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Proteomic identification of apoplastic proteins from rice, wheat, and barley after *Magnaporthe oryzae* infection

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Abstract

The fungal pathogen *Magnaporthe oryzae* causes devastating blast disease in various cereals, including rice (*Oryza sativa*), wheat (*Triticum aestivum*), maize (*Zea mays*), and barley (*Hordeum vulgare*). Despite previous reports on fungal host specificity, the mechanisms underlying differential host infection strategies remain unclear. This study aimed to identify differentially abundant proteins (DAPs) in the apoplast of rice, barley, and wheat following infection with two *M. oryzae* pathovars using liquid chromatography-tandem mass spectrometry (LC–MS/MS). LC–MS/MS analysis revealed an enrichment of both *M. oryzae* and host proteins in the apoplast during the compatible reaction compared to the incompatible reaction. DAPs from *M. oryzae* involved in the host interaction included secreted extracellular enzymes (e.g., hydrolases), which were significantly increased in the *M. oryzae Oryzae* (MoO)-infected rice apoplast. Among host proteins, the proportion of protein-modifying enzymes increased in the *M. oryzae Triticum* (MoT)-infected rice and MoO-infected wheat apoplastic fluids, particularly rice glycosidases, peroxidases, and serine proteases, as well as wheat serine proteases. Furthermore, DAPs from MoL-infected rice were enriched in carbohydrate metabolism, suggesting that carbohydrate metabolism-related proteins may play a vital role in rice resistance to MoL. Additionally, protein-modifying and cytoskeletal proteins, as well as stress-responsive proteins, were enriched in the MoO-infected wheat apoplastic fluid. Finally, DAPs from both MoO- and MoL-infected barley were enriched in hydrogen peroxide catabolism, suggesting that peroxidases may be vital for barley resistance to *M. oryzae*. The identification of DAPs from both *M. oryzae* strains and the three host plants offers valuable insights into the host specificity mechanisms of *M. oryzae* in cereal crops.

Keywords *Magnaporthe oryzae*, Rice blast, Wheat blast, Disease resistance, Proteomics and apoplast protein

Background

The hemibiotrophic fungal pathogen *Magnaporthe oryzae* (anamorph: *Pyricularia oryzae*) causes blast disease in a wide variety of graminaceous species, including cereals such as rice (*Oryza sativa*), wheat (*Triticum aestivum*), and barley (*Hordeum vulgare*) (Wang and Valent 2017). Rice blast is one of the most destructive diseases caused by the *M. oryzae Oryzae* (MoO) pathotype and has existed for centuries (Ou 1985). It is responsible for 10–30% of cultivated rice yield losses in nearly 80 countries worldwide (Liu et al. 2014). Wheat blast, caused by the pathotype *M.*

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oryzae Triticum (MoT), was first identified in Brazil in 1985 and has recently been observed in Asia and Africa, causing substantial crop losses of up to 100% under favorable conditions for the pathogen (Ceresini et al. 2018; Tembo et al 2020). Additionally, it has been demonstrated to affect seed germination and nutritional quality after harvest (Cruz and Valent 2017; Sadat and Choi 2017; Islam et al. 2020; Surovy et al. 2020). The pathotype *M. oryzae Lolium* (MoL) causes gray leaf spot disease on turfgrasses and perennial ryegrass (*Lolium perenne*), annual ryegrass (*L. multiflorum*), and tall fescue (*Festuca arundinacea*) (Cruz and Valent 2017). Although the MoL pathotype does not typically infect wheat, some American MoL isolates infect this species, albeit to a lesser degree than MoT strains from Brazil (Farman et al. 2017). It is also worth noting that *M. oryzae* was reported to infect maize (*Zea mays*) plants in Iran, posing a significant concern for this crop's future (Pordel et al. 2021).

Although the host specificity of *M. oryzae* strains has been reported in many studies (Yoshida et al. 2016; Chung et al. 2020; Li et al. 2022), the underlying mechanisms remain unclear. Based on sequence analysis of several genomic loci, *M. oryzae* isolates can be divided into multiple clades, each with a limited number of host species (Couch et al. 2005). A comparative genomic analysis classified *M. oryzae* strains into lineages associated with virulence in specific plant species, such as a lineage infecting wheat in Bangladesh (Islam et al. 2016). Sequencing of 76 *M. oryzae* isolates sampled from 12 grass and cereal genera in many countriesrevealed multiple divergent lineages within *M. oryzae*, each preferentially associating with one host genus, suggesting incipient speciation following host shift or host range expansion (Gladieux et al. 2018). However, the specific mechanisms through which *M. oryzae* expands its host range in monocots and the fungal and host genes involved in this shift require further in-depth investigation.

In this study, we inoculated rice, barley, and wheat with MoL isolate PL2-1 or MoO isolate RO1-1, which show different host specificities. PL2-1 can only infect barley and wheat, whereas RO1-1 can only infect rice and barley. Proteomic analysis of the apoplastic proteins from MoL- and MoO-infected plants via liquid chromatography-tandem mass spectrometry (LC–MS/MS) identified many differentially abundant proteins (DAPs) from both the pathogen and the host. The DAPs from both the pathogens and hosts are excellent candidates for molecular analysis of their roles in host specificity to *M. oryzae.*

Results

MoO and MoL isolates show host infection specificity to rice, wheat, and barley

Because wheat blast has not yet been reported in the United States, we used the MoL isolate PL2-1, which can infect wheat (Farman et al. 2017). Preliminary inoculations showed that PL2-1 can infect both wheat cv. Everest and barley cv. Hockett, whereas the MoO isolate RO1-1 can infect both rice cv. Nipponbare and barley cv. Hockett. To validate these results, we grew all cultivars in the same growth chamber and inoculated them with either RO1-1 or PL2-1 by spraying. Typical susceptible lesions appeared on RO1-1-inoculated rice and barley leaves by three days post-inoculation (dpi) but not on the inoculated wheat leaves (Fig. 1a, c). By contrast, PL2-1 induced susceptible lesions only on wheat and barley leaves, but not on rice. Notably, many typical susceptible lesions appeared on barley leaves infected with either RO1-1 or PL2-1, and wheat leaves infected with PL2-1 at 3 dpi (Fig. 1a, c).

Compatible reactions induce more protein release from the cytoplasm into the apoplast

To explore the mechanisms that determine host-specific resistance to different *M. oryzae* pathotypes, we extracted apoplastic fluid from infected rice, wheat, and barley leaves and isolated proteins using the infiltration-centrifugation extraction method (Gentzel et al. 2019). β-Actin was not detected in the apoplast fractions but was visible in the total protein fraction (Fig. 1b), indicating that there was little or no contaminating cytoplasmic protein in the apoplastic protein preparations. To avoid high salt concentration extraction buffer affecting subsequent tests, we desalted protein samples via SDS-PAGE, followed by in-gel trypsin digestion. We then conducted label-free quantification of the digested peptides through LC–MS/MS. This proteomic analysis identified 81 and 25 host DAPs in MoO- and MoL-infected rice, 303 and 208 host DAPs in MoO- and MoL-infected barley, and 43 and 147 host DAPs in MoO- and MoL-infected wheat apoplastic fluid, respectively (Fig. 1d and Additional file 1: Table S1). SignalP-6.0 was used to predict the subcellular localizations of the identified DAPs (Additional file 1: Table S1). These results suggest that compatible reactions lead to cell death and, therefore, induce more protein release from the cytoplasm into the apoplast.

