1 **Comparative secretome of *Magnaporthe oryzae* identified** 2 **proteins involved in virulence and cell wall integrity**

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Abstract

Plant fungal pathogens secrete numerous proteins into the apoplast at the plant–fungus contact sites to facilitate colonization. Only a few secreted proteins were functionally characterized in *Magnaporthe oryzae*, the fungal pathogen causing rice blast disease worldwide. ALG3 is an α -1, 3-mannosyltransferase function in N-glycan synthesis for secreted N-glycosylated proteins, and the $\Delta alg3$ mutants show strong defects in cell wall integrity and fungal virulence, indicating a potential effect on the secretion of multiple proteins. In this study, we compared the secretome of wild type and $\Delta alg3$ mutants, and identified 51 proteins that require ALG3 for proper secretion. These are predicted to be involved in metabolic processes, interspecies interactions, cell wall organization, and response to chemicals. The tested secreted proteins localized at the apoplast region surrounding the fungal infection hyphae. Moreover, the N-glycosylation of candidate proteins was significantly changed in the $\Delta alg3$ mutant, leading to the reduction of protein secretion and abnormal protein localization. Furthermore, we tested the function of two genes, one is a previously reported *M. oryzae* gene *Invertase 1* (*INV1*) encoding a secreted invertase, and the other one is a gene encoding an Acid mammalian chitinase (*AMCase*). The fungal virulence was significantly reduced and the cell wall integrity was altered in the $\Delta inv1$ and $\Delta amcase$ mutant strains. Elucidation of the comparative secretome of *M. oryzae* improves our understanding of the proteins that require ALG3 for secretion, and of their function in fungal virulence and cell wall integrity.

Key words: Rice blast fungus; Secretome; N-glycosylation; INV1; AMCase

55 Introduction

56 The fungal pathogen *Magnaporthe oryzae* causes rice blast disease, one of the most
 57 devastating diseases of cultivated rice (*Oryza sativa*) worldwide [1]. Due to its
 58 agronomic and scientific importance, *M. oryzae* has become a model fungus to study
 59 plant–pathogen interactions. As a hemibiotrophic fungus, *M. oryzae* undergoes an
 60 initial biotrophic infection stage and then switches to a necrotrophic stage [2, 3]. Once
 61 in contact with the plant surface, the conidium germinates and the germ tip forms a
 62 dome-shape appressorium on the leaf surface. The appressorium matures and
 63 develops a penetration peg to rupture the plant cuticle and invade the epidermal cell.
 64 After penetration, the fungus develops biotrophic invasive hyphae in the initial leaf
 65 cell and branches into neighboring cells. Eventually, the fungus generates a visible
 66 necrotic lesion with numerous, newly formed conidia ready for the next infection
 67 cycle [4]. *M. oryzae* mutants impaired in appressorium and/or infection hyphae
 68 formation also showed defects in pathogenicity, indicating that the formation of the
 69 fungal appressorium and biotrophic growth are essential for successful infection
 70 [5-10]. Therefore, exploring the molecular basis underlying these processes enable us
 71 to illustrate the underlined mechanisms of plant–fungus interactions and develop new
 72 anti-fungal strategies.

73 During the initial biotrophic infection stage, *M. oryzae* secretes numerous proteins
 74 into the plant–fungus contact sites to facilitate colonization. One class of secreted
 75 proteins are enzymes including cutinase2, endoglucanase, endo- β -1,4 xylanase and
 76 cellulases that break down the plant cell wall; the other class of secreted proteins are
 77 small effector proteins that suppress the host immune system or manipulate host
 78 metabolism [11-15]. *M. oryzae* effector proteins include cytoplasmic effectors and
 79 apoplastic effectors, which are secreted through distinct pathways [16]. The
 80 cytoplasmic effectors, such as PWL2 and BAS1, are secreted through exocyst
 81 components to the biotrophic interfacial complex, a specific plant membrane-rich
 82 structure associated with invasive hyphae. Many cytoplasmic effector proteins have
 83 been studied, especially the avirulent effector proteins including AvrPia, Avr1-CO39,

84 AvrPita, AvrPik, and AvrPiz-t [17-23]. The apoplastic effectors, such as Slp1 and Bas4,
85 are secreted from invasive hyphae into the extracellular compartment surrounding
86 invasive hyphae via the conventional secretory pathway from the endoplasmic
87 reticulum (ER) to the Golgi apparatus [8, 12, 16]. So far, only few apoplastic effector
88 proteins have been characterized. The virulence effector Slp1 functions in host
89 immune suppression by binding chitin oligosaccharides to avoid the chitin-induced
90 plant immune response [12]. Since proteins secreted at early biotrophic fungal growth
91 stages contribute to fungal pathogenicity, characterization of additional secreted
92 proteins during invasive hyphae growth may help us to better understand the
93 mechanisms of the rice-*M. oryzae* interaction.

94 *N*-glycosylation of protein is a post-translational modification commonly found in
95 eukaryotic organisms. Many *N*-glycosylated proteins are plasma
96 membrane-associated proteins or secreted proteins. The proper folding of these
97 proteins relies on correct *N*-glycosylation process, which then related to a proper
98 secretion route to their functional sites. *N*-glycosylation starts with the synthesis of the
99 core oligosaccharide-- NAcGlc₂Man₉Glc₃--short for two N-acetylglucosamines
100 (NAcGlc), nine mannoses (Man) and three glucose (Glc) molecules. After that, the
101 core oligosaccharide is added to an asparagine residue (*N*) in the consensus sequence
102 Asn-x-Ser/Thr (x is any amino acid apart from Proline). The *N*-glycosylation
103 modification takes place in the ER and is modified in the Golgi apparatus, following
104 the conventional secretion route for those target proteins. Defects in the early step of
105 *N*-glycosylation results in an improper folding of target proteins, leading to protein
106 breakdown through ER-associated degradation. Correct *N*-glycosylation is necessary
107 for the proper function of secreted proteins in eukaryotes. For instance, impaired plant
108 *N*-glycosylation or quality control for glycoprotein folding in the ER results in
109 reduced plant resistance to bacterial pathogens. In pathogenic fungi, defects of
110 *N*-glycosylation modification result in a reduction of fungal pathogenicity [24, 25]. In
111 *M. oryzae*, loss of *ALG3*, an α -1, 3-mannosyltransferase functioning in core
112 oligosaccharide synthesis, results in a significant reduction of fungal virulence and
113 defects in fungal cell wall integrity. The apoplastic effector Slp1 was the target protein

114 required ALG3 for its proper *N*-glycosylation [26]. Since $\Delta alg3$ exhibits a much
 115 stronger phenotype than that of the loss-of-function $\Delta slp1$ mutant, indicating apart
 116 from Slp1, other target proteins should also be affected in the $\Delta alg3$ mutant. Therefore,
 117 we attempted a proteomics approach to identify the other secreted proteins affected in
 118 the $\Delta alg3$ mutant, which should play essential roles in fungal virulence and cell wall
 119 integrity.

120 In this study, we used a comparative secretome analysis to identify secreted
 121 *N*-glycosylated proteins whose secretion required ALG3 function. Comparing the *in*
 122 *vitro* secretome of *M. oryzae* wild-type strain P131 and the knockout mutant $\Delta alg3$,
 123 we found 51 proteins that were not secreted, or secreted in reduced amounts in the
 124 $\Delta alg3$ mutant. By testing 9 out of those 51 proteins, we confirmed that in the $\Delta alg3$
 125 mutant, the *N*-glycosylation level of those proteins, as well as their localization in the
 126 infection hyphae were affected. We confirmed that two genes, *INV1* and *AMCase*
 127 function in fungal pathogenicity and cell wall integrity. Our study provided new
 128 insight about the role of secreted *N*-glycosylated proteins in fungal virulence and cell
 129 wall integrity.

