

# ***N*-Glycosylation of Effector Proteins by an $\alpha$ -1,3-Mannosyltransferase Is Required for the Rice Blast Fungus to Evade Host Innate Immunity**<sup>WJOPEN</sup>

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**Plant pathogenic fungi deploy secreted effectors to suppress plant immunity responses. These effectors operate either in the apoplast or within host cells, so they are putatively glycosylated, but the posttranslational regulation of their activities has not been explored. In this study, the *ASPARAGINE-LINKED GLYCOSYLATION3 (ALG3)*-mediated *N*-glycosylation of the effector, Secreted LysM Protein1 (Slp1), was found to be essential for its activity in the rice blast fungus *Magnaporthe oryzae*. *ALG3* encodes an  $\alpha$ -1,3-mannosyltransferase for protein *N*-glycosylation. Deletion of *ALG3* resulted in the arrest of secondary infection hyphae and a significant reduction in virulence. We observed that  $\Delta alg3$  mutants induced massive production of reactive oxygen species in host cells, in a similar manner to  $\Delta slp1$  mutants, which is a key factor responsible for arresting infection hyphae of the mutants. Slp1 sequesters chitin oligosaccharides to avoid their recognition by the rice (*Oryza sativa*) chitin elicitor binding protein CEBiP and the induction of innate immune responses, including reactive oxygen species production. We demonstrate that Slp1 has three *N*-glycosylation sites and that simultaneous Alg3-mediated *N*-glycosylation of each site is required to maintain protein stability and the chitin binding activity of Slp1, which are essential for its effector function. These results indicate that Alg3-mediated *N*-glycosylation of Slp1 is required to evade host innate immunity.**

## **INTRODUCTION**

Plants defend themselves from microbial attack using pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMPs) to initiate innate immune responses (Chisholm et al., 2006; Jones and Dangl, 2006). Immune reactions include the regulated synthesis of reactive oxygen species (ROS), which can be induced by the recognition of cell wall PAMPs, such as oligosaccharides originating from chitin and glucans, released by invading pathogens during infection (Kaku et al., 2006). In response to these defenses, plant pathogens have developed strategies to avoid or overcome PAMP-triggered immunity. The most important structural protection of an invading fungus is the outer layer of the cell wall, by which fungal pathogens can avoid host recognition of inner chitin and glucans (Fujikawa et al., 2012). Pathogens can also counter PAMP-triggered immunity mechanisms by effector-mediated suppression of these immunity

responses, leading to effector-triggered susceptibility (Jones and Dangl, 2006).

Secreted proteins, including cell wall glycoproteins and effector proteins, are synthesized in the endoplasmic reticulum (ER), where they often undergo *N*-glycosylation and/or O-glycosylation (Helenius and Aebi, 2004). *N*-Linked glycosylation of proteins is one of the most ubiquitous posttranslational modifications and provides proteins not only with distinct folding, quality control, sorting, degradation, and secretion characteristics but also with a recognition tag (Helenius and Aebi, 2001, 2004). The *N*-linked glycosylation of proteins has been extensively studied in higher eukaryotes. In mammals, for instance, *N*-linked glycosylation is involved in cell adhesion, molecular trafficking and clearance, receptor activation, signal transduction, and endocytosis and is important for human health and disease (Ohtsubo and Marth, 2006). In higher plants, enzymes involved in protein *N*-glycosylation and glycoproteins that are *N*-glycosylated have been widely identified and found to play vital roles in diverse aspects of development and physiology, including salt tolerance and plant immunity (Lerouge et al., 1998; Kang et al., 2008; Qin et al., 2008; Liebming et al., 2009; Saijo et al., 2009; Häweker et al., 2010; Liu and Howell, 2010; Ruiz-May et al., 2012).