M. oryzae proteins are enriched in the MoO-infected rice apoplast

The LC–MS/MS analysis above revealed that DAPs from *M. oryzae* were not detectably present in the apoplast of MoL-infected rice leaves, likely due to their incompatible interaction. By contrast, we detected 92 DAPs from *M. oryzae* in the apoplast of MoO-infected rice leaves (Additional file 1: Table S2). Singular enrichment analysis (SEA) of the 92 DAPs suggested that they are enriched in biological processes associated with host interaction (Fig. 2a). Notably, we identified several known *M.*

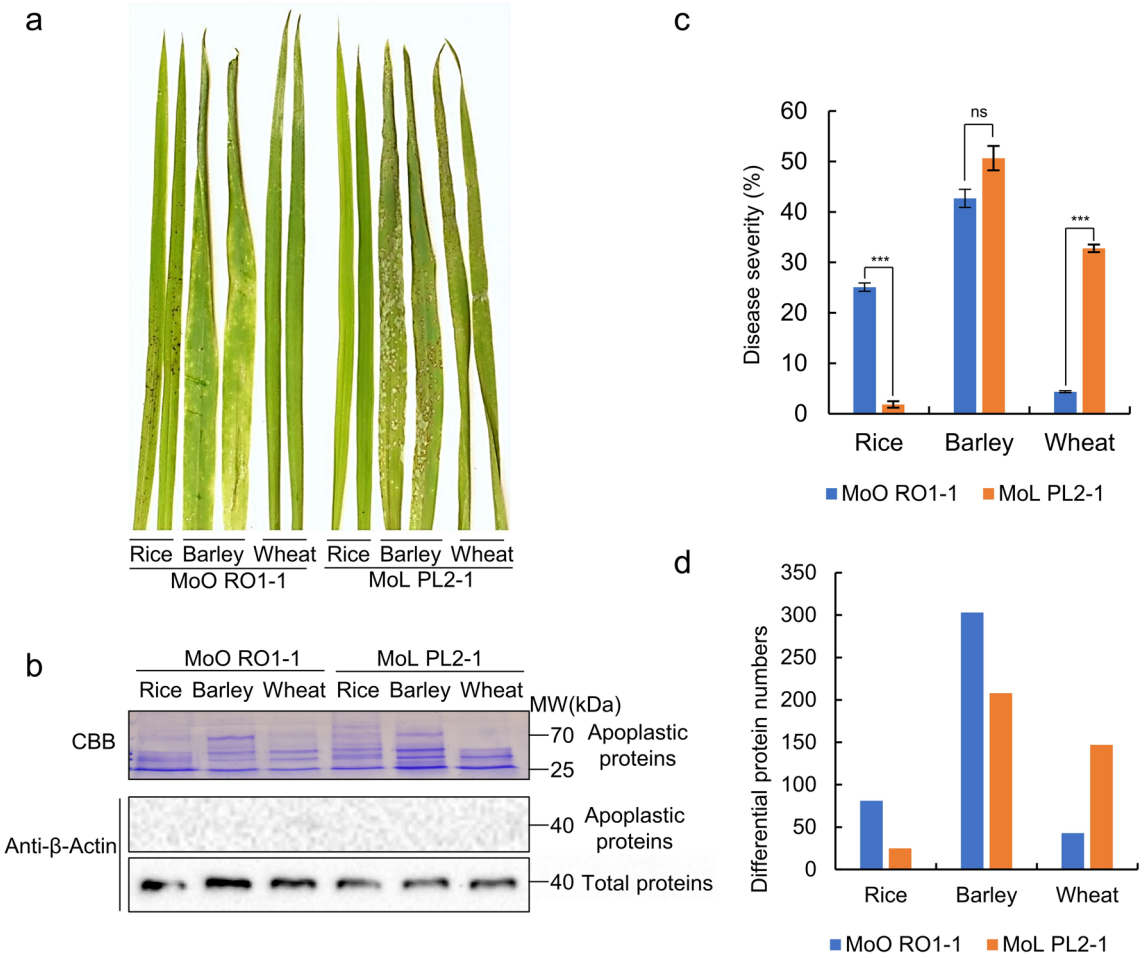
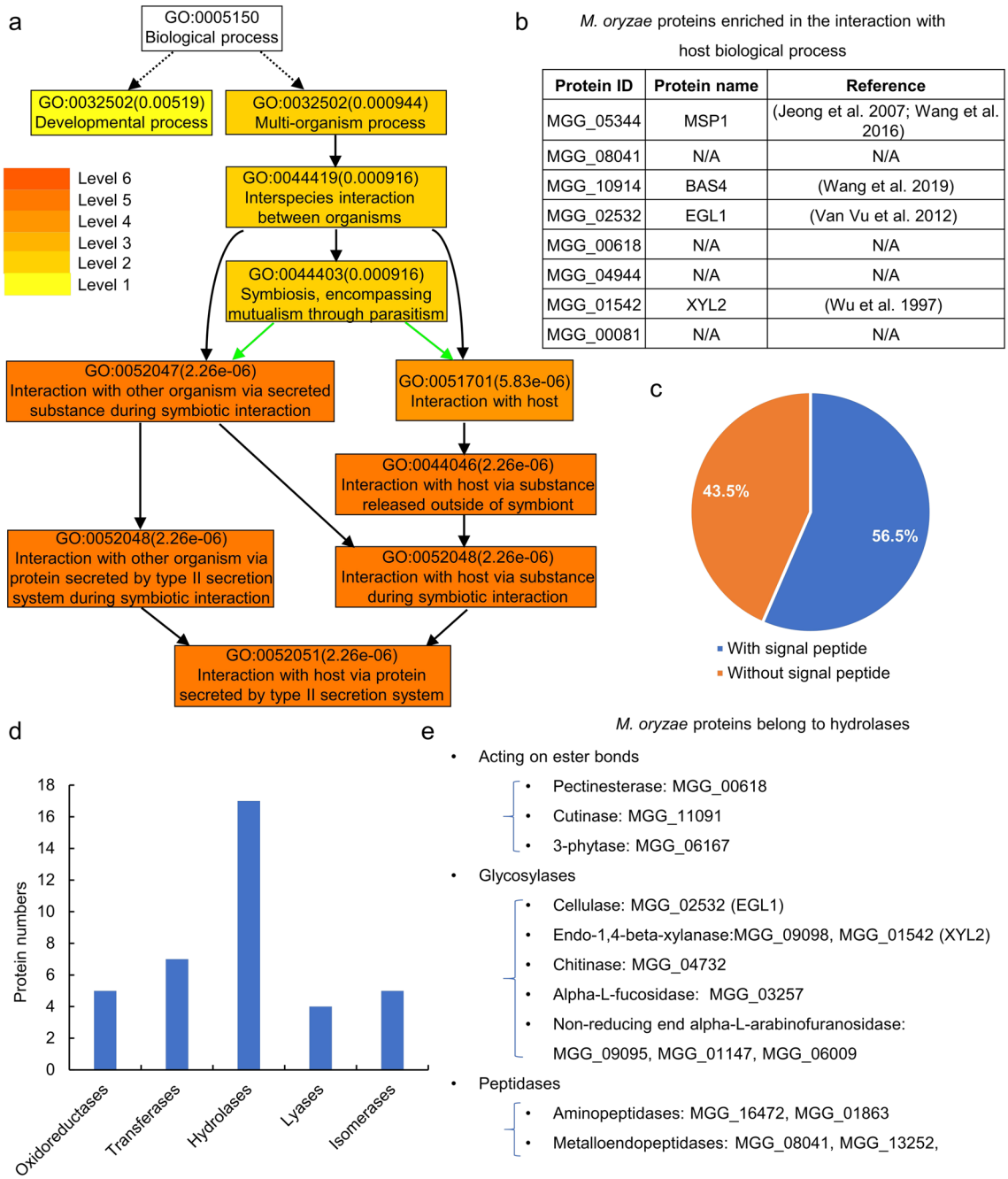


