 2. Function of effectors associated with the cell wall of plant-colonizing fungi. Left panels indicate the situation where the respective effector is present, and right panels indicate the situation where the respective effector is absent. The same symbols are used as in FIG. 1. In (A–E) only relevant cell wall constituents are given in full color whereas others are shaded. (A) Function of chitin-binding proteins. The cell wall–bound effectors protect the chitin layer from plant chitinases. In the absence of the effectors, the chitin layer is degraded by plant chitinases with adverse effects of fungal viability and chitin fragments released from cell wall activate plant defenses. (B) Function of β-glucan-binding lectins. The cell wall–bound proteins organize the β-glucan chains. In the absence of the effector, the composition of cell wall is altered. (C) Function of hydrophobins and repellents. The proteins confer cell wall hydrophobicity (indicated by showing that a water droplet, shown in gray, remains at the surface of the colony), and this provides for surface attachment, surface recognition, hyphal aggregation, and aerial growth. In the absence of the effector, hyphal cell walls lose hydrophobicity (indicated by increased wettability of the colony, indicated in light gray throughout) and surface attachment is reduced. (D) Function of cerato-platanin family proteins. The cell wall–bound effectors protect the chitin layer from plant chitinases, reducing fungal viability and increasing plant defense responses. In the absence of these effectors, the chitin layer is degraded by plant chitinases. (E) Function of proteins protecting from antifungal proteins. The effector protects the fungal cell wall from mannose-binding antifungal proteins secreted from plant cells. In the absence of the effector, binding of antifungal proteins to mannose leads to fungal cell death.
Proteins related to FGB1 are found specifically in fungi but remain to be characterized. Biochemical analysis reveals that FGB1 shows high affinity to laminarin, a β-1,3-glucan with β-1,6-glucan branches, indicating that FGB1 is a β-glucan-binding lectin. Overexpression of FGB1 in Ustilago maydis increases tolerance to the cell wall stressor Congo red and alters β-glucan and chitin levels in the fungal cell wall, suggesting that FGB1 changes fungal cell wall composition (FIG. 2B). Although the addition of FGB1 fails to protect hyphae from β-glucanases, the added effector protein effectively suppresses the production of reactive oxygen species induced by β-glucan and facilitates plant colonization by P. indica (Wawra et al. 2016). The WSC3 protein from P. indica is also identified as a β-glucan-binding lectin (TABLE 1) (Wawra et al. 2019). The 39-kDa WSC3 protein carries three WSC domains, which are known to have carbohydrate-binding capacity. The genome of P. indica encodes 36 such WSC-domain-containing proteins, and 23 of them are either up-regulated or down-regulated during plant colonization. When heterologously expressed in Pichia pastoris, WSC3 is incorporated into the cell wall and enhances resistance to cell wall stressors (Wawra et al. 2019). Biochemical analysis demonstrates that WSC3 binds long β-1,3-glucan chains regardless of the presence or absence of β-1,6-glucan branches but lacks affinity to short-chain β-1,3- or β-1,6-linked glucose oligomers. The addition of recombinant WSC3 protein to P. indica, Bipolaris sorokiniana, or U. maydis leads to fungal cell aggregation, suggesting an agglutination function. However, in contrast to FGB1, the addition of WSC3 protein does not increase fungal colonization, suggesting that this effector is not involved in suppression of plant immune responses. Since WSC proteins exist as a gene family in P. indica, their contribution to plant colonization by P. indica has not yet been assessed. Attempts to gain insight into the function of WSC3 by overexpression were unsuccessful, because such P. indica strains do not show enhanced root colonization (Wawra et al. 2019). Therefore, one is left with the speculation that this effector might reinforce the fungal cell wall, which could provide protection from environmental stress, a property not yet assessed. β-Glucan-binding effectors have not been identified in fungal plant pathogens yet; hence, it remains largely unresolved how plant pathogens suppress glucan-triggered immunity.