*N*-Glycosylation of proteins in eukaryotic cells is accomplished in two distinct organelles, the ER and the Golgi (Helenius and Aebi, 2001), and initially involves *N*-glycan biosynthesis. *N*-Glycan biosynthesis starts with the assembly of a precursor, Man<sub>5</sub>-GlcNAc<sub>2</sub>, linked to dolicholphosphate at the cytoplasmic side of

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the ER. This precursor is flipped to the luminal side of the ER, where four Man and three Glc residues are added by different glycosyltransferases in a stepwise manner (Snider et al., 1980; Helenius and Aebi, 2002). The oligosaccharide  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  is then transferred en bloc by the oligosaccharyltransferase complex onto specific Asn residues of secreted proteins (Hubbard and Ivatt, 1981; Kelleher and Gilmore, 2006). Once glycoproteins have folded and oligomerized, they are moved to the Golgi complex for further modification of *N*-linked glycans (Dean, 1999). Additional saccharides are added to form complex *N*-glycans in mature glycoproteins (Roth, 2002).

The budding yeast *Saccharomyces cerevisiae* has been used as a model for investigating eukaryotic *N*-glycan synthesis. Studies with mutants defective in the biosynthesis of *N*-glycans have led to the characterization of the *ASPARAGINE-LINKED GLYCOSYLATION (ALG)* genes (Huffaker and Robbins, 1983; Lehle et al., 2006). Yeast *alg* mutants, for instance, are defective in the biosynthesis of donor substrates or the assembly of lipid-linked oligosaccharides (Burda and Aebi, 1999; Kelleher and Gilmore, 2006) and have been used to uncover the function of *N*-glycosylation. Deletion of some *ALG* genes involved in the earliest steps of *N*-glycosylation results in lethal, or extremely severe, phenotypes and the secretion of underglycosylated proteins, demonstrating the biological importance of *N*-linked glycosylation (Kelleher and Gilmore, 2006). In *Candida albicans*, *N*-glycosylation is important for both the structure and function of certain proteins that play vital roles in cell-cell interactions, adhesion, and reaction to host immune responses (Poulain and Jouault, 2004; Mora-Montes et al., 2010; Hiller et al., 2011). However, the role of *N*-glycosylation in the pathogenesis of filamentous fungi remains largely unexplored. To date, there are only three reports with glycosylation-related mutants in *Ustilago maydis* and *Mycosphaerella graminicola* that implicate *N*-glycosylation in the pathogenesis of plant pathogenic fungi (Schirawski et al., 2005; Motteram et al., 2011; Fernández-Álvarez et al., 2013). However, the underlying mechanism was not characterized in those studies; therefore, it is not clear why *N*-glycosylation is important for fungal pathogenesis.

Rice (*Oryza sativa*) blast disease is caused by the filamentous fungus *Magnaporthe oryzae* and is one of the most destructive diseases of cultivated rice worldwide (Dean et al., 2012). The fungal pathogen initiates infection by the attachment of conidium to the plant surface (Hamer et al., 1988). The conidium then germinates and the germ tube tip develops into a dome-shaped structure called an appressorium, in which high turgor is generated (Howard et al., 1991; de Jong et al., 1997). Both mitogen-activated protein kinase and cAMP-dependent protein kinase A signaling pathways are essential for appressorium morphogenesis and penetration (Mitchell and Dean, 1995; Xu and Hamer, 1996; Xu et al., 1998; Thines et al., 2000). After penetration, the pathogen colonizes host cells in a biotrophic manner (Kankanala et al., 2007) and the thin primary penetration hypha differentiates into bulbous and branched infection hyphae (IH; Kankanala et al., 2007). IH possess a cap-like structure known as the biotrophic interfacial complex, which has been implicated in the release of cytoplasmic effectors into plant cells (Kankanala et al., 2007; Mosquera et al., 2009; Giraldo et al., 2013). Recently, a secreted LysM protein, Slp1, was shown to

function in *M. oryzae* as an effector protein that suppresses host immunity by binding chitin oligosaccharides in the apoplast (Mentlak et al., 2012), thereby preventing activation of the chitin elicitor binding protein (CEBiP), a pattern recognition receptor present in the plasma membrane of rice cells (Shimizu et al., 2010).