Fig. 1 Total apoplastic protein isolation from *Magnaporthe oryzae*-infected rice, wheat, and barley plants. **a** Photos of rice, wheat, and barley leaves infected with MoO and MoL. Photographs were taken at 3 dpi. **b** 150 µg each of the apoplastic proteins were used for CBB staining (top panel). Immunoblot analysis of apoplastic and total proteins. The antibody against β-Actin was used for the test (bottom panel). **c** The percentage of lesion areas. Disease severity was evaluated by calculating disease lesion areas using Photoshop software. Asterisks indicate significant differences in the disease lesion area between the MoO and MoL inoculated plants (Student's *t*-test; ***, $P < 0.001$), "NS" indicates that no significant difference was found. **d** Number of differentially abundant proteins identified in the apoplastic fluid of rice, wheat, and barley. A ten-fold cut-off was set to detect candidate differentially abundant proteins

oryzae functional proteins or effectors, such as *M. oryzae*-secreted protein 1 (MSP1), biotrophy-associated secreted protein 4 (BAS4), the endoglucanase EGL1, and the endo-β-1,4 xylanase XYL2 (Fig. 2b).

MSP1 belongs to the Snodprot1 protein family; Snodprot1 is a small secreted protein from the wheat pathogen *Stagonospora nodorum*; MSP1 is required for *M. oryzae* pathogenicity and can induce excessive cell death in plants (Jeong et al. 2007; Wang et al. 2016). BAS4 is an extra-invasive hyphal membrane (EIHM) matrix protein that facilitates the transition from the biotrophic to necrotrophic phase during the *M. oryzae*-rice interaction (Wang et al. 2019). EGL1 is a glycosyl hydrolase that enables *M. oryzae* to penetrate the host epidermis and promotes invasion (Van Vu et al. 2012). The hydrolase XYL2 slightly affects *M. oryzae* growth (Wu et al. 1997). These results suggest that those *M. oryzae* proteins that mediate plant cell death and necrotrophic phase transition may play essential roles in the *M. oryzae* invasion progression. In addition, we observed that approximately 56.5% (52 of 92) of the identified *M. oryzae* proteins contain a signal peptide based on the Uniprot database (https://www.uniprot.org/) (Fig. 2c), indicating that most of these proteins may be secreted into the apoplast during infection.

During host infection, fungi secrete diverse extracellular enzymes, particularly hydrolases, which aid in acquiring nutrients from the environment (Zhou et al.



Protein ID	Protein name	Reference
MGG_05344	MSP1	(Jeong et al. 2007; Wang et al. 2016)
MGG_08041	N/A	N/A
MGG_10914	BAS4	(Wang et al. 2019)
MGG_02532	EGL1	(Van Vu et al. 2012)
MGG_00618	N/A	N/A
MGG_04944	N/A	N/A
MGG_01542	XYL2	(Wu et al. 1997)
MGG_00081	N/A	N/A

Fig. 2 *M. oryzae* proteins exclusively identified in the apoplast of MoO-infected rice leaves. **a** GO enrichment analysis of the *M. oryzae* proteins isolated from the rice apoplast. The black dotted arrows represent "one significant node", the solid black arrows represent "is a", and the green arrows represent "negatively regulates". The GO term color represents its significance level. **b** *M. oryzae* proteins likely involved in host interaction. **c** Proportion of *M. oryzae* proteins with or without a signal peptide. **d** Enzyme types identified from *M. oryzae* in the apoplast of MoO-infected rice leaves. **e** *M. oryzae* hydrolases identified in the apoplast of MoO-infected rice leaves

2013). Enzyme class analysis of the DAPs showed that many are hydrolases. In addition, we detected oxidoreductases, transferases, lyases, and isomerases among the DAPs (Fig. 2d). Most identified hydrolases belonged to three major classes: glycosylases, peptidases, and ester hydrolases (Fig. 2e). Notably, EGL1 and XYL2, two *M. oryzae* proteins associated with the host interaction (Van Vu et al. 2012; Wang et al. 2019), are glycosylases (Fig. 2e). These results suggest that *M. oryzae*-secreted hydrolases might mediate its interaction with rice.

DAPs involved in protein modification and metabolite interconversion are enriched in MoL-infected rice apoplast

We characterized the host proteins from the apoplast of MoO- and MoL-infected rice leaves with the PANTHER Classification System (Mi et al. 2019). PANTHER GO-Slim molecular function analysis indicated that proteins with binding and catalytic activities are the most significant classes in MoO- or MoL-infected rice leaves. Within the MoO-infected rice apoplast, we also identified proteins with molecular adaptor activity, molecular function regulator activity, molecular transducer activity, and structural molecule activity (Additional file 2: Figure S1). The DAPs identified in the apoplast of MoO-infected rice leaves can be grouped into 11 protein classes: metabolite interconversion enzyme, protein-modifying enzyme, protein-binding activity modulator, translational protein, transmembrane signal receptor, transporter, RNA metabolism protein, chaperone, chromatin/chromatin-binding or -regulatory protein, cytoskeletal protein, and membrane traffic protein (Fig. 3a). By contrast, we only identified three protein classes for the DAPs in the MoL-infected rice apoplast (Fig. 3b). These three protein classes, metabolite interconversion enzyme, protein-modifying enzyme, and translational protein, were also the most abundant in the apoplast of MoO-infected rice leaves (Fig. 3a, b).