HYDROPHOBS AND REPELLENTS

Hydrophobins are fungus-specific small surface-active proteins with eight conserved cysteine residues (FIG. 1)
Table 1. Effectors associated with the cell wall of plant-colonizing fungi.

| Effector protein | Plant-colonizing fungus | Function | Contribution to virulence or colonization | Binding substrate | Characterized domain | Reference |
|------------------|--------------------------|----------|------------------------------------------|------------------|---------------------|-----------|
| PfAv4            | Cladosporium fulvum      | Hyphal protection from chitinase | Yes            | Chitin            | CBM14               | van den Burg et al. 2004, 2006 |
| PfAv4            | Pseudocercospora fuliginea | Hyphal protection from chitinase | Yes            | Chitin            | CBM14               | Kohler et al. 2016 |
| MAAv4            | Mycosphaerella fijiensis | Hyphal protection from chitinase | Not assessed  | Chitin            | CBM14               | S tergiopoulos et al. 2010 |
| SnTox1           | Parastagonospora nodorum | Hyphal protection from chitinase | Yes            | Chitin            | Similar to CBM14    | Liu et al. 2012 |
| VnaChBP          | Verticillium nonalfalae  | Hyphal protection from chitinase | No             | Chitin            | CBM18               | Volk et al. 2019 |
| Mg1LysM          | Mycosphaerella graminicola| Hyphal protection from chitinase | No             | Chitin            | CBM50/LysM          | Marshall et al. 2011 |
| Mg3LysM          | Mycosphaerella graminicola| Hyphal protection from chitinase | Yes            | Chitin            | CBM50/LysM          | Marshall et al. 2011 |
| RISLM            | Rhizopus irregularis     | Hyphal protection from chitinase | Yes            | Chitin            | CBM50/LysM          | Zeng et al. 2020 |
| ChELP1           | Colletotrichum           | Cell wall maintenance          | Yes            | Chitin            | CBM50/LysM          | Takahara et al. 2016 |
| ChELP2           | Higginssianum            | Hyphal protection from chitinase | Yes            | Chitin            | CBM50/LysM          | Seidl-Seiboth et al. 2013; Romero-Contreras et al. 2019 |
| FGB1             | Piriformospora indica    | Cell wall maintenance          | Yes            | Laminarin         | Unknown             | Wawra et al. 2016 |
| WSC3             | Piriformospora indica    | Cell adhesion                  | No             | Long β-1,3-glucan chain | WSC              | Wawra et al. 2019 |
| MPG1             | Magnaporthe oryzae       | Attachment to hydrophobic surface | Yes            | Unknown           | Class I hydrophobin | Talbot et al. 1993, 1996 |
| MHP1             | Magnaporthe oryzae       | Conidial development and viability | Yes            | Unknown           | Class II hydrophobin | Kim et al. 2005 |
| FgHyd1           | Fusarium graminearum     | Unknown                        | No             | Unknown           | Class I hydrophobin | Quarantin et al. 2019 |
| FgHyd2           | Fusarium graminearum     | Attachment to hydrophobic surface | Yes            | Unknown           | Class I hydrophobin | Quarantin et al. 2019 |
| FgHyd3           | Fusarium graminearum     | Unknown                        | No             | Unknown           | Class I hydrophobin | Quarantin et al. 2019 |
| FgHyd5           | Fusarium graminearum     | Unknown                        | No             | Unknown           | Class I hydrophobin | Quarantin et al. 2019 |
| HYD1             | Fusarium verticillioides | Microconidial chain formation  | No             | Unknown           | Class II hydrophobin | Fuchs et al. 2004 |
| HYD2             | Fusarium verticillioides | Unknown                        | No             | Unknown           | Class I hydrophobin | Fuchs et al. 2004 |
| HYD4             | Fusarium verticillioides | Unknown                        | No             | Unknown           | Class II hydrophobin | Fuchs et al. 2004 |
| HYD5             | Fusarium verticillioides | Unknown                        | No             | Unknown           | Class I hydrophobin | Mosbach et al. 2011 |
| Bhp1             | Batrytis cinerea         | Unknown                        | No             | Unknown           | Class II hydrophobin | Mosbach et al. 2011 |
| Bhp2             | Batrytis cinerea         | Unknown                        | No             | Unknown           | Class I hydrophobin | Mosbach et al. 2011 |
| Bhp3             | Batrytis cinerea         | Unknown                        | No             | Unknown           | Class II hydrophobin | Mosbach et al. 2011 |
| CPHH1            | Claviceps purpurea       | Unknown                        | No             | Unknown           | Class II hydrophobin | Mey et al. 2003 |
| Cerato-ulmin     | Ceratoctys ulmi          | Unknown                        | No             | Unknown           | Class I hydrophobin | Bowden et al. 1996 |
| Hum2 Hum3        | Ustilago maydis          | Unknown                        | No             | Unknown           | Class I hydrophobin | Tettstra et al. 2006 |
| Rsp1             | Ustilago maydis          | Unknown                        | No             | Unknown           | Repetitive sequences | Müller et al. 2008 |
| Rep1             | Ustilago maydis          | Attachment to hydrophobic surface | No             | Unknown           | Repetitive sequences | Wösten et al. 1996 |
| Cerato-platanin  | Ceratoctys platani       | Elicitor                        | Not assessed  | Chitin            | Cerato-platanin family | Pazzagli et al. 1999; Boddi et al. 2004; Baccelli et al. 2014a |
| Snodprot1        | Phaeosphaeria nodorum    | Unknown                        | Not assessed  | Chitin            | Cerato-platanin family | Hall et al. 1999 |
| Sp1              | Leptosphaeria maculans   | Elicitor                        | No             | Unknown           | Cerato-platanin family | Wilson et al. 2002 |
| MxCP1-5          | Monilophthora perniciosa | Elicitor                        | Not assessed  | Chitin            | Cerato-platanin family | Zappori et al. 2009 |
| Sm1              | Trichoderma virids       | Elicitor                        | No             | Unknown           | Cerato-platanin family | Djonović et al. 2006, 2007 |
| ELP1             | Trichoderma atrovireide  | Elicitor                        | Not assessed  | Chitin            | Cerato-platanin family | Seidl et al. 2006 |
| Pop1             | Ceratoctys polypilicola  | Elicitor                        | Not assessed  | Chitin            | Cerato-platanin family | Comparini et al. 2009 |