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133 Results

134 Identification of *M. oryzae* secreted proteins requiring ALG3 for proper 135 secretion

136 In order to identify *N*-glycosylated proteins that require ALG3 for their secretion, we
137 collected *M. oryzae* secreted proteins from the wild-type strain P131 and the knockout
138 mutant $\Delta alg3$ grown in liquid growth medium (**Fig 1A**). *M. oryzae* may experience
139 nutrient-deficient conditions when infecting susceptible plants; therefore, to better
140 mimic the nutrient-deficient conditions during *M. oryzae* infection *in vitro*, we first
141 compared the secreted proteins isolated from hyphae grown on nutrient-sufficient
142 complete medium (CM) and nutrient-deficient minimal medium (MM). However,
143 immunoblotting analysis using GFP fusion proteins found that Slp1-- a known
144 effector protein whose secretion is regulated by ALG3-- only detected from *M. oryzae*
145 grown in liquid CM but not from cultures grown in liquid MM (Supplemental Fig.
146 1A). Therefore, secreted proteins were collected from *M. oryzae* grown in liquid CM.
147 Three replicates of extracted P131 and $\Delta alg3$ protein samples were separated by
148 sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and protein
149 bands were clearly detected using silver staining (Supplemental Fig. 1B), indicating
150 that those samples were suitable for subsequent analysis. The same amounts of
151 secreted proteins were then used to the liquid chromatography with tandem mass
152 spectrometry (LC-MS/MS) analysis for comparison.

153 Using a label-free quantitative (LFQ) proteomic analysis, we identified 411 proteins
154 from the wild-type P131 strain and 539 proteins from the $\Delta alg3$ mutant strain
155 (Supplemental Table S1). The replication ratio was >74% for both samples (**Table 1**
156 and Supplemental Table S1). We used Principal component analysis (PCA) to analyze
157 the correlation and distribution of those secreted proteins from P131 and $\Delta alg3$
158 samples based on the protein intensity. In the PCA plot, P131 samples were clearly
159 separated from $\Delta alg3$ samples (Fig. 1B), confirming that the isolated secreted proteins

160 are different between P131 and $\Delta alg3$ strains.

161 Based on the protein LFQ intensities, we calculated the ratio of proteins in P131 to
 162 $\Delta alg3$ and selected proteins for which the fold-change ratio was >2.0 or <0.5 and the
 163 p value was <0.05 . The identified proteins could be differentiated into three groups: 1)
 164 51 proteins with a higher abundance in the secretome of P131 than in $\Delta alg3$; 2) 295
 165 proteins with an equal abundance in both secretomes; and 3) 210 proteins with a
 166 higher abundance in the secretome of $\Delta alg3$ than in P131 (**Table 2** and Supplemental
 167 Table S1). ALG3 is an α -1, 3-mannosyltransferase, which functions in the
 168 biosynthesis of the core oligosaccharide of N-glycan, and loss of ALG3 alters protein
 169 N-glycosylation modifications and reduces client protein steady-state levels, as shown
 170 previously for the effector protein Slp1. Therefore, proteins with significant lower
 171 abundance in $\Delta alg3$ (Group 1 proteins) are putative target proteins for
 172 N-glycosylation specifically require ALG3 for their secretion (**Table 3**).

173 We used SignalP (<http://www.cbs.dtu.dk/services/SignalP-4.1/>) , SecretomeP
 174 (<http://www.cbs.dtu.dk/services/SecretomeP/>) , and Transmembrane Hidden Markov
 175 Model analyses (TMHMM, <http://www.cbs.dtu.dk/services/TMHMM/>) to validate the
 176 secreted nature of the proteins identified from secretome. SignalP predicts the
 177 N-terminal signal peptide for proteins secreted through the ER-Golgi pathway,
 178 SecretomeP predicts proteins non-classical secreted proteins, while TMHMM predicts
 179 possible transmembrane domains for membrane-anchored proteins. For Group 1
 180 proteins, 43 proteins contain a SP and 7 proteins were predicted with SecretomeP,
 181 among them 11 proteins were predicted to have one TM domain. Therefore, 62.7% of
 182 Group 1 proteins are secreted proteins and 21.6% are membrane-localized proteins.
 183 Following the same calculation, 35.9% of Group 2 proteins are secreted proteins and
 184 7.5% are membrane proteins; only 9.0% of Group 3 proteins are secretory proteins
 185 and 6.7% are membrane proteins. From the groups 3 proteins that specifically
 186 detected with higher abundance, a much smaller proportion are secreted proteins,
 187 indicating that many non-secreted proteins were abnormally secreted in the $\Delta alg3$

188 strain, possibly because the cell wall integrity was impaired in $\Delta alg3$ mutant; however,
189 those proteins were not of interest in this study (Table 2 and Supplemental Table S2).

190 In further analysis, we focus on the 51 proteins listed in Table 3. We used *N*-GlycoSite
191 analysis (<http://www.cbs.dtu.dk/services/NetNGlyc/>) to predict the possible *N*-linked
192 glycosylation sites for them. Among them, 28 proteins (55%) have more than two
193 predicted *N*-glycosylation sites, while 6 (12%), 11 (21%), and 6 (12%) proteins
194 contain two, one, or no predicted *N*-glycosylation sites, respectively (**Figure 2A** and
195 Supplemental Table 2). Thus, most proteins regulated by ALG3 contain predicted
196 *N*-glycosylation sites.

197 Gene ontology enrichment analysis was performed for the 51 putative
198 ALG3-regulated secreted proteins. Apart from eight hypothetical proteins with
199 unknown functions, many proteins were detected with hydrolase, ion binding, and
200 oxidoreductase activity (Figure 2B). Furthermore, many proteins were predicted to
201 play a role in metabolic processes, eg: organic substance metabolites, cellular
202 metabolites, and single-organism metabolic (Figure 2C), suggesting that those
203 secreted proteins strongly influence *M. oryzae* metabolic processes. Other proteins
204 were associated with cell wall organization, response to chemicals, or interaction
205 between organisms (Figure 2C).

206 **Validation of secreted proteins regulated by ALG3**

207 To further confirm the secretion of the identified proteins specifically regulated by
208 ALG3, proteins were expressed in the *M. oryzae* P131 strain and $\Delta alg3$ strain, with a
209 C-terminal GFP tag, under the control of the promotor from a strongly expressed *M.*
210 *oryzae* gene (**Figure 3A**). We selected the following proteins for the validation tests:
211 nine proteins including MGG_05785, MGG_01956, MGG_08772, MGG_09460,
212 MGG_03826, MGG_10209, MGG_10466, MGG_00592 and MGG_04732 were
213 identified with a significantly higher abundance in the medium of P131 compared
214 with that of $\Delta alg3$ (Group 1 proteins, table 3), and one protein MGG_13764 identified

215 at a similar level in the medium of both strains was chosen as a control sample for the
216 secretion assay (Group 2 protein, Supplemental Table 2).

217 We cultured various fungal strains in liquid CM and detect the level of proteins in the
218 secreted CM medium using anti-GFP antibody. We found that 05785-GFP, 01956-GFP,
219 08772-GFP, 09460-GFP, 03826-GFP, 10209-GFP and 10466-GFP were detected in
220 the CM medium of P131 strain but not of $\Delta alg3$ strain; while 00592-GFP and
221 04732-GFP were detected in the CM medium of P131 strain, and barely detectable in
222 that of $\Delta alg3$ strain, and 13764-GFP were detected in the CM of both P131 and $\Delta alg3$
223 strains (Figure 3B). All the proteins were detectable in the mycelia protein extracts
224 (see below), indicating efficient protein expression in those fungal strains. The
225 immunoblotting assays confirmed our secretome data that tested secreted proteins
226 from Group 1 require ALG3 for proper secretion.