It is worth noting that although the MoO-infected rice apoplast contains more protein classes overall, the metabolite interconversion and protein-modifying enzymes were significantly abundant in the MoL-infected apoplastic fluid, suggesting that these proteins may be important modulators of rice MoL resistance (Fig. 3a, b). Further analysis indicated that rice glycosidases, peroxidases and serine proteases are the main metabolite

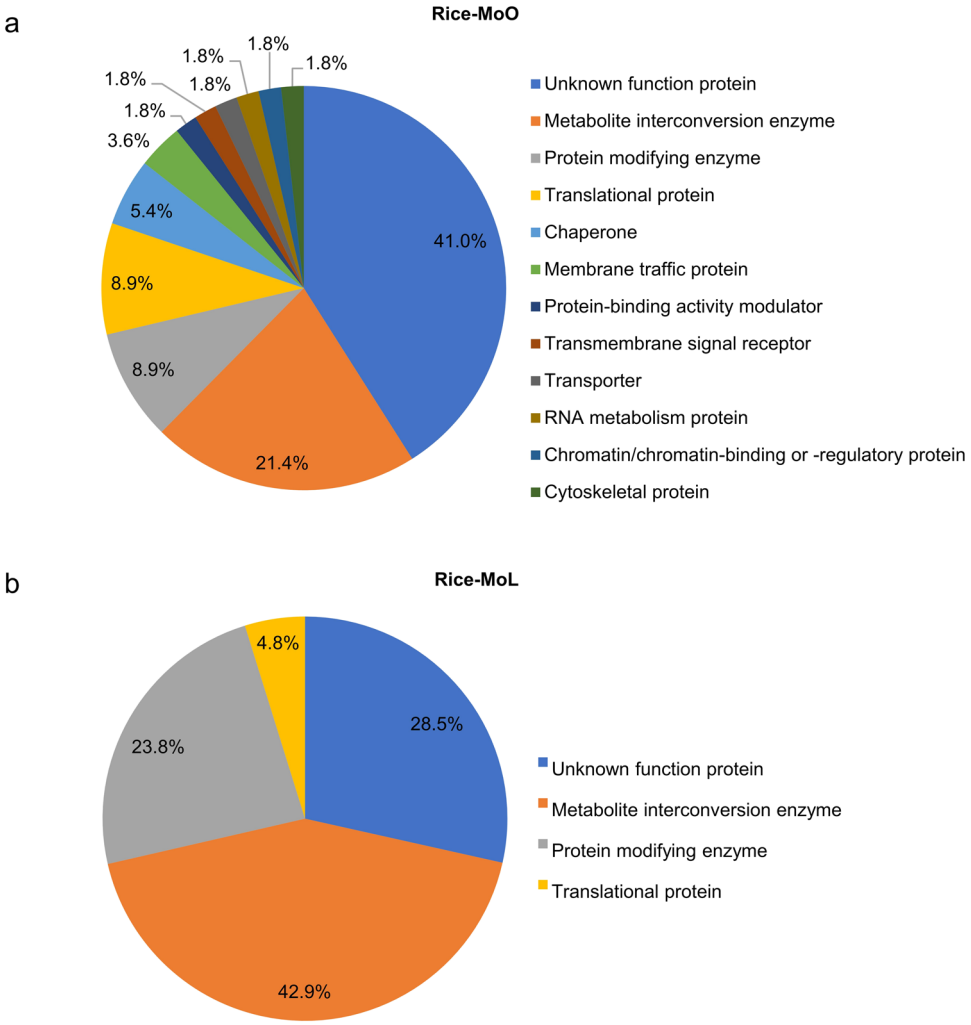


Fig. 3 Protein classes identified in the apoplast of MoO- and MoL-infected rice leaves. **a, b** Protein classes determined by PANTHER protein class analysis in the apoplast of MoO- and MoL-infected rice leaves

interconversion and protein-modifying enzymes, some of which are involved in pathogen resistance (Additional file 1: Table S3). For example, the glycosidase OsGLN1 is important in plant defense, with recombinant glutathione S-tranferase (GST)-tagged OsGLN1 rapidly hydrolyzing *M. oryzae* cell wall β-glucans (Akiyama and Pillai 2001). In addition, the peroxidase gene *OsPrx30*, which is transcriptionally modulated by the AT-hook protein OsATH1, mediates reactive oxygen species (ROS) accumulation induced by rice bacterial blight (Liu et al. 2021). However, the functions of the other identified proteins within these two classes in plant immunity remain unknown.

Proteins related to carbohydrate metabolism are enriched in the MoL-infected rice apoplast

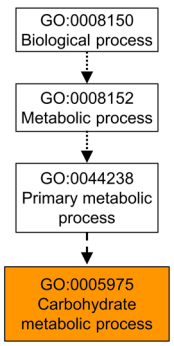
To characterize the host proteins that may regulate rice resistance to MoL, we performed SEA to identify enriched GO terms among the DAPs. Notably, eight proteins detected in the MoL-infected rice apoplast were enriched for carbohydrate metabolism (Fig. 4a, b). Although we identified more than triple as many DAPs in MoO-infected rice apoplasts than in MoL-infected rice apoplasts, only five were related to intracellular protein transport, protein folding, and alcohol metabolism (Additional file 2: Figure S2). Carbohydrate metabolism is a fundamental biochemical set of reactions that ensures a constant energy supply to living cells. Upon pathogen infection, primary metabolism is thus downregulated to divert energy toward defense responses.

Previous studies have suggested that carbohydrate metabolism promotes the expression of defense-related genes and that genes involved in carbohydrate metabolism are induced by pathogens or pathogen-derived elicitors (Rojas et al. 2014). Of the DAPs identified from MoL-infected rice apoplasts, we analyzed the function of eight associated with carbohydrate metabolism (Fig. 4b), several of which are also involved in plant immunity. For instance, the chitinase OsCHIB2 has 91% amino acid identity to the pathogenesis-related protein OsCHIB1 (Park et al. 2004). *PHOTOSENSITIVE LEAF ROLLING 1* (*PSL1*, also named *OsPG1*) encodes a cell wall-localized polygalacturonase (PG), a type of pectin-degrading enzyme (Zhang et al. 2021). A loss-of-function mutation in *PSL1* results in spontaneous cell death and an auto-activated defense response, including ROS burst and *PATHOGENESIS-RELATED* (*PR*) expression, as well as enhanced resistance to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (Surovy et al. 2020). Glycoside hydrolases (GH) catalyze the hydrolysis of glycosidic bonds in cell wall polymers and greatly affect cell wall architecture (Sharma et al. 2013). We identified two proteins, encoded by LOC_Os01g43160 and LOC_Os07g35350, that belong to the OsGH28 and OsGH17 families, respectively. LOC_Os07g35350 is upregulated in rice plants infected with rice stripe virus (Sharma et al. 2013). *OsPR4b* encodes a pathogenesis-related protein with antifungal activity in vitro (Zhu et al. 2006). Whether these proteins are involved in rice MoL resistance must be investigated using genetic approaches.