(Continued)
Hydrophobins are highly expressed proteins that self-assemble at hydrophilic-hydrophobic interfaces into amphipathic monolayers. This provides hyphae or spores with a hydrophobic protein coating that aids spore dispersal and aerial growth of hyphae during escape from an aqueous environment (Fig. 2C) (Wösten 2001; Bayry et al. 2012). Based on differences in structure and properties, hydrophobins are subdivided into three classes (Wösten 2001; Littlejohn et al. 2012). Class I hydrophobins are surface-assembled into sodium dodecylsulfate (SDS)-resistant fibrillar structures called rodlets that can be solubilized by treatment with trifluoroacetic acid (Talbot et al. 1996; Wessels 1999). Class II hydrophobins are also bound to hyphae but do not form a fibrillar rodlet structure and can be solubilized with organic solvents and detergents. Class III hydrophobins have intermediate features of class I and class II hydrophobins (Jensen et al. 2010; Littlejohn et al. 2012) but have not yet been characterized in plant-colonizing fungi.

A characterized class I hydrophobin from plant-colonizing fungi is MPG1 in *P. oryzae* (Table 1) (Talbot et al. 1993, 1996). MPG1 is highly induced during the early infection stages (Soanes et al. 2002). *mpg1* deletion mutants show a reduction in virulence, which is caused by defects in attachment to a hydrophobic surface (Fig. 2C) and subsequent appressorium formation. It is speculated that hydrophobins might act as sensors of the hydrophobic plant surface and trigger appressorial development (Talbot et al. 1996). Hyphal surface coating with MPG1 has been shown to allow efficient recruitment and retention of cutinase 2, which aids in appressorium differentiation and penetration (Skamnioti and Gurr 2007; Pham et al. 2016).

An example for a class II hydrophobin is MHP1 of *P. oryzae* (Table 1) (Kim et al. 2005). Mutants are attenuated in appressorium development, show significantly reduced biotrophic growth inside host cells, and are affected in conidiation, conidial viability, as well as conidial germination (Kim et al. 2005). In the presence of MHP1, MPG1 fails to assemble, possibly caused by altered surface properties conferred by bound MHP1 (Pham et al. 2016). The genome of the head blight fungus *Fusarium graminearum* contains four class I hydrophobin genes (FgHyd1 to FgHyd4) and one class II gene (FgHyd5) (Table 1) (Quarantin et al. 2019). Mutants carrying single gene deletions in these genes demonstrate that FgHyd2, FgHyd3, and FgHyd4 have an impact on virulence, and this is attributed to reduced attachment of fungal cells to the hydrophobic plant surface (Fig. 2C) (Quarantin et al. 2019).