Here, we report the identification of a virulence gene in *M. oryzae* designated *ALG3*, which encodes an  $\alpha$ -1,3-mannosyltransferase involved in protein *N*-glycosylation in the ER. Disruption or deletion of *ALG3* leads to the arrest of infection hypha development and a significant reduction in virulence. Strikingly,  $\Delta alg3$  mutants induce massive production of ROS in host cells. We show that *ALG3* is essential for *N*-glycosylation of the Slp1 effector and that simultaneous *N*-glycosylation at three sites is required for Slp1 to be functional. We also show that incomplete *N*-glycosylation of Slp1 leads to a dramatic reduction in the chitin binding capability and the protein level of Slp1. Together, our results provide evidence that Alg3-mediated *N*-glycosylation of Slp1 in the rice blast fungus is required to prevent chitin-triggered innate immunity in host plants.

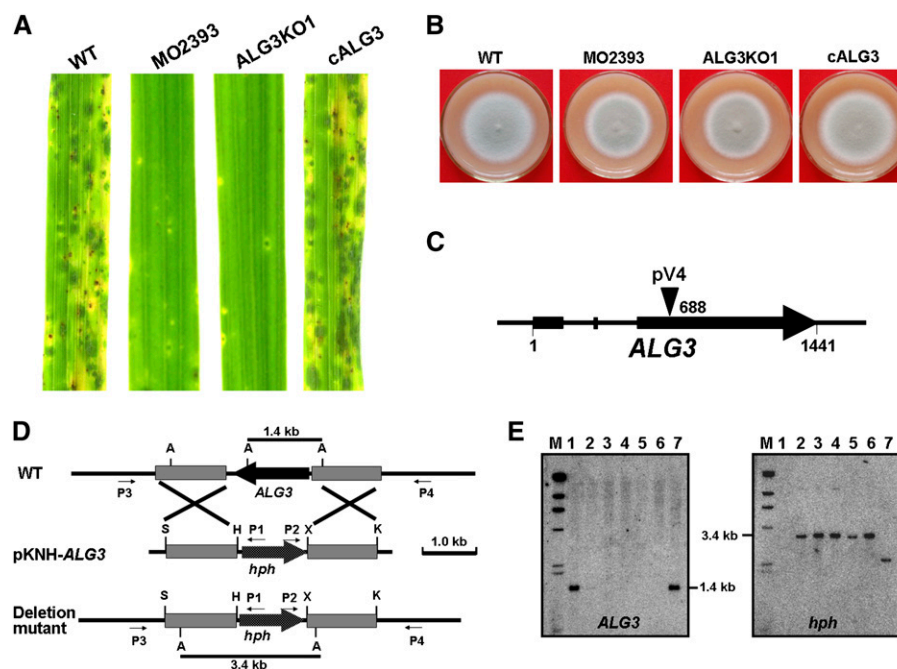
## RESULTS

### Isolation and Characterization of *ALG3* Mutants

To identify novel pathogenicity mutants of the rice blast fungus, an insertional library of *M. oryzae* P131 was generated by restriction enzyme-mediated integration (REMI), as described previously (Sweigard et al., 1998). After screening a library of 13,500 hygromycin-resistant transformants, a mutant, MO2393, was found that was significantly reduced in virulence. It developed fewer and smaller lesions on leaves of a susceptible rice cultivar in comparison with the wild-type strain P131 (Figure 1A). The mutant was also slightly reduced in colony growth (Figure 1B). For cosegregation analysis, a total of 49 F1 progeny were obtained by crossing MO2393 (*MAT1-1-2*) with the wild-type strain S1528 (*MAT1-1-1*). Whereas 24 of the progeny were resistant to hygromycin and exhibited defects similar to MO2393 in pathogenesis and growth, the remaining 25 were sensitive to hygromycin and showed normal growth and virulence. Thus, the disruption of a single genetic locus in mutant MO2393 must be responsible for its defects in vegetative growth and virulence.

To identify the gene disrupted in MO2393, genomic regions flanking the insertion site were isolated by plasmid rescue (Sweigard et al., 1998). Sequence analysis revealed that the transforming vector pV4 was inserted in the coding region of a gene, MGG\_08010.6, at a position 688 bp downstream of the start codon (Figure 1C). This predicted gene was named *ALG3* because of its sequence similarity to *ALG3* of *S. cerevisiae*, which encodes an  $\alpha$ -1,3-mannosyltransferase (Supplemental Figure 1).