Protein-modifying and cytoskeletal proteins are enriched in the MoO-infected wheat apoplast

PANTHER GO-Slim molecular function analysis revealed that DAPs from the MoL-infected wheat apoplastic fluid likely exhibit catalytic activity, molecular

a



b

Gene ID	Description	Gene name	Reference
LOC_Os01g71830	Glycosyl hydrolases family 17, putative, expressed	OsGLN1	(Akiyama and Pillai. 2001)
LOC_Os10g28120	Glycosyl hydrolase, putative, expressed	OsCHIB2	(Chan-Ho et al. 2004)
LOC_Os01g19170	Polygalacturonase, putative, expressed	PSL1/OsPG1	(Cao et al. 2021)
LOC_Os01g43160	Polygalacturonase, putative, expressed	OsGH28	(Sharma et al. 2013)
LOC_Os07g35350	Glucan endo-1,3-beta-glucosidase precursor, putative, expressed	OsGH17	(Sharma et al. 2013)
LOC_Os11g37960	WIP4 - Wound-induced protein precursor, expressed	OsPR4b	(Zhu et al. 2006)
LOC_Os05g46200	beta-galactosidase 8 precursor, putative, expressed	N/A	N/A
LOC_Os08g44020	Rhamnogalacturonate lyase, putative, expressed	N/A	N/A

Fig. 4 Carbohydrate metabolism-related proteins enriched in the apoplast of MoL-infected rice leaves. **a** Singular enrichment analysis of the enriched Gene Ontology (GO) terms for the DAPs identified in the apoplast of MoL-infected rice leaves. The dotted arrows represent "one significant node" and the dashed arrow represents "two significant nodes". The orange color represents the GO term significance level; the *P* value of GO:0005975 is $6.48e^{-06}$. **b** The eight carbohydrate metabolism-related genes encoding the DAPs identified in the apoplast of MoL-infected rice leaves

function regulator activity, molecular transducer activity, structural molecule activity, translation regulator activity, transporter activity, ATP-dependent activity, and binding activity. Although we only identified binding activity, catalytic activity, structural molecule activity, and translation regulator activity among the MoO-infected wheat apoplast DAPs, proteins with binding and catalytic activities were present in both MoO and MoL interactions (Additional file 2: Fig. S3). PANTHER protein class analysis grouped the host DAPs from the MoO- and MoL-infected wheat apoplasts into seven and 13 classes, respectively (Fig. 5a, b). Notably, the protein-modifying enzymes and cytoskeletal proteins occupy an important proportion in the MoO-infected wheat apoplastic fluid, suggesting that they may function in wheat MoO resistance (Fig. 5a, b). Additional analysis revealed that three of the five identified protein-modifying enzymes are serine proteases (Additional file 1: Table S4). However, the functions of these proteases in wheat disease resistance have yet to be investigated.

Stress-responsive proteins are enriched in the MoO-infected wheat apoplast

We employed SEA to identify enriched GO terms among the host DAPs from the MoO and MoL-infected wheat apoplastic fluid. Stress-responsive proteins were enriched in the MoO-infected wheat apoplast (Fig. 6a); the annotations of the five identified proteins, TaTPC1, PR4A, GLC1, POD1, and XIP1, are shown in Fig. 6b. TWO-PORE CHANNEL 1 (TaTPC1) is a calcium channel that regulates plant responses to environmental change (Wang et al. 2005). *PR4A* expression is responsive to infection by the wheat soil-borne pathogen *Fusarium culmorum*, and PR4A has ribonuclease and antifungal

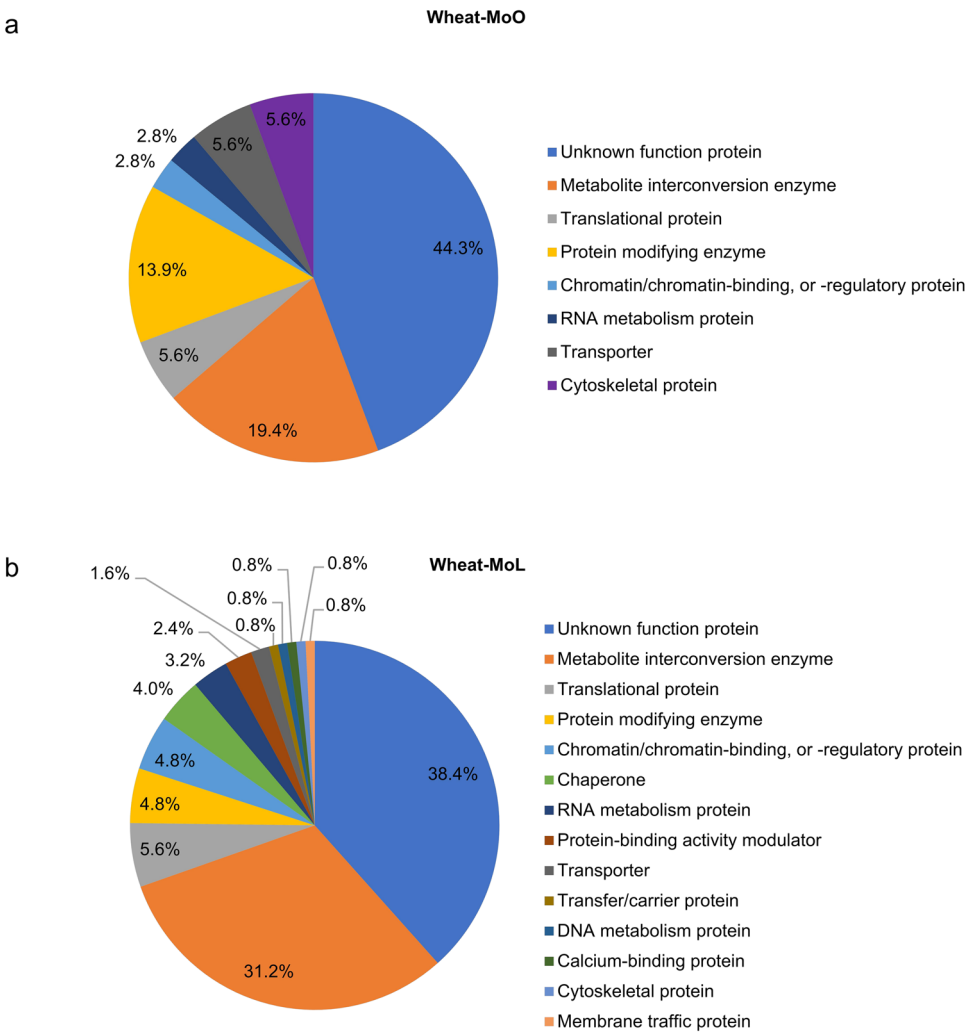
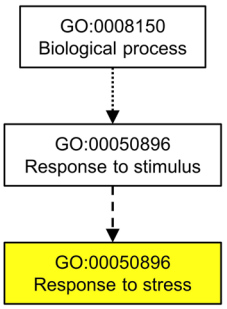