However, hydrophobins do not always contribute to virulence (Table 1) (Bowden et al. 1996; Mey et al. 2003; Fuchs et al. 2004; Mosbach et al. 2011; Quarantin...
et al. 2019). A special situation is described in U. maydis, which possesses two hydrophobins: Hum2 and Hum3 (TABLE 1) (Teertstra et al. 2006). Whereas Hum2 is a typical class I hydrophobin, Hum3 consists of a class I hydrophobin domain preceded by 17 repeated sequences of 41–46 amino acids, which are likely processed by Kex2 protease (Teertstra et al. 2006; Müller et al. 2008). Whereas mutant hyphae lacking hum2 and hum3 are unaltered in surface hydrophobicity and unaffected in virulence (Teertstra et al. 2006), double mutants lacking the repetitive Hum2 effector as well as the secreted repetitive effector Rsp1 are strongly affected in virulence (Müller et al. 2008). This might suggest functional redundancy between these processed effectors during plant colonization. However, the underlying mechanism awaits elucidation.

In U. maydis, surface hydrophobicity is largely provided by repellents (FIG. 1) (Wösten et al. 1996). Repellents are produced from the repetitive precursor protein Rep1, consisting of 12 repeats ranging in size between 37 and 55 amino acids. Ten of these repeats are liberated after processing by Kex2 protease (TABLE 1) (Wösten et al. 1996). These peptides lack cysteine residues and are SDS insoluble but can be dissociated from hyphae with trifluoroacetic acid, a property shared with type I hydrophobins. The Rep1 peptides are required for the formation of aerial hyphae (Wösten et al. 1996) and aid in hyphal attachment to artificial hydrophobic surfaces (FIG. 2C) (Teertstra et al. 2006). Synthetic Rep1-1 peptide reduces water surface tension and forms amyloid-like fibrils (Teertstra et al. 2009). Hyphae of rep1 deletion mutants show reduced surface hydrophobicity and fail to extend into the air but are unaffected in virulence (Wösten et al. 1996). It is speculated that repellents have functionally replaced hydrophobins in U. maydis (Wösten et al. 1996; Teertstra et al. 2006), with the caveat that this hydrophobic surface layer does not seem to play a prominent role in attachment to the host surface.