Because the disrupted *ALG3* in MO2393 is not a null allele, we generated the *ALG3* deletion mutants by homologous recombination (Figure 1D). Five *ALG3* deletion mutants, named *ALG3KO1* to *ALG3KO5*, were identified by PCR verification and confirmed by DNA gel blot analysis (Figure 1E). Vegetative growth of the  $\Delta alg3$  mutants was reduced ( $\Phi 3.76 \pm 0.02$  cm) in



**Figure 1.** *ALG3* Is Important for Virulence and Vegetative Growth of *M. oryzae*.

(A) Rice leaves were sprayed with conidium suspensions ( $1 \times 10^5$  spores/mL) of the wild-type P131, REMI mutant MO2393, *ALG3* deletion mutant ALG3KO1, and complemented transformant cALG3. The inoculated leaves were photographed at 5 d post inoculation.

(B) Five-day-old oatmeal agar cultures of P131, MO2393, ALG3KO1, and cALG3.

(C) Schematic diagram of the *ALG3* gene and the integration site of pV4 in the REMI mutant MO2393.

(D) Schematic diagram of the *ALG3* deletion strategy. Letters A, S, H, K, and X mark the recognition sites of *Apal*, *SpeI*, *HindIII*, *KpnI*, and *XhoI*, respectively. *hph*, hygromycin phosphotransferase gene; P1 to P4, PCR primers.

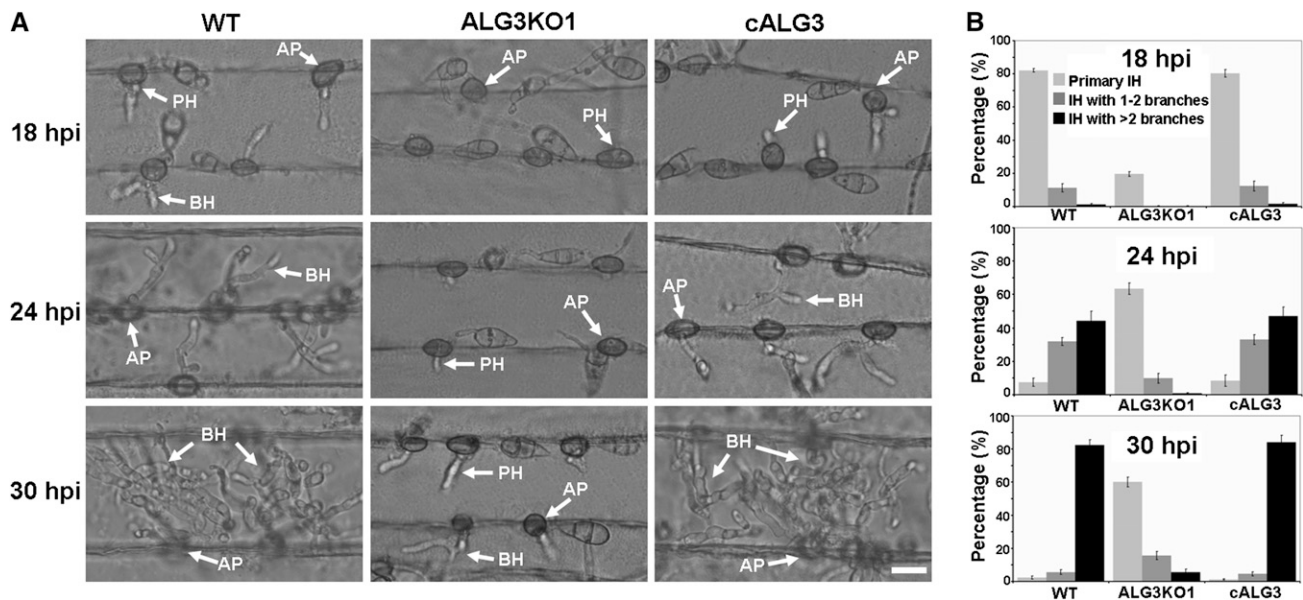
(E) DNA gel blot analysis of the *ALG3* deletion mutants. *Apal*-digested genomic DNAs were hybridized with a deleted fragment of *ALG3* (left) and *hph* (right). Lane M, *HindIII*-digested fragments of  $\lambda$ DNA; lane 1, wild-type strain P131; lanes 2 to 6, *ALG3* deletion mutants ALG3KO1, ALG3KO2, ALG3KO3, ALG3KO4, and ALG3KO5; lane 7, ectopic transformant 08010-E1.