Fig. 5 Protein classes identified in the apoplast of MoO- and MoL-infected wheat leaves. **a, b** Protein classes determined by PANTHER protein class analysis in the apoplast of MoO- and MoL-infected wheat leaves

a



b

Uniprot ID	Protein description	Protein name	Reference
Q6YLX9	Two pore calcium channel protein	TaTPC1	(Wang et al. 2005)
O64392	Pathogenesis-related protein 4a	PR4A	(Bertini et al. 2003; Bertini et al. 2009)
P52409	Glucan endo-1,3-beta-glucosidase	GLC1	N/A*
C3VQ52	L-ascorbate peroxidase	POD1	N/A
Q8L5C6	Fungal xylanase inhibitor	XIP1	(Belien et al. 2005; Tundo et al. 2015)

*N/A: Not available

Fig. 6 Stress-related proteins enriched in the MoO-infected wheat apoplast. **a** Singular enrichment analysis of the enriched Gene Ontology (GO) terms for the DAPs identified in the apoplast of MoO-infected wheat leaves. The dotted arrow represents "one significant node" and the dashed arrow represents "two significant nodes". The yellow color represents the GO term significance level; the *P* value of GO:00050896 is 0.0234. **b** The five stress-related genes encoding the DAPs identified in the apoplast of MoO-infected wheat leaves

activity against several pathogenic fungi (Bertini et al. 2003, 2009). The wheat xylanase inhibitor Xylanase inhibitor protein 1 (XIP1) limits *Fusarium graminearum* infection by directly inhibiting xylanase activity and/or preventing host cell death in wheat (Belien et al. 2005; Tundo et al. 2015). How these proteins are involved in wheat MoO resistance remains unknown. Notably, DAPs in the MoL-infected wheat apoplastic fluid were enriched in several metabolic processes, such as organonitrogen compound biosynthesis, carbohydrate derivative metabolism, and cellular protein metabolism (Additional file 2: Figure S4). These results suggest that the incompatibility of MoO and compatibility of MoL on wheat induce different sets of defense proteins into the apoplast.

Different classes of apoplastic proteins are involved in MoO- and MoL-barley interactions

We identified 303 and 208 host DAPs from the MoO- and MoL-infected barley apoplastic fluids, respectively (Fig. 1d). We also compared the protein classes and functions of the DAPs in the two samples. Although barley is compatible with both MoO and MoL, the protein functions identified in the two groups differed. Besides the proteins with binding and catalytic activities, the DAPs from the MoO-infected barley apoplastic fluid had molecular function regulator activity (Additional file 2: Figure S5a), whereas the DAPs in the MoL-infected barley apoplastic fluid showed functions related to structural molecule activity and transcription regulator activity (Additional file 2: Figure S5b). In addition, MoO-infected barley apoplastic host proteins belonged to ten classes, compared to six in the MoL-infected barley apoplast (Fig. 7a, b). The ten classes comprising the MoO-infected barley apoplastic proteins were metabolite interconversion enzyme, protein-binding activity modulator, translational protein, cytoskeletal protein, protein-modifying enzyme, transporter, chaperone, RNA metabolism protein, cell adhesion molecule, and gene-specific transcriptional regulator (Fig. 7a). The six protein classes among DAPs in the MoL-infected barley apoplast were metabolite interconversion enzyme, chaperone, translational protein, chromatin/chromatin-binding or -regulatory protein, protein-modifying enzyme, and transporter (Fig. 7b).

The SEA showed that the host DAPs in MoO-infected barley apoplasts are enriched in glucose metabolism, small GTPase-mediated signal transduction, and hydrogen peroxide catabolism (Additional file 1: Table S5, Additional file 2: Figure S6). By contrast, the host DAPs in MoL-infected barley apoplasts were enriched in translation, protein folding, small molecule catabolism, and hydrogen peroxide catabolism (Additional file 1: Table S6, Additional file 2: Figure S7). Notably, the host DAPs in *M. oryzae*-infected barley apoplasts were all enriched in hydrogen peroxide catabolism and most were peroxidases (Additional file 1: Table S5 and Table S6), suggesting that peroxidases may play a vital role in barley resistance to *M. oryzae*.

Discussion

Host specificity refers to the capacity of pathogens to cause diseases on only particular plant species or only in some members of a specific plant species (Borah et al. 2018). The differential infection specificity of MoO, MoL, and MoT toward rice, barley, wheat, and other monocots is an excellent example of such host specificity (Yoshida et al. 2016; Chung et al. 2020; Li et al. 2022). However, the virulence and host factors that determine *M. oryzae* host specificity remain unknown.

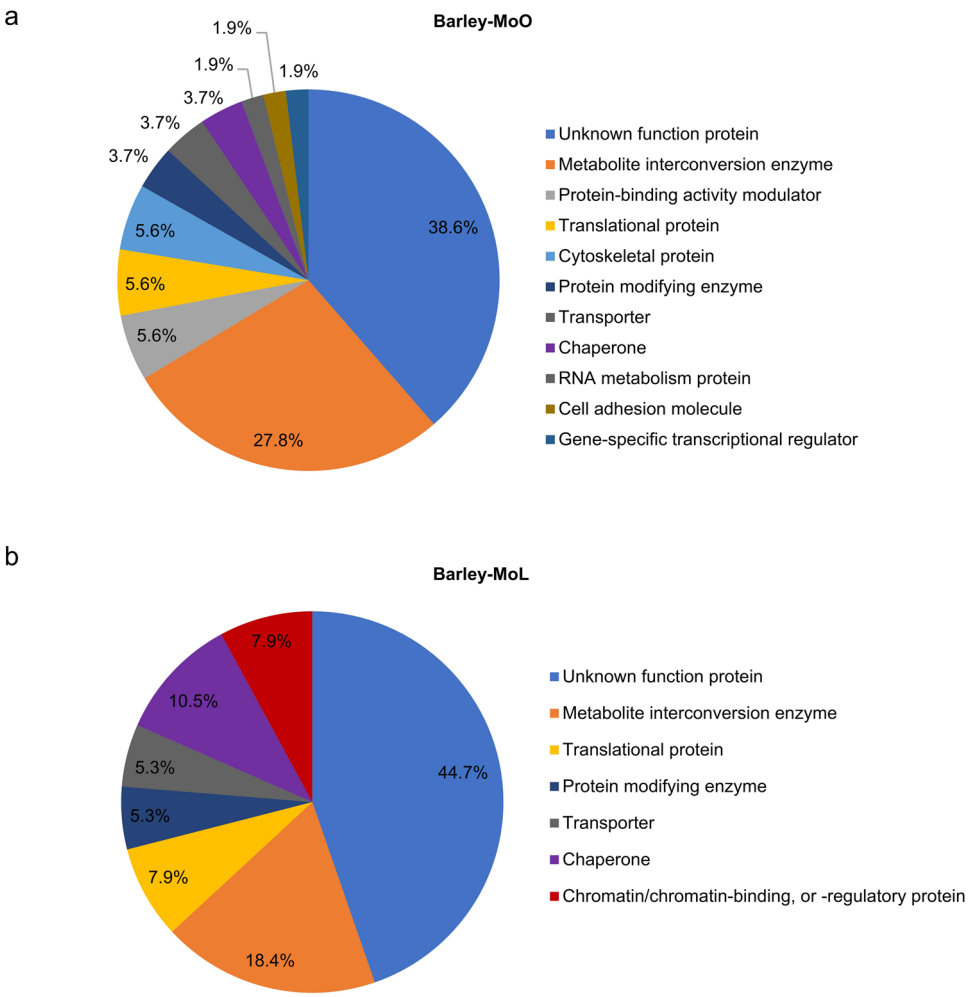


Fig. 7 Protein classes identified in the apoplast of MoO- and MoL-infected barley leaves. **a, b** Protein classes determined by PANTHER protein class analysis in the apoplast of MoO- and MoL- infected barley leaves