227 We developed another method to directly test protein secretion in the liquid medium
228 without a protein extraction step. The fluorescence signal from a functional GFP
229 fusion can be easily measured using a microplate reader, and the fluorescence
230 intensity correlates to the protein expression level (Figure 3C). Therefore, the liquid
231 CM was loaded in the microplate reader and the fluorescence intensity was measured
232 to detect secreted GFP fusion proteins. Considering that the same vector might
233 integrate into different genomic regions in different strains, we could not directly
234 compare the measured fluorescence intensity between P131 and $\Delta alg3$ strains
235 harboring the same GFP fusion protein. Therefore, the fluorescence intensity of
236 protein extracts from a certain weight (0.1g) of *M. oryzae* mycelia were also measured
237 for normalization. For each strain, the fluorescence signals of the liquid CM were
238 divided by that of mycelium protein extracts and calculated as a ratio to determine the
239 level of secretion of each protein (Fig. 3D). To test whether this measurement works
240 for secreted proteins, the fluorescence intensity of the P131 strain expressing control
241 GFP alone was measured and the rate of secretion was calculated as 0.08, indicating
242 that unfused GFP was not secreted into the liquid CM. Moreover, Slp1-GFP was

secreted in the P131 strain at a rate of 0.36, while the nuclei-localized protein MoGRP1-GFP was secreted at a rate of 0.06, supporting the observation that Slp1-GFP but not MoGRP1-GFP was secreted into the liquid CM in the P131 strain [27] (Supplemental Fig. 2B). Furthermore, in the $\Delta alg3$ strain, Slp1-GFP was secreted at a rate of 0.06, confirming the finding that the secretion of Slp1-GFP is impaired in the $\Delta alg3$ strain. These controls confirm that this measurement and calculation is suitable to detect secreted GFP fusion proteins in *M. oryzae* strains.

Using the method described above, we validated that proteins encoded by MGG_05785, MGG_01956, MGG_08772, MGG_09460, MGG_03826, MGG_10209, MGG_10466, MGG_00592 were secreted in the liquid CM from the P131 strains, but were not secreted from the $\Delta alg3$ strains (Fig. 3E), indicating that those proteins were indeed secreted proteins regulated by ALG3. Protein MGG_04732 were secreted in the liquid CM of the P131 strains but with a reduced level in the liquid of $\Delta alg3$ strains. The control protein encoded by MGG_13764, were secreted in the liquid CM of both P131 and $\Delta alg3$ strains (Fig. 3E), further confirmed our secretome data on Group1 proteins.

We next tested whether those secreted proteins contain the *N*-glycosylation modification. Our previous study demonstrated that the client protein Slp1 were *N*-glycosylated with Man₅GlcNAc₂ (but not Man₉GlcNAc₂) in the $\Delta alg3$ mutants, and the size difference was observed using immunoblot analysis. We found that all the tested proteins differed in size between the P131 and $\Delta alg3$ strains (**Fig. 4**), indicating that complete *N*-glycosylation of the above-mentioned proteins occurs in the P131 strain, while only partial *N*-glycosylation takes place in the $\Delta alg3$ strain. We also applied the peptide *N*-glycosidase F (PNGase F) [28]--an amidase that cuts the GlcNAc group and the Asn residues from *N*-glycosylated proteins--and detected the de-glycosylated form of each protein in the P131 strain (Fig. 4). The different protein bands between the minus and plus PNGase F treatment in the $\Delta alg3$ strain suggested that the partial *N*-glycosylation of glycoproteins was also cleaved by PNGase F. Our

findings revealed that the identified secretory proteins contained the *N*-glycosylation modification and depend on ALG3 for a complete *N*-glycosylation.

Since those secreted proteins were identified from liquid medium during fungal vegetative growth, we wondered whether they also localized in the infection hypha during the plant–fungus interaction. We investigated their subcellular localization in the infection hyphae using a fluorescence microscope. In P131 strains transformed with GFP-labeled MGG_05785, MGG_01956, MGG_08772, MGG_09460, MGG_03826, MGG_10209, MGG_10466, MGG_00592, MGG_04732 and MGG_13764, the GFP signals were distributed in the cytoplasm and at the plant–fungus interface (**Fig. 5**), similar to Slp1-GFP in the P131 strain. In $\Delta alg3$ strains transformed with GFP-labeled MGG_05785, MGG_01956, MGG_08772, MGG_09460, MGG_03826, MGG_10209, MGG_10466, MGG_00592 and MGG_04732, the GFP fusion proteins were restricted in small dot-like structures within the infection hyphae and only a small portion of GFP signals located at the plant–fungus interface (Fig. 5), indicating that *N*-glycosylation is important for the subcellular localization of those proteins. In $\Delta alg3$ strains expressing MGG_13764-GFP, the fluorescent signals were similar to that in P131 strains, indicating that *N*-glycosylation modification is not essential for the subcellular localization of MGG_13764 protein.

MGG_05785 is important for fungal pathogenicity and cell wall integrity

From the LC-MS data, INV1, encoded by *MGG_05785*, is the most abundant protein identified only in liquid CM of the P131 strain (Table 3). The *N*-glycosylation modification of INV1 was confirmed in the mycelium proteins of *M. oryzae* strain P131 expressing INV1-GFP fusion proteins, and this process was mediated by ALG3 (Fig. 4). Invertase is a major enzyme present in plants and microorganisms [29], and carries out the irreversible conversion of sucrose to glucose and fructose (**Fig. 6A**). A previous study showed that *M. oryzae* $\Delta inv1$ mutants had reduced fitness during plant infection [30]. INV1 has a length of 660 amino acids and contains 13 predicted

299 *N*-glycosylation sites (Supplemental Table 2 and Fig. 5B). INV1 belongs to the
300 glycosyl hydrolase family 32, containing a GH32 N-terminal domain, a GH32
301 C-terminal domain, and a signal peptide as a leading sequence (Fig. 6B).

302 We further characterized the function of INV1 in cell wall integrity and virulence by
303 generating two independent knockout mutants in the P131 background, $\Delta inv1-1$ and
304 $\Delta inv1-2$, both mutants were confirmed with Southern blot analysis (Supplemental Fig.
305 3A and B), and we generated one complementation strain. The $\Delta inv1$ mutant strains
306 grew normally on oatmeal-tomato agar plates (Supplemental Fig. 3C). To confirm its
307 invertase function, the $\Delta inv1$ mutant strains were grown on solid MM without a
308 carbon source or were supplied with sucrose, glucose, or fructose as the only carbon
309 source. Since the size of the colonies was similar among the P131 strain and $\Delta inv1$
310 mutant strains, the thickness of the fungal growth reflects the carbon absorption
311 efficiency. The mycelium of the P131 strain was very thin on the solid MM without a
312 carbon source, but was much thicker on the solid MM with sucrose, glucose, or
313 fructose. By contrast, the mycelium of the $\Delta inv1$ mutant strain was thicker only on the
314 solid MM with glucose or fructose, but not with sucrose, confirming that the $\Delta inv1$
315 mutants could not utilize sucrose for fungal mycelium growth (Supplemental Fig. 3D).
316 Introducing a functional copy of *INV1* into the $\Delta inv1$ mutant strain restored the
317 sucrose utilization in the resulting $\Delta inv1/INV1$ complementation strain (Supplemental
318 Fig. 3D). The fungal mycelial weights of $\Delta inv1$ mutant strains were also measured in
319 the liquid MM supplied with different carbon sources and the same results were
320 observed (Supplemental Fig. 3D). Therefore, we concluded that INV1 plays an
321 important role in sucrose utilization.

322 In our previous report, we observed defects of cell wall integrity in the $\Delta alg3$ strains,
323 but not in the $\Delta slp1$ mutant, suggesting that other secreted proteins regulated by
324 ALG3 should function in cell wall integrity. Since INV1 is the most abundant protein
325 secreted in CM during mycelium growth, we wondered whether INV1 plays a role in
326 cell wall integrity. We grew $\Delta inv1$ mutant strains on common perturbing reagents used

in cell wall integrity test, including Calcofluor white (CFW), Congo red (CR), and SDS [26]. When the P131 strain and the complementation strain $\Delta invI/INV1$ were grown on CM with those agents, the vegetative growth was reduced by 41% with CFW, 23% with CR, and 28% with SDS, in comparison with their growth on CM. By contrast, growth in the mutant strains $\Delta invI-1$ and $\Delta invI-2$ was reduced by 52% with CFW, 39% with CR, and 37% with SDS, suggesting an increased sensitivity to cell wall perturbing reagents (Fig. 6C and D). We noticed that the sugar source commonly used in CM is sucrose, which cannot be utilized by the $\Delta invI$ mutants. When we replaced the sucrose with glucose in this experiment, we found that all strains had similar reductions in growth rates on plates with CFW, CR, and SDS (Supplemental Fig. 3F and G). The growth assays in different CM revealed that fungal cell wall integrity is associated with normal carbon utilization in $\Delta invI$ mutants.