CERATO-PLATANIN-RELATED EFFECTORS

Cerato-platanin was initially identified in the plant-pathogenic fungus Ceratocystis platani infecting plane trees (TABLE 1) (Pazzagli et al. 1999). Subsequently, related proteins have been reported in many filamentous fungi and include Snodprot1 from Phaeochaeta nodorum, Sp1 from Leptosphaeria maculans, Sm1 from Trichoderma virens, EPL1 from T. atroviride, Pop1 from Ceratocystis populicola, MpCP1-5 from Moniliophthora perniciosa, and VdCP1 from Verticillium dahiae (TABLE 1) (Hall et al. 1999; Wilson et al. 2002; Djonovic et al. 2006, 2007; Seidl et al. 2006; Comparini et al. 2009; Zaporolli et al. 2009; Zhang et al. 2017). Most of these cerato-platanin family proteins act as elicitors that induce hypersensitive responses in plants (Pazzagli et al. 2014). Botrytis cinerea is such an example where the cerato-platanin family protein BeSp1 induces a hypersensitive response after protein infiltration (TABLE 1) (Frias et al. 2011), and it is speculated that this response is beneficial for virulence of this necrotrophic fungus. A hypersensitive response can be also induced by other cerato-platanin family proteins, such as MSP1 from P. oryzae (TABLE 1) (Yang et al. 2009; Hong et al. 2017) and ScCP1 from the necrotrophic pathogen Sclerotinia sclerotiorum (TABLE 1) (Yang et al. 2018). The cerato-platanin protein consists of 120 amino acid residues with four cysteines, and nearly 40% of its amino acids are hydrophobic (Pazzagli et al. 1999). Therefore, it was speculated that it might be a hydrophobin-like protein. Indeed, EPL1 from T. atroviride has the ability to self-assemble into a protein layer at the water-air interface (Frischmann et al. 2013). However, in contrast to hydrophobins, EPL1 increases wetting of fungal hyphae and facilitates fungal growth in aqueous condition (Frischmann et al. 2013; Gaderer et al. 2014). Furthermore, the structural analysis of cerato-platanin protein revealed that the protein does not expose a large hydrophobic patch like hydrophobins (Gaderer et al. 2014; Sunde et al. 2017) and the adopted fold is similar to those found in expansins, endoglucanases, and the plant defense protein barwin (de Oliveira et al. 2011). Several cerato-platanin family proteins are detected in the apoplast but also remain bound to the fungal cell wall where they bind chitin (FIG. 1) and likely alter fungal cell wall properties (Boddi et al. 2004; de O Barsottini et al. 2013; Frischmann et al. 2013; Baccelli et al. 2014). Some cerato-platanin family proteins also have the ability to loosen cellulose paper, a property shared with expansins, suggesting that this activity on plant cellulose could aid in host colonization (de O Barsottini et al. 2013; Baccelli et al. 2014). However, VdCP1 does not display such a cellulose-loosening activity; the protein binds to chitin and VdCP1 deletion mutants show increased sensitivity to chitinase, indicating a protective role (FIG. 2D) (Zhang et al. 2017). This suggests that cerato-platanin family proteins are functionally diversified. Several cerato-platanin family proteins from plant-pathogenic fungi contribute to virulence (TABLE 1) (Jeong et al. 2007; Frias et al. 2011; Zhang et al. 2017; Yang et al. 2018; Liu et al. 2019), although the distinction of whether this occurs by the cell wall–bound form, the apoplastic form, or a plant cell wall–loosening activity remains to be elucidated in most systems.

EFFECTORS PROVIDING PROTECTION FROM ANTIFUNGAL PROTEINS/COMPOUNDS

Upon pathogen infection, plants secrete antifungal proteins/compounds that kill fungal cells (Osbourn 1996;
Selitrennikoff 2001). To prevent this, U. maydis secretes the virulence-promoting Rsp3 effector protein (FIG. 1; TABLE 1) (Ma et al. 2018). Rsp3 is highly expressed upon plant colonization and decorates the surface of fungal hyphae. The C-terminal domain of Rsp3 consists of a complex array of repetitive sequences, which vary in length in field isolates. Rsp3 binds the antifungal maize proteins AFP1 and AFP2, which have sequence similarity to Gnk2, a secreted antifungal mannose-binding protein from Ginkgo biloba. Overexpression of Rsp3 in U. maydis increased resistance to AFP1, whereas silencing of AFP1 and AFP2 increased virulence, suggesting that Rsp3 protects fungal cells from the toxic activity of this class of antifungal proteins (FIG. 2E). Ustilago maydis also secretes the virulence-promoting Sta1 effector (TABLE 1) (Tanaka et al. 2020). Although Sta1 protein lacks characterized domains implying carbohydrate binding, the protein is specifically detected on the surface of U. maydis hyphae but fails to attach to budding cells.

It is presently unknown whether Sta1 binds to oligosaccharide components or proteins of the hyphal cell wall that differ between budding cells and hyphae. Expression of the sta1 gene peaks at an early stage of plant infection and goes down at later time points. Shifting early expression of sta1 to late expression or making sta1 expression constitutive reduced virulence, suggesting that the Sta1 effector is needed in a specific time window during colonization. When expressed either too early or too late, Sta1 actually negatively impacts colonization (Tanaka et al. 2020). Overexpression of the sta1 gene rendered fungal hyphae more susceptible to chitinases and β-glucanase, suggesting that Sta1 has a role in maintenance of the fungal cell wall. After infection with sta1 mutants, the plant cell wall exhibited increased autofluorescence, suggesting an accumulation of phenolic compounds, including lignin. Therefore, it is speculated that Sta1 might prevent the release of fungal cell wall-derived elicitors and act as a stage-specific stealth molecule.