The plant cell apoplast is a major battleground for plant-pathogen interactions (Du et al. 2016; Wang and Wang 2018). To facilitate infection, plant pathogenic fungi secrete an arsenal of enzymes and effectors into the apoplast during the early stages of infection (Quoc and Chau 2017). To defend against an attack, plants deliver numerous defense-related proteins into the apoplast. Some of these proteins may recognize pathogen-derived proteins and lead to the activation of plant innate immunity, while other proteins may directly inhibit pathogen growth.

In this study, we inoculated rice, barley, and wheat with host-specific *M. oryzae* isolates and extracted apoplastic fluids from infected leaves. Proteomic analysis identified 807 proteins from the three hosts and 92 *M. oryzae* proteins from the apoplast of infected leaves. It should be noted that all 92 fungal proteins were identified from the MoO-rice interaction. Fungal proteins may not have been identified in the other *M. oryzae*-host interactions due to the low abundance of *M. oryzae*-secreted proteins in the intercellular space of wheat and barley, which may be difficult to detect by mass spectrometry. Nevertheless, to our knowledge, this is the first study to identify approximately 900 host and pathogen apoplastic proteins in a plant-fungus pathosystem. Several candidate proteins may play important roles in determining the host specificity of the three *M. oryzae* pathotypes.

Plant cell walls consist primarily of cellulose, hemicellulose, pectin, and other components such as lignin, water, and proteins (Quoc and Chau 2017). Fungal pathogens secrete various enzymes to degrade this physical barrier during early infection. For example, cutinases can degrade the plant cuticle by hydrolyzing cutin polymer ester bonds, representing a pivotal step in pathogen penetration and carbon acquisition (Kolattukudy 1985; Deising et al. 1992). Cellulases are known as the major

cell wall-degrading enzymes by converting cellulose to glucose via cleavage of the internal linkages of cellulose chains and destroying both the crystalline and amorphous regions of cellulose (Teeri 1997). Moreover, xylanases can degrade the linear molecules of polysaccharide β-1,4-xylan into xylose to break down hemicellulose (Paixao et al. 2021). Alpha-L-fucosidase releases L-fucose from plant xyloglucan and contributes to xyloglucan degradation together with other enzymes (Ajisaka et al. 1998). In this study, we identified 17 hydrolases from *M. oryzae* in MoO-infected apoplast (Fig. 2d, e), which may be crucial for *M. oryzae* virulence during infection. Further functional analysis of these 17 candidate hydrolases during the *M. oryzae*-rice interaction and identification of their host targets will provide new insights into the importance of these fungus-secreted proteins.

A large portion of host proteins in the MoO- and MoL-infected rice apoplasts were protein-modifying and metabolite interconversion enzymes (30.3% and 66.7%, respectively) (Fig. 3a, b). Glycosidases, peroxidases, and serine proteases were the main proteins in these two categories (Additional file 1: Table S3). Peroxidases are frequently associated with plant defense against pathogens and are the main source of apoplastic ROS (Survila et al. 2016). A double mutant in two *Arabidopsis thaliana* peroxidase genes (*PRX33* and *PRX34*) blocks the ROS burst in response to a fungal elicitor, causing enhanced susceptibility to a broad range of fungal and bacterial pathogens (Daudi et al. 2012). Notably, metabolite interconversion enzymes were the most abundant DAP type in the apoplast of all three infected hosts (21.4% in rice-MoO, 42.9% in rice-MoL, 19.4% in wheat-MoO, 31.4% in wheat-MoL, 27.8% in barley-MoO, and 18.4% in barley-MoL) (Figs. 3, 5, and 7). Previous studies showed an enrichment of plant-derived proteases within the apoplastic region, which play a role in bolstering host resistance against various types of pathogens (Kim et al. 2013; Wang et al. 2017; Grosse-Holz et al. 2018). How these enzymes interact with fungal effectors and activate subsequent immune responses warrants investigation.

Apoplast-localized pathogen suppression is a widely employed immune strategy of plant apoplastic proteins (Wang and Wang 2018). Among these proteins, secreted proteases are essential for plant pathogen resistance and are highly enriched in the apoplast, suggesting that their apoplastic localization may be crucial for their function (Wang et al. 2020). Serine proteases are one of the most abundant protease types in plants and mediate peptide bond cleavage. Serine proteases from different plant species play important roles in plant disease resistance. For instance, the tomato (*Solanum lycopersicum*) serine protease genes *P69B* and *P69C* are induced upon pathogen infection, and *P69B* is necessary for matrix metalloproteinase-mediated plant cell death (Jorda et al. 1999; Zimmermann et al. 2016). In our study, we identified three serine proteases (SERINE CARBOXYPEPTIDASE 23 [OsSCP23], OsSub28, and OsSCP3) in the MoL-infected rice apoplast (Additional file 1: Table S3). Serine proteases participate in numerous vital cellular responses, such as immunity and programmed cell death (Balakireva and Zamyatnin 2018). The functions of OsSCP23, OsSub28, and OsSCP3 in rice immunity against *M. oryzae* should be explored.

Lastly, more than one-fourth of the DAPs (8 in 25) in the MoL-infected rice apoplasts were involved in the carbohydrate metabolic process, implying that primary metabolism-related proteins may also play a critical role in rice MoL resistance (Fig. 4a, b). In the incompatible reaction between wheat and MoO, the proportion of protein-modifying enzymes, cytoskeletal proteins, and serine proteases increased in the apoplastic fluid. In addition, several stress-responsive proteins were also enriched. These results indicate that the above-mentioned proteins may be involved in wheat resistance to MoO.

Conclusions

Our study established a rapid and efficient strategy to isolate virulence factors and defense-related host proteins from the apoplast during fungal infection in three important cereals. We demonstrated that protein-modifying and metabolite interconversion enzymes, as well as cytoskeletal proteins, are core components in establishing plant resistance to *M. oryzae*. Among these proteins, serine proteases and peroxidases were significantly enriched in the apoplast after *M. oryzae* infection. This study unveils valuable candidate proteins for a deep understanding of the host specificity of *M. oryzae* towards cereals.