We questioned whether the reduced pathogenicity of $\Delta invI$ mutants was also associated with carbon uptake. Therefore, we investigated the role of INV1 on *M. oryzae* pathogenicity. Consistent with our previous report, the $\Delta invI$ mutants showed significantly reduced virulence on rice and barley (*Hordeum vulgare*) leaves (Supplemental Fig. 3H, I, J, and K). Furthermore, we added an additional carbon source to see whether it could restore the mutant's virulence. In drop infection assays, the symptom lesions from $\Delta invI$ strains were much narrower than from the P131 strains on the unwounded barley leaves (Fig. 6E). When 0.2% glucose and 0.2% fructose were applied in the drop infection assays, the pathogenicity of $\Delta invI$ mutants was partially restored (Fig. 6E and F). Therefore, carbon utilization is partially linked to the fungal virulence in $\Delta invI$ mutants.

350 **MGG_04732 is important for fungal pathogenicity and cell wall integrity**

We next tested the gene *MGG_04732*, encoding a protein with homologue to Acid mammalian chitinase (named **AMCase** accordingly) containing a signal peptide followed with a glycosyl hydrolase family 18 (GH_18) domain (**Fig. 7A**) [31, 32]. *Magnaporthe oryzae* genome have 15 genes encoding GH_18 family chitinase, in

355 which only *Chia1* was characterized to be important for fungal pathogenicity [33].
 356 The *N*-glycosylation of AMCase was also confirmed in the mycelium proteins of
 357 P131 expressing AMCase -GFP fusion proteins, and this process was mediated by
 358 ALG3 (Fig. 4E).

359 We wondered whether *AMCase* also plays a role in cell wall integrity and
 360 pathogenicity, three independent knockout mutant $\Delta amcase-1$, $\Delta amcase-2$ and Δ
 361 *amcase-3* were generated and confirmed by PCR analysis (Supplemental Fig. 4A and
 362 B). Those knockout mutant strains grew normal on the oatmeal-tomato agar (OTA)
 363 plates (Supplemental Fig. 4C). In the cell wall integration test, when the P131 strain
 364 was grown on CM with cell wall perturbing reagents, vegetative growth was reduced
 365 by 36% with CFW, 16% with CR, and 31% with SDS, in comparison with their
 366 growth on CM. By contrast, growth in the mutant strains $\Delta amcase-1$, $\Delta amcase-2$, and
 367 $\Delta amcase-3$ was reduced by 50, 13, and 30% on plates with CFW, CR, and SDS,
 368 respectively, suggesting an increased sensitivity to CFW, but reduced sensitivity to
 369 CR (Fig. 7B and C). In the pathogenicity assay, we sprayed rice and barley leaves
 370 with spore solution. The knockout mutants showed significantly reduced
 371 pathogenicity on rice and barley (*Hordeum vulgare*) leaves (Fig. 7D, E, F and G),
 372 suggesting that AMCase functions in fungal pathogenicity. Since AMCase is related to
 373 the decomposition of chitin, we speculated AMCase might be also involved in chitin
 374 decomposition. We grew the wild type P131 and knockout strains on the solid
 375 minimal medium (MM) without carbon source or supplied with glucose and chitin as
 376 sole carbon source. The colony diameter of knockout strains slightly decreased
 377 compared with P131 on MM without carbon source, but no growth difference on MM
 378 supplied with glucose and chitin (Supplemental Fig 4F and G).

379

380 Discussion

381 Many studies have used liquid culture medium to identify the secretome from
 382 pathogenic microbes, eg. *Xanthomonas oryzae*, *Botrytis cinerea*, and *Staphylococcus*
 383 *aureus* [34-37]. In the present study, we used a quantitative proteomics approach to
 384 analyze the secretome of *M. oryzae* and identified 558 proteins from the liquid culture
 385 medium, including 411 proteins of the wild-type P131 strain and 539 proteins of the
 386 $\Delta alg3$ mutant strain. Among the 558 proteins, 190 were found in previous *M. oryzae*
 387 secretome studies (Supplemental Table 2). In a previous study, 51 proteins were
 388 identified by comparative secretome analyses of *M. oryzae* grown in CM, MM, and
 389 MM-N (MM lacking N) media, of which 34 proteins (66.7%) were also identified in
 390 our data set [38]. In another study, the secretome of conidiospores grown in glass
 391 plates, PVDF membrane, and liquid culture medium were analyzed and 52 proteins
 392 were identified [39], of which 32 proteins (61.5%) overlapped with our secretome
 393 (Supplemental Table 2). Therefore, our *in vitro* secretome analysis detected many
 394 more secreted proteins compared with the two previous studies, possibly because we
 395 used gel-free rather than gel-based proteomics. Although it is a much greater
 396 challenge, *in vivo* apoplastic fluid of rice after *M. oryzae* infection was analyzed and a
 397 total of 441 secreted proteins were identified, of which 52 proteins were from a 2-DE
 398 gel-based approach and 425 proteins were from a gel-free MudPIT analysis [40].
 399 When comparing this *in vivo* secretome with our *in vitro* secretome data, 171 proteins
 400 overlapped, accounting for 38.8–41.6% of the proteins identified in the two studies
 401 (Supplemental Table 2), indicating that a portion of pathogen infection-related
 402 proteins were expressed and secreted when grown in the liquid culture. Interestingly,
 403 many previously characterized secreted proteins were identified from our secretome
 404 dataset common in the P131 strain and the $\Delta alg3$ strain. Secreted proteins including
 405 MSP1 (MGG_05344), EMP1 (MGG_00527), MoHrip1 (MGG_15022), and MoHrip2
 406 (MGG_16187) were found to be important for fungal pathogenicity [41-43]. Two
 407 secreted chitin deacylates MoCDA1 (MGG_14966), and MoCDA 2 (MGG_08774)
 408 were found to be dispensable for *Magnaporthe* virulence but important for fungal

409 vegetative growth under stress conditions [44]. Notably, apart from EMP1, which
 410 contains four potential N-glycosylation sites, the other above-mentioned secreted
 411 proteins have only one or even no potential N-glycosylation sites, which could be
 412 explained as that N-glycosylation is dispensable for secretion of those proteins, thus
 413 they were detected both from P131 strain and the $\Delta alg3$ strain. Therefore, in the
 414 common list of proteins secreted from both strains, some uncharacterized secreted
 415 proteins might also function in fungal pathology or vegetative growth in different
 416 stress conditions. Further investigation of those commonly secreted proteins might
 417 identify additional virulence effectors in the future.

418 Protein N-glycosylation is a significant protein modification essential for fungal
 419 infection. Mutants of protein N-glycosylation showing defect in fungal virulence
 420 are reported not only for *M. oryzae* [45], but also for other plant pathogens such as
 421 *Penicillium digitatum*, *Botrytis cinerea* and *Ustilago maydis* [24, 25, 46-52]. Recent
 422 study on glycoprotein proteomics in *Ustilago maydis* using wildtype and mutants
 423 $\Delta gls1$, a mutant also involved in N-glycosylation process, had identified several
 424 protein with N-glycosylation modification [53]. In our study, comparing the
 425 secretome of P131 and $\Delta alg3$, 17 proteins were totally absent in the $\Delta alg3$ strain and
 426 34 proteins had a significantly higher abundance in P131 samples than in $\Delta alg3$
 427 samples; thus, these 51 proteins were considered secreted proteins that specifically
 428 require ALG3 for their secretion (Table 3). Since ALG3 functions in the early step of
 429 oligosaccharide synthesis for N-glycosylated proteins, the modified proteins should
 430 contain potential N-glycosylation sites. Among those 51 proteins, the 45 proteins that
 431 contain at least one predicted N-glycosylation site should be direct targets of ALG3
 432 (Table 3); the other 6 proteins that do not have a predicted N-glycosylation site could
 433 be indirect targets of ALG3, as their secretion may be influenced by other
 434 ALG3-regulated proteins. It is worth to notice that our proteomics approach identified
 435 previously reported proteins involved in cell wall integrity, such as Gel3 (MGG_
 436 08370) and Gel4 (MGG_11861) [44]. Thus, a reduced secretion level of Gel3 and
 437 Gel4 in the $\Delta alg3$ mutant might account for the defects of cell wall integrity in $\Delta alg3$

438 mutants.