**GPI-ANCHORED CELL WALL PROTEINS**

Glycosylphosphatidyl inositol (GPI)-anchored proteins are known as fungal cell wall components and have an essential role in cell wall integrity (Lima et al. 2019). The GPI anchor becomes covalently attached to the C-terminus of proteins in the endoplasmic reticulum via a series of enzymatic reactions after a proteolytic cleavage occurring at the so called ω site (Kinoshita 2016). In Saccharomyces cerevisiae, approximately 60 GPI-anchored proteins are predicted (Pittet and Conzelmann 2007). Although several GPI-anchored proteins are biosynthetic enzymes for the construction of the cell wall, others seem to be structural components of the cell wall or are of unknown function. In S. cerevisiae, several GPI-anchored proteins are processed and become integrated into the cell wall by covalent attachment to β-glucan through remnants of their GPI anchor (Klis et al. 2006; Lesage and Bussey 2006). GPI-anchored proteins could in principle function as effectors by shielding fungal hyphae from specific attack by plant enzymes. The hemibiotrophic fungi F. graminearum and P. oryzae have 84 and 80 proteins predicted to be GPI-anchored. In contrast, the biotroph U. maydis is predicted to have only 16 GPI-anchored proteins (Oliveira-Garcia and Deising 2016b), suggesting a possible link to the different pathogenic strategies. GPI-anchored protein biogenesis genes were shown to be critical for cell wall integrity and virulence in L. maculans (Remy et al. 2008), C. graminicola (Oliveira-Garcia and Deising 2016b), and P. oryzae (Liu et al. 2020). Although this is likely due to pleiotropic effects of respective mutations, it remains to be shown whether singular GPI-anchored proteins can be identified that contribute to virulence in plant-pathogenic fungi. One such example could be the EMP1 protein of P. oryzae (Ahn et al. 2004). EMP1 shows high identity to FEM1, a covalently linked cell wall protein from the tomato pathogen F. oxysporum (TABLE 1) (Schoffelmeer et al. 2001; Ahn et al. 2004). EMP1 is induced during appressorium formation, and EMP1 deletion mutants show reduced conidial adhesion and appressoria formation on a hydrophobic surface and are attenuated in virulence. However, the EMP1 deletion mutant can induce appressoria on a hydrophilic surface where the wild-type cannot form appressoria (Ahn et al. 2004). This suggests that the GPI-anchored EMP1 protein might be involved in perception of surface properties. However, the detailed biochemical function of EMP1 and related proteins remains to be elucidated.

**CONCLUDING REMARKS AND FUTURE CHALLENGES**

From many plant-colonizing fungi, cell wall-associated effectors have been identified during the past decades. Although many such proteins possess characteristic domains involved in carbohydrate binding, several effector proteins detected on the cell wall lack known domains for carbohydrate binding. To understand why and how these effectors contribute to virulence on the molecular level, it will be necessary to determine their binding targets—which could be cell wall carbohydrates or cell wall proteins—and to elucidate the structural basis for binding as well as specificity. These are nontrivial experiments, which might need sophisticated biochemical expertise. In our view, it is very likely that many secreted effectors without characteristic domains will turn out to be localized to the fungal cell wall. Since the fungal cell wall is in direct contact with host
plant cells and the molecules secreted by the host, placing effector proteins in the fungal cell wall or even covalently attaching them to the cell wall seems like a way to provide the necessary high local concentration that may be required for acting as a protective shield. In contrast to extensive studies of LysM effectors, we have relatively little knowledge with respect to the detailed biochemical functions and underlying structures of other fungal cell wall–associated effectors. Consequently, the mechanisms of how those proteins contribute to virulence remain an open question. In particular in those systems where effectors can be multi-functional and can exist in a fungal cell wall–bound, an apoplastic, or even a host cell wall–bound form, the biological functions need to be separated genetically to elucidate which function is relevant for promoting virulence and how the relative abundance of the different forms is regulated. Another aspect that has not received sufficient attention is the many cell wall–bound effectors that appear not to affect virulence. In this regard, it will be necessary to consider either redundancy or compensation and transcriptomic studies and/or synthetic virulence screens may be necessary to sort this out. We believe that the study of cell wall–bound fungal effectors will provide a wealth of novel insights for understanding the molecular basis of plant-fungus interactions.

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