Methods

Fungal and plant materials

M. oryzae Oryzae (MoO) strain RO1-1 and *M. oryzae Lolium* (MoL) strain PL2-1 were used to inoculate rice (*Oryza sativa* cv. 'Nipponbare'), wheat (*Triticum aestivum* cv. 'Everest'), and barley (*Hordeum vulgare* cv. 'Hockett') plants in this study. Plants were grown in a growth chamber at 26/20°C and 80/60% relative humidity with a 12-h light/12-h dark photoperiod.

M. oryzae inoculation of host plants

Host plant inoculation with *M. oryzae* was conducted as previously described (Wang et al. 2021). Briefly, *M. oryzae* isolates were cultivated on oat/V8 medium in a dark incubator set to 28°C for one week and then moved to the benchtop at room temperature under fluorescent lighting for at least one week for spore induction. A suspension

of 5×10^5 conidia/mL containing 0.025% (v/v) Tween-20 was sprayed onto the leaves of 14-day-old wheat, 12-day-old barley, and 21-day-old rice seedlings, respectively. Leaves were collected for imaging and subsequent assays at three days post-inoculation (dpi). Disease severity was evaluated using Adobe Photoshop to calculate the pixel number of the leaves infected with blast strains.

Apoplastic fluid extraction

Apoplastic fluid extraction was performed as described previously with minor modifications (Gentzel et al. 2019). Briefly, leaves were placed in a 60-mL syringe containing apoplast wash solution (0.05 M sodium phosphate, 0.3 M NaCl, pH 7.5). After a pull-and-release vacuum cycle from the syringe, the leaves were removed from the wash solution and centrifuged at 2500 *g* for 10 min at 4°C. The apoplast liquid released from the leaves was gently resuspended and centrifuged at 2320 *g* for 5 min at 4°C. The supernatant was then transferred to a fresh tube for analysis.

Sample preparation and LC–MS/MS analyses

The same amount of total proteins (10 µg) isolated from apoplastic fluids was digested using the in-gel digestion method described previously (Shevchenko et al. 2006). Peptides were separated on an EASY-Spray™ nano column (Pepmap™ RSLC, C18, 3 µm, 100 Å, 75 µm * 250 mm, Thermo Scientific) using an HPLC system (Thermo Scientific). Mobile phases were 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B). The flow rate was set to 300 nL/min. Mobile phase B was increased from 2 to 16% over 45 min, then increased from 16 to 25% over 10 min, and again from 25 to 90% over 1 min. The flow rate was then kept at 90% for 2 min before being returned to 2% over 1 min. MS/MS analysis was performed on a Thermo Scientific Orbitrap Fusion mass spectrometer equipped with nanospray FAIMS Pro™ Sources in positive-ion mode and data-dependent mode. The full scan was obtained in the range of 375–1500 m/*z* with a resolution of 120,000. Three compensation voltages (–50 V, –65 V, and –80 V) were used for sample acquisition. MS^n was performed using higher-energy collisional dissociation (HCD) in ion trap mode to ensure the highest signal intensity of MS^n spectra. Dynamic exclusion was enabled with a repeat count of 1 within 60 s and a low and high mass width of 10 ppm.

Database processing

Raw MS data were queried against the most recent Uniprot databases (https://www.uniprot.org/) using Mascot Daemon (Matrix Science, version 2.7.0) via Proteome Discoverer (version 2.4, Thermo Scientific). The mass accuracy of the precursor ions was set to 10 ppm. The fragment mass tolerance was set to 0.5 Da. Carbamidomethylation (Cys) was set as a fixed modification, while oxidation (Met) and deamidation (N and Q) were set as variable modifications. A maximum of four missed cleavage sites for the enzyme were permitted. A decoy database was also searched to determine the false discovery rate (FDR), and peptides were filtered according to a 1% false discovery rate (FDR) threshold. Proteins identified with at least two unique peptides were considered reliable. Modified peptides were manually assessed for validation.

Bioinformatic analysis

DAPs are proteins exhibiting a minimum ten-fold difference between samples of the same cultivar after inoculation with two different *M. oryzae* isolates. SignalP-6.0 (https://services.healthtech.dtu.dk/service.php?SignalP-6.0) was used to predict the subcellular localizations of the identified DAPs (Teufel et al. 2022). PANTHER GO-Slim molecular function and PANTHER protein class analyses were performed using the PANTHER database (http://PANTHERdb.org/) (Mi et al. 2019; Thomas et al. 2022). Gene lists were uploaded in MSU gene ID format for rice and UniProtKB-ID format for wheat and barley, and the default whole-genome lists from the respective species were used as references. Singular enrichment analysis (SEA) for Gene Ontology (GO) enrichment was performed with agriGO (version 2.0, http://systemsbiology.cpolar.cn/agriGOv2/) (Tian et al. 2017).

Abbreviations

DAP	Differentially abundant protein
dpi	Days post-inoculation
EIHM	Extra-invasive hyphal membrane
FDR	False discovery rate
GH	Glycoside hydrolase
GO	Gene ontology
MoL	*Magnaporthe oryzae Lolium*
MoO	*Magnaporthe oryzae Oryzae*
MoT	*Magnaporthe oryzae Triticum*
PG	Polygalacturonase
PR	Pathogenesis-related
ROS	Reactive oxygen species
SEA	Singular enrichment analysis
Xoo	*Xanthomonas oryzae* pv. *oryzae*

Supplementary Information

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Additional file 1. Table S1. Different host proteins in MoO- and MoL-infected plants. **Table S2**. Differential M. oryzae proteins were identified in the apoplast of the MoO-infected rice leaves. **Table S3**. List of rice protein-modifying and metabolite interconversion enzymes in the apoplastic fluid of MoO- and MoL-infected rice leaves. **Table S4**. List of protein-modifying enzymes and cytoskeletal proteins in the apoplast fluid of MoO-infected wheat leaves. **Table S5**. Enriched GO terms among the DAPs in the

MoO-infected barley apoplast. **Table S6**. Enriched GO terms among the DAPs in the MoL-infected barley apoplast.

Additional file 2. Figure S1. Host protein function of identified proteins from MoO/MoT infected rice apoplastic fluid. **Figure S2.** Singular enrichment analysis is conducted to identify enriched Gene Ontology terms in identified differential proteins from MoO-infected rice apoplasts. **Figure S3.** Host protein function of identified differential proteins from MoO/MoT infected wheat apoplastic fluid. **Figure S4.** SEA is conducted to identify enriched GO terms in identified differential proteins from MoL-infected wheat apoplasts. **Figure S5.** Host protein function of identified differential proteins from MoO/MoT infected barley apoplastic fluid. **Figure S6.** Singular enrichment analysis of identified differential proteins in the MoO-infected barley apoplast. **Figure S7.** Singular enrichment analysis of identified differential proteins in the MoL-infected barley apoplast.

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Author contributions
JW and GW conceived and designed the study. JW and KH collected the data. JW curated the data and conducted the analyses. JW, LQ, and GW drafted the manuscript. JW, LZ, and MQ prepared the figures. JW and GW revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding authors upon reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
The authors declare no competing interests.

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