439 A subset of secreted proteins containing one predicted *N*-glycan site (protein encoded
440 by MGG_09460), two *N*-glycan sites (MGG_13764), and more than three *N*-glycan
441 sites (MGG_05785, MGG_01956, MGG_08772, MGG_03826, MGG_10209, and
442 MGG_10466) were chosen for a series of validation assays. We confirmed that all
443 candidate proteins indeed had *N*-glycosylation modifications (Fig. 4). The secretion
444 test results suggest that all proteins were secreted in the growth medium. The
445 secretion and localization of most of the tested proteins were affected in the $\Delta alg3$
446 mutant, validating that *N*-glycosylation is important for protein secretion and proper
447 localization. Both the secretion and the localization of the protein encoded by
448 MGG_13764 were not affected in the $\Delta alg3$ mutant, suggesting that for this specific
449 protein, *N*-glycosylation is not necessary for its secretion. The validation tests on
450 those selected proteins indicate that our *in vitro* comparative secretome analysis is a
451 powerful approach to identify novel proteins that undergo *N*-glycosylation mediated
452 by ALG3.

453 *M. oryzae* effectors consist of cytoplasmic effectors and apoplastic effectors [16].
454 Interestingly, apoplastic effectors Slp1 and Bas4 both contain predicted
455 *N*-glycosylation sites, but none of the cytoplasmic effectors had a predicted
456 *N*-glycosylation site. Thus, it is possible that *N*-glycosylation is a common feature of
457 apoplastic effectors. We observed that all tested secreted proteins localized at the
458 plant–fungus interface surrounding the infection hyphae (Fig. 5), which resembles the
459 localization of Slp1, further supporting that the *N*-glycosylation modification is
460 important for protein secretion via the conventional secretory pathway.

461 *M. oryzae* is a hemibiotrophic fungus, which, similar to other biotrophic fungi,
462 depends on the nutrients provided by host plants at its early infection stage. Sugar is
463 one of the most abundant nutrients a pathogen can get from living plants. Indeed,
464 invertase homologs have been reported in several obligate biotrophic pathogens. The
465 invertase from the rust fungus *Uromyces fabae* was expressed during infection and
466 localized in the extrahaustorial matrix membrane and was important in breaking down
467 sucrose into D-glucose and D-fructose [54]. In addition, a report on wheat stripe rust

(*Puccinia striiformis* f. sp. *tritici*) revealed that the invertase gene *PsINV* plays a role in *Pst* pathogenicity [55]. The *M. oryzae* genome contains four genes encoding invertases, which were clustered in a subclade when compared with other fungal invertases, but only *INV1* was identified as an abundant protein in our secretome dataset. We found that *N*-glycosylation and the localization of *INV1* were severely affected in the $\Delta alg3$ mutant; moreover, *INV1* was also identified as secreted proteins in all the other *M. oryzae* secretome datasets. A previous report confirmed that a $\Delta invI$ mutant showed impaired growth on sucrose-containing media, including impaired biomass formation and virulence [30]. In addition, we showed that the cell wall integrity was affected in $\Delta invI$ mutants when grown in CM with sucrose, but this phenotype was completely restored when we replaced the sucrose with glucose. In a fungal virulence test, adding 0.2% glucose and 0.2% fructose also partially restored the pathogenicity of the $\Delta invI$ mutants. Taken together, these observations show that carbon utilization is important for fungal cell wall integrity and virulence in *M. oryzae*.

Chitin, a homopolymer of 1, 4- β -linked N-acetyl-D-glucosamine (GlcNAc), is a structural component of fungal cell wall [56]. Chitin fragments are well-characterized elicitors that induce plant immune responses in many plant species [57, 58]. Chitinases are chitin-degrading enzymes that ubiquitous exist in a wide range of organisms including viruses, bacteria, fungi, insects, plant and animals [59]. *M. oryzae* has 15 genes that are annotated as GH_18 family chitinases. MoChial is involved in the fungal virulence through degrading chitin into small fragments, which would escape the recognition of plant immune receptors [33, 60]. Different chitinases had their specific expression in different cell types, *AMCase* maintains the highest expression level in appressorium among 15 chitinases, indicating *AMCase* is likely to be associated with fungal infection [33]. Indeed, our study exhibited that $\Delta amcase$ mutants were less virulent on both rice and barley leaves, confirming that *AMCase* play an important role in pathogenicity. In addition, we showed that the cell wall integrity was affected in $\Delta amcase$ mutants, but with different sensitivity in CFW and CR, indicating that the mechanism underline the cell wall remodeling in response to

498 CFW differs to that to CR.

499 Notably, our secretome analysis did not identify the well characterized apoplastic
 500 effector protein Slp1, which is known to undergo *N*-glycosylation mediated by ALG3.
 501 Since our research was conducted on vegetative mycelium growth, the expression of
 502 Slp1 might be lower than that in the *in vivo* infection condition. Many
 503 pathogenicity-related genes, especially effectors, have no or a low level of expression
 504 during vegetative growth, and higher expression specifically during infection [61, 62].
 505 Recent studies reported the functions of various transcription factors and epigenetic
 506 control in regulating effector expression in phytopathogenic fungi. For example,
 507 methylation of lysine 9 and/or lysine 27 of histone H3 (H3K9me3, H3K27me3) seems
 508 to be related with heterochromatin and effector gene silencing, and methylation of
 509 lysine 4 of histone H3 (H3K4me2) with euchromatin and effector gene expression
 510 [63-66]. Further proteomics studies using related histone modification mutants would
 511 enhance our understanding of secreted effector proteins in *M. oryzae*.

512

513

514 **Materials and Methods**

515 **Fungal strains and Growth Conditions**

516 *M. oryzae* wild-type strain P131 (field isolate) and the mutant strains $\Delta alg3$ which
 517 lacking the α -1, 3-mannosyltransferase were used in this study. The $\Delta alg3$ strain was
 518 generated and verified previously [26]. *M. oryzae* strains with different transformants
 519 generated in this study were listed in supplemental Table 3. All *M. oryzae* strains were
 520 maintained on oatmeal tomato agar (OTA) medium at 28°C. [67]. The liquid complete
 521 medium (CM) contains 6g/L yeast extract, 3g/L casein acid hydrolysate, 3g/L casein
 522 enzymatic hydrolysate and 10g/L sucrose. And the liquid minimal medium (MM)
 523 contains 6g/L NaNO₃, 0.502g/L KCl, 0.502g/L MgSO₄·7H₂O, 1.52g/L KH₂PO₄, 1000
 524 X trace element, 10g/L D-glucose, 1% thiamine and 0.05% biotin (PH 6.5). For cell
 525 wall integrity test, 5mm mycelial blocks of different strains were placed on CM agar
 526 with 0.1 mg/mL CFW (Sigma-Aldrich, Shanghai, China), 0.2 mg/mL CR
 527 (Sigma-Aldrich, Shanghai, China) and 0.005% SDS. For the carbohydrate supplement
 528 test, 5mm mycelial blocks of different strains were placed on solid minimal medium
 529 without or with different carbohydrate supplements at 28°C for 5 days. For the
 530 mycelial wet-weight measurement, the 0.1g mycelia were grown on liquid minimal
 531 medium without or with different carbohydrate supplements at 28°C for 1 day.

532 **Virulence Test**

533 Rice (*Oryza sativa* cv LTH) seedlings at the third leaf stage and barley (cv E9)
 534 seedlings with 7-d-old were used for virulence test. Different *M. oryzae* strains with
 535 10⁵/mL conidia suspension were used for spray inoculation as described previously
 536 [68]. Leaves were photographed at 5 days after inoculation and relative lesion area on
 537 leaves was calculated. To test the effect of exogenous glucose and fructose on
 538 infection of the $\Delta inv1$ mutant, 0.2% glucose and 0.2% fructose was added onto the
 539 fungal inoculation site at 18 hpi after the drop inoculation, and subsequently
 540 photographs and statistics of lesion area were taken at 5 days after inoculation.

541 **Secretome sample extraction**

542 Secreted proteins were extracted from liquid medium. One gram of mycelia was
543 collected and grown in liquid culture for 18 hours, then the liquid medium was
544 filtrated through Miracloth (Merck millipore, Beijing, China) and centrifugated at
545 12,000rpm for 10 min. Two hundred-milliliters supernatants were collected and added
546 with 12.5% (v/v) trichloroacetic acid at 4°C overnight to precipitate proteins. The
547 pellets were collected after centrifuging for 30 min at 12,000rpm, washed twice with
548 100% acetone and dried. The protein samples were stored at -80 °C for further
549 analyses.

550 **LC-MS/MS analyses and data processing**

551 Proteins were digested using FASP method [69]. Peptides were separated with a
552 Waters (Milford, MA, USA) nanoAcquity nano-HPLC. Mobile phases were 0.1% FA
553 in water (A) and 0.1% FA in acetonitrile (B). We used linear gradient from 1% B to 35%
554 B in 65 min. The trap column was Thermo Acclaim PepMap 100 (75 µm×20 mm,
555 C18, 3 µm). The analytical column was homemade (100 µm×200 mm C18 stationary
556 phase, Phenomenex, Aqua 3 µm 125Å). nanospray ESI-MS was performed on a
557 Thermo Q Exactive high resolution mass spectrometer (Thermo Scientific, Waltham,
558 MA, USA) in a data-dependent mode.

559 Raw MS files were subjected to MaxQuant software (Version 1.6.0.1) for protein
560 identification and quantification as described before [70]. Peak list was generated by
561 Andromeda which is a built-in engine in MaxQuant and searched against a
562 *Magnaporthe oryzae* database (download from <https://www.broadinstitute.org>)
563 augmented with the reversed sequence. Trypsin/P was set as the enzyme for digestion.
564 Carbamidomethyl (C) was set as a fixed modification, while Oxidation (M) and
565 Acetyl (protein N-term) were set as variable modifications. The maximum of missed
566 cleavage was set as 2. Main search peptide tolerance and MS/MS match tolerance
567 were both set as 20 ppm. False discovery rates of peptide and protein were both set at
568 1%.

569 **Vector Construction**

To generate fluorescent protein fused with GFP, coding regions of candidate proteins were cloned into pGTN under a *MoEF* (MGG_03641) promoter [71]. The resulting constructs were digested with *EcoRI* and delivered into the protoplasts of P131 and *Δalg3* strains as described [68]. Media with 400 g/ml neomycin (Ameresco, Ohio, USA) were used to select neomycin-resistant transformants.

To generate the gene replacement constructs, flanking sequences with 1.5-kb upstream and downstream of targeted genes (*INVI* and *AMCase*) were amplified using genomic DNA of P131. The two flanking sequences were cloned into pKOV21 [68] as the deletion vector pKO-*genes*. The resulting constructs were linearized by *NotI* and delivered into the protoplasts of P131 to generate deletion mutants, which were confirmed by southern blot hybridization.

Fluorescence intensity and Subcellular localization Analysis

To analysis fluorescence intensity, the transformants of candidate proteins fused with GFP was grown in liquid CM cultured at 160 rpm for 22 h. Then fluorescence intensity of 100ul filtered medium and 0.1g mycelium proteins was measured with microplate reader (Molecular Devices i3x, Shanghai, China), respectively. To observe subcellular localization, these transformants of these validated proteins fused with GFP inoculated in barley leaves were observed with an epifluorescence microscope (Nikon, Toyko, Japan).

Protein N-Glycosylation Analysis

For glycosylation analysis, total proteins were extracted from mycelia using the protein lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40). Proteins with or without PNGase F (NEB, USA) digestion were mixed with the loading buffer (250Mm Tris-HCl PH 6.8, 10% SDS, 0.5% bromophenol blue, 50% glycerol, 5% β -mercaptoethanol) for Western Blot analysis. Antibodies of anti-GFP (1:5000; ABclonal, Hubei, China) and anti-Actin (1:10,000; ABclonal, Hubei, China) were used to detect GFP fusion proteins and actin control, respectively.

597 **Authors' contributions**

598 XL, YP, JY and NL conceived the project; LQ, XL, NL, CY and RW undertook
599 proteomic analysis; NL, XL, MH, DC, YZ, XW and GY conducted experiments; XL,
600 YP, NL and LQ wrote the article.

601

602 **Competing interest**

603 The authors declare that they have no conflict of interest.

604

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793

794 **Figure legends**

795 **Figure 1 Quantitative secretome analysis of *Magnaporthe oryzae* secreted** 796 **proteins from wild type P131 and $\Delta alg3$ mutant strains**

797 **A.** Experimental strategy to identify secreted proteins of *M. oryzae* from liquid
798 medium. **B.** PCA plot indicates the identified proteins from wild-type P131 strains and
799 $\Delta alg3$ strains were grouped separately.

800

801 **Figure 2 Bioinformatic analysis of secreted proteins requiring ALG3 for proper** 802 **secretion**

803 **A.** Predicted *N*-glycosylation sites using NetNGlyc analysis for the secreted proteins
804 requiring ALG3 for proper secretion. Gene Ontology analysis of those secreted
805 proteins classified with molecular function category (**B**) and biological process
806 category (**C**).

807

808 **Figure 3 Secretion of the tested proteins requiring ALG3 for proper secretion**

809 **A.** Schematic diagram of constructed vectors for tested genes. MoEF pro, *M. oryzae*
810 elongation factor promoter; Gene, genomic DNA sequences of target genes. Each
811 constructed vector was transformed into wild-type P131 and $\Delta alg3$ strains. **B.**
812 Immunoblotting analysis of tested proteins from culture supernatants from wild-type
813 P131 and $\Delta alg3$ strains. Three replicates were performed for each strain, and one
814 representative figure was shown. **C.** Experimental strategy to measure the GFP
815 intensity from mycelial protein extracts and CM liquid culture. **D.** The GFP intensity

ratio was calculated as the GFP intensity from 100µl CM medium versus that from 0.1g mycelium protein extracts. **E.** The GFP intensity ratio calculated for the tested secreted proteins in wild-type P131 and $\Delta alg3$ strains. Slp1, a known effector protein regulated by ALG3, was used as a positive control. Error bars denote standard deviations from three biological replicates. **, statistically significant differences between the wild type and mutants ($P < 0.01$, Student's t test).

822

Figure 4 Assays for N-glycosylation of secreted proteins in the wild-type P131 and $\Delta alg3$ strains

Mycelial protein lysates with (+) or without (–) PNGase F treatment were immunoblotted with anti-GFP antibody. Protein loading control was detected using anti-Actin antibody.

828

Figure 5 The localization of secreted proteins in the infected hyphae differs in wild-type P131 and $\Delta alg3$ strains

Left panel, Fluorescence signal is imaged in infected hyphae growing in barley epidermal cells for the tested proteins. White lines in the figures show the path for fluorescence intensity measurement. Middle panel, the brightfield image for the infection hyphae. Right panel, the fluorescence intensity measured using ImageJ.

Scale bar=20 µm

836

Figure 6 *INV1* is important for fungal virulence and cell wall integrity

838 **A.** The schematic diagram shows that INV1 functions in decomposition of sucrose. **B.**
839 Deduced protein domains of INV1. Red dots indicate predicted *N*-glycosylation sites.
840 **C.** Colony growth of strains P131, $\Delta inv1-1$, $\Delta inv1-2$, and $\Delta inv1/INV1$ on CM medium
841 supplemented with the cell wall-disturbing agents 0.1 mg/ml CFW, 0.2 mg/ml Congo
842 Red and 0.005%SDS. The cultures were incubated at 28°C for 5 days before being
843 photographed. **D.** Calculation of the growth reduction rates of mycelia growth on CM
844 supplemented with cell wall-disturbing agents. Growth reduction rate (%) = [diameter
845 (CM) – diameter (stress)]/diameter (CM). Error bars denote standard deviations from
846 three biological replicates. **, statistically significant differences between the wild
847 type and mutants ($P < 0.01$, Student's *t* test). **E.** Barley leaves drop-inoculated with
848 conidium suspensions (2×10^4 spores/mL) of the strains P131, $\Delta inv1$ and $\Delta inv1/INV1$
849 without or with exogenous glucose or fructose. The leaves were photographed at 5 dpi
850 and the absolute lesion area was calculated (**F**). Error bars denote standard deviations
851 from three biological replicates with at least 9 leaves. The letters indicate significantly
852 different statistical groups ($P < 0.05$, one-way ANOVA with post-hoc Turkey tests) for
853 corresponding strains or treatments.

854
855 **Figure 7 *AMCase* is important for fungal virulence and cell wall integrity**

856 **A.** Deduced protein domains of AMCase. Red dots indicate predicted *N*-glycosylation
857 sites. **B.** Colony growth of strains P131, $\Delta amcase-1$, $\Delta amcase-2$, and $\Delta amcase-3$
858 on CM medium supplemented with the cell wall-disturbing agents 0.1 mg/ml CFW,
859 0.2 mg/ml Congo Red and 0.005%SDS. The cultures were incubated at 28°C for 5

860 days before being photographed. **C.** Calculation of the growth reduction rates of
 861 mycelia growth on CM supplemented with cell wall-disturbing agents. Growth
 862 reduction rate (%) = [diameter (CM) – diameter (stress)]/diameter (CM). Error bars
 863 denote standard deviations from three biological replicates. *, statistically significant
 864 differences between the wild type and mutants ($P < 0.05$, Student's t test). **,
 865 statistically significant differences between the wild type and mutants ($P < 0.01$,
 866 Student's t test). **D.** Rice leaves were sprayed with conidium suspensions (1×10^5
 867 spores/mL) of the strains P131, *Δamcase-1*, *Δamcase-2*, and *Δamcase-3*. The leaves
 868 were photographed at 5dpi and the relative lesion area was calculated (**E**). Error bars
 869 denote standard deviations from three biological replicates with at least 9 leaves. **,
 870 statistically significant differences between the wild type and mutants ($P < 0.01$,
 871 Student's t test). **F.** Barley leaves were sprayed with conidium suspensions (1×10^4
 872 spores/mL) of the strains P131, *Δamcase-1*, *Δamcase-2*, and *Δamcase-3*. The leaves
 873 were photographed at 5dpi and the relative lesion area was calculated (**G**). Error bars
 874 denote standard deviations from three biological replicates with at least 9 leaves. **,
 875 statistically significant differences between the wild type and mutants ($P < 0.01$,
 876 Student's t test).

877 **Tables**

878 **Table 1 An overview of the identified secreted proteins of *Mo* from three**
 879 **biological replicates**

880 **Table 2 An overview of the number of proteins of different LFQ intensity**
 881 **groups**

882 **Table 3 The putative secreted proteins regulated by ALG3**

883

884 **Supplementary material**

885 **Supplemental Figure 1 Secreted proteins from *Magnaporthe oryzae* isolate P131** 886 **and $\Delta alg3$ mutant**

887 **A.** Western blot shows the secretion of Slp1-GFP from P131 in CM and MM liquid
888 medium. Total secreted protein was used as a loading control. **B.** Silver staining of
889 secreted protein samples from the CM liquid culture of P131 and $\Delta alg3$ strains. Three
890 replicates were performed for each strain.

891

892 **Supplemental Figure 2.** The GFP signal from control samples.

893 **A.** Immunoblotting analysis of protein 03670-GFP, 10234-GFP and 10318-GFP
894 (Group 3) from culture supernatants and mycelial extracts from wild-type P131 and
895 $\Delta alg3$ strains. **B.** GFP fluorescent was measured in 96 well plates (upper panel) and
896 the ratio of GFP intensity from CM liquid culture versus mycelial protein extracts was
897 calculated (lower panel) for Slp1-GFP, MoGRP1-GFP, control GFP samples and
898 background control P131 samples. Error bars denote standard deviations from three
899 biological replicates. **, statistically significant differences between the wild type and
900 mutants ($P < 0.01$, Student's t test). **C.** The fluorescence signal from control GFP
901 samples. Left panel, Fluorescence signal is imaged in infected hyphae growing in
902 barley epidermal cells for the tested proteins. White lines in the figures show the path
903 for fluorescence intensity measurement. Middle panel, the brightfield image for the
904 infection hyphae. Right panel, the fluorescence intensity measured using ImageJ.
905 Scale bar=20

906

907 **Supplemental Figure 3 The characterization of *M. oryzae* *INV1* gene function**

908 **A.** Schematic diagram of the *INV1* deletion strategy. **B.** DNA gel blot analysis of the
909 *INV1* deletion mutants. **C.** Colony growth of wild-type strain P131, *INV1* deletion
910 mutant $\Delta inv1-1$ and $\Delta inv1-2$, and complemented strain $\Delta inv1/INV1$ on OTA medium.
911 **D.** *M. oryzae* stains P131, $\Delta inv1-1$, $\Delta inv1-2$ and $\Delta inv1/INV1$ were grown on minimal
912 medium (MM) with different carbon sources (sucrose, glucose and fructose) and the
913 picture were taken at day 5 grown in 28°C. **E.** The mycelia fresh weight of strains
914 P131, $\Delta inv1-1$, $\Delta inv1-2$ and $\Delta inv1/INV1$ grown in MM liquid media was measured
915 without or with different carbon sources for 24 hours. Error bars denote standard
916 deviations from three biological replicates. **, statistically significant differences
917 between the wild type and mutants ($P < 0.01$, Student's t test). **F.** Colony growth of
918 strains P131, $\Delta inv1-1$, $\Delta inv1-2$, and $\Delta inv1/INV1$ on CM with glucose supplemented
919 with the cell wall-disturbing agents 0.1 mg/ml CFW, 0.2 mg/ml Congo Red and
920 0.005%SDS. The cultures were incubated at 28°C for 5 days before being
921 photographed. **G.** Statistical analysis of the growth reduction rates of mycelia growth
922 on CM with glucose supplemented with cell wall-disturbing agents. Growth reduction
923 rate (%) = [diameter (CM) – diameter (stress)]/diameter (CM). Error bars denote
924 standard deviations from three biological replicates. **H.** Rice leaves were sprayed with
925 conidium suspensions (1×10^5 spores/mL) of the strains P131, $\Delta inv1-1$, $\Delta inv1-2$, and
926 $\Delta inv1/INV1$. The leaves were photographed at 5dpi and the relative lesion area was
927 calculated (**I**). Error bars denote standard deviations from three biological replicates
928 with at least 9 plants. **, statistically significant differences between the wild type
929 and mutants ($P < 0.01$, Student's t test). **J.** Barley leaves were sprayed with conidium

suspensions (1×10^4 spores/mL) of the strains P131, $\Delta inv1-1$, $\Delta inv1-2$, and $\Delta inv1/INV1$. The leaves were photographed at 5dpi and the relative lesion area was calculated (**K**). Error bars denote standard deviations from three biological replicates with at least 9 plants. **, statistically significant differences between the wild type and mutants ($P < 0.01$, Student's t test).

935

936

937 **Supplemental Figure 4 The characterization of *M. oryzae* *AMCase* gene** 938 **function**

939 **A.** Schematic diagram of the *AMCase* deletion strategy. **B.** DNA gel blot analysis of
940 the *AMCase* deletion mutants. **C.** Colony growth of wild-type strain P131, *AMCase*
941 deletion mutant $\Delta amcase-1$, $\Delta amcase-2$ and $\Delta amcase-3$ on OTA medium. **D.** The
942 colony diameter was calculated for wild-type P131 and *AMCase* deletion mutants
943 grown on OTA medium. **E.** Statistical analysis of the sporulation of wild-type P131
944 and *AMCase* deletion mutants. **F.** *M. oryzae* stains P131, $\Delta amcase-1$, $\Delta amcase-2$ and
945 $\Delta amcase-3$ were grown on minimal medium (MM) with different carbon sources
946 (glucose and chitin) and the picture were taken at day 5 grown in 28°C. **G.** The colony
947 diameter was calculated for wild-type P131 and *AMCase* deletion mutants grown on
948 minimal medium (MM) with different carbon sources. Error bars denote standard
949 deviations from three biological replicates.

950 **Supplemental Tables**

951 **Supplemental Table 1 The identified secretory proteins of P131 and *Aalg3***

952 **Supplemental Table 2 Bioinformatics analysis identified secretory proteins**

953 **Supplemental Table 3 List of strains used in this study**

954 **Supplemental Table 4 List of oligonucleotide primers used in this study**

955

Figure 1

Figure 2

A

B

C

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Table 1 An overview of the identified secreted proteins of Mo from three biological replicates

samples	rep1	rep2	rep3	common proteins in three replicates	Replication ratio (%)
P131	360	276	310	233	74.77%
<i>Δalg3</i>	471	503	455	371	78.02%

Table 2 An overview of the number of proteins of different LFQ intensity groups

Groups	LFQ intensity	Total	SignalP	SecretomeP	TM
Group 1	P131> $\Delta alg3$	51	43	7	11
Group 2	P131= $\Delta alg3$	295	128	69	22
Group 3	P131< $\Delta alg3$	210	33	43	14

Table 3 The secreted proteins regulated by ALG3

Protein name	Protein ID	Unique peptides ^a	Sequence coverage	MW ^b [kDa]	Average LFQ ^c intensity		P131/ Δ alg3 ratio	P-value ^d
					P131	Δ alg3		
invertase	MGG_05785	18	34.6	71.804	1.48E+10	0	+ ∞	N ^e
endoglucanase c	MGG_01956	9	24.8	36.915	1.73E+08	0	+ ∞	N
hypothetical protein	MGG_00210	5	30.7	29.468	1.72E+08	0	+ ∞	N
protease inhibitor	MGG_08772	4	22.2	31.713	1.19E+08	0	+ ∞	N
carbohydrate esterase	MGG_12016	4	27.5	25.041	1.47E+08	0	+ ∞	N
cell wall protein	MGG_09460	3	16.7	25.663	1.09E+08	0	+ ∞	N
endothiapepsin	MGG_13598	5	13.4	48.017	8.95E+07	0	+ ∞	N
cell wall mannoprotein	MGG_05092	4	19	39.375	1.00E+08	0	+ ∞	N
mitochondrial iron uptake protein	MGG_02435	4	30.2	21.905	6.09E+07	0	+ ∞	N
galactose oxidase	MGG_03826	4	16.3	37.936	4.40E+07	0	+ ∞	N
hypothetical protein	MGG_06523	5	30.6	24.388	2.79E+07	0	+ ∞	N
serine-threonine rich protein	MGG_01009	2	20.3	15.298	3.25E+07	0	+ ∞	N
hsp70-like protein	MGG_03983	4	25.3	23.384	2.09E+07	0	+ ∞	N
1,2-dihydroxy-3-keto-5-methylthio opentene dioxygenase 2	MGG_06443	4	27.7	20.661	1.90E+07	0	+ ∞	N
cellobiose dehydrogenase	MGG_07949	2	13	30.51	1.04E+08	0	+ ∞	N
alpha-amylase 3	MGG_10209	6	18	61.346	7.99E+07	0	+ ∞	N
surface protein 1	MGG_07791	2	18.7	13.953	1.09E+08	0	+ ∞	N
hypothetical protein	MGG_08971	4	41.1	20.736	9.22E+09	1.16E+08	79.21	0.029
dj-1 family protein	MGG_10466	5	21.1	28.217	2.50E+09	3.86E+07	64.91	0.009
phytase	MGG_06167	10	25.9	57.625	1.51E+09	1.03E+08	14.68	0.043
hypothetical protein	MGG_08655	8	40.8	42.037	5.12E+09	7.09E+08	7.21	0.002
ligninase H2	MGG_07790	15	42.2	51.333	1.94E+09	3.01E+08	6.45	0.014
1,3- β -glucanosyltransferase gel3	MGG_08370	10	27.4	55.871	4.46E+08	6.96E+07	6.41	0.004
ecm33	MGG_06189	12	32.8	40.639	4.86E+09	8.56E+08	5.68	0.000
gds1-like lipase acylhydrolase	MGG_05879	3	19.8	26.702	4.28E+07	7.72E+06	5.54	0.035
peptidase a1 protein	MGG_09818	11	44.5	48.357	2.93E+09	5.49E+08	5.34	0.034
eukaryotic aspartyl protease	MGG_03014	5	13.1	53.58	1.62E+08	3.06E+07	5.30	0.013
endoglucanase c	MGG_13993	7	26.2	33.254	1.17E+09	2.34E+08	4.99	0.011
cell wall glucanosyltransferase	MGG_00592	16	54	38.442	7.25E+09	1.62E+09	4.47	0.000
FAD dependent oxidoreductase	MGG_02295	10	28.5	50.565	5.89E+08	1.35E+08	4.38	0.033
acidic mammalian chitinase	MGG_04732	15	39.3	45.789	3.80E+09	8.72E+08	4.37	0.042
hypothetical protein	MGG_00283	7	56.8	16.073	5.32E+10	1.32E+10	4.04	0.025
acid phosphatase	MGG_03439	17	68.3	31.695	5.56E+09	1.43E+09	3.90	0.040
acetylxyylan esterase 2	MGG_09840	8	27.8	30.582	2.65E+09	6.99E+08	3.80	0.000
hypothetical protein	MGG_08275	4	29.2	21.252	1.99E+08	5.36E+07	3.71	0.047
ThiJ/PfpI family protein	MGG_01085	8	51.4	26.67	3.39E+10	9.15E+09	3.70	0.046
hypothetical protein	MGG_04944	8	39.3	27.294	3.12E+09	8.70E+08	3.59	0.018
chitinase	MGG_17153	13	40.2	46.36	5.92E+09	1.72E+09	3.45	0.003
phosphorylcholine phosphatase	MGG_08342	6	24.4	39.875	1.95E+08	5.76E+07	3.38	0.017
acid phosphatase	MGG_00552	8	25.9	50.763	3.57E+08	1.08E+08	3.31	0.013

septation protein SUN4	MGG_00505	10	38.2	43.597	2.28E+10	7.78E+09	2.93	0.028
cell wall glucanase	MGG_01134	4	14.6	46.89	7.15E+07	2.51E+07	2.85	0.047
lipocalin-like domain-containing protein	MGG_03369	3	23	20.849	7.53E+07	2.65E+07	2.85	0.028
calcineurin-like phosphoesterase	MGG_05689	9	33.8	49.561	3.47E+08	1.29E+08	2.68	0.000
glycoside hydrolase	MGG_04582	9	22.6	55.891	1.71E+09	6.43E+08	2.66	0.034
carboxypeptidase S1	MGG_03995	4	18.4	63.027	1.15E+08	4.44E+07	2.59	0.050
hypothetical protein	MGG_09321	12	57.7	38.315	9.25E+09	3.58E+09	2.58	0.044
adhesin protein Mad1	MGG_12523	7	13.7	72.224	1.42E+09	6.08E+08	2.33	0.022
1,3- β -glucanosyltransferase gel4	MGG_11861	14	29.7	56.562	7.08E+09	3.17E+09	2.23	0.011
endothelin-converting enzyme	MGG_06643	31	47.8	85.844	3.65E+09	1.67E+09	2.19	0.012
glucan 1,3- β -glucosidase	MGG_04689	11	42.1	42.225	3.17E+09	1.50E+09	2.11	0.008

Note:

a Unique peptides indicated the number of different peptides assigned to the proteins.

b MW, the molecular weight of each identified protein.

c LFQ, label free quantification

d P-value < 0.05

e N, Non.