comparison with the wild type ( $\Phi 4.01 \pm 0.05$  cm) but similar to MO2393 ( $\Phi 3.75 \pm 0.02$  cm) on 5-d-old oatmeal–tomato agar (OTA) cultures (Figure 1B). In infection assays,  $\Delta alg3$  mutants were significantly reduced in their ability to cause blast lesions (Figure 1A). Moreover, disease lesions caused by  $\Delta alg3$  mutants were smaller than those of the wild type (Figure 1A). However, the conidiation capacity of  $\Delta alg3$  mutants was not significantly affected (Supplemental Table 1). Because all five *ALG3* deletion mutants were indistinguishable in phenotype, only mutant ALG3KO1 was used for further analyses. When the full-length *ALG3* gene was reintroduced into ALG3KO1, all 25 resulting neomycin-resistant transformants displayed wild-type phenotypes (Figure 1), indicating that deletion of *ALG3* was directly responsible for the observed phenotypes in the  $\Delta alg3$  mutant. Therefore *ALG3* is important for virulence and vegetative growth in *M. oryzae*.

#### ***ALG3* Is Required for Invasive Growth of the Rice Blast Fungus**

To investigate the role of *ALG3* in pathogenesis, we compared the infection process on barley (*Hordeum vulgare*) leaves of the  $\Delta alg3$  mutant with the wild type and a complemented strain,

cALG3. Conidial germination and appressorium formation were unaffected (Supplemental Table 1), but the development of secondary IH in plant tissue was severely impaired in the  $\Delta alg3$  mutant (Figure 2; Supplemental Table 2). At 18 h postinoculation (hpi), over 90% of appressoria of the wild type penetrated and developed primary IH, but only  $19.5\% \pm 1.5\%$  of appressoria of the  $\Delta alg3$  mutant had formed IH (Figure 2A, left panels). Furthermore, at 24 hpi, more than 85% of IH of the wild type formed branched (Figure 2A, middle panels). At 30 hpi, more than 90% of wild-type appressoria formed IH with at least two branches. Under the same conditions, less than 20% ( $15.1\% \pm 2.9\%$ ) of the  $\Delta alg3$  appressoria formed multiple-branched IH (Figure 2A, right panels). However, we noticed that at 30 hpi,  $86.1\% \pm 2.9\%$  of appressoria of the  $\Delta alg3$  mutant had developed primary IH (Figure 2B), suggesting that the development of primary IH was not completely blocked but, rather, was delayed by the deletion of *ALG3*. Further observation at 36 hpi also showed that although most of the  $\Delta alg3$  appressoria produced multiple-branched IH, the invasive hyphae were completely restricted to the initially penetrated plant cell, whereas most appressoria of the wild-type strain and the complemented strain extended the IH into neighboring plant cells (Supplemental Figure 2).



**Figure 2.** *ALG3* Deletion Mutants Are Defective in Invasive Growth.

Barley leaves were inoculated with conidium suspensions of the wild-type P131, *ALG3* deletion mutant *ALG3KO1*, and complemented transformant *cALG3*.

(A) Penetration and infectious hyphae were examined at 18, 24, and 30 hpi. AP, PH, and BH indicate appressoria, primary IH, and branched IH, respectively. Bar = 20  $\mu$ m.

(B) Percentages of appressoria with distinct types of IH, primary IH, IH with one or two branches, and IH with more than two branches, at 18, 24, and 30 hpi.

REMI mutant MO2393 showed similar defects in the infection process to the  $\Delta alg3$  mutant, which also could be fully complemented by reintroduction of *ALG3* (data not shown). We conclude that *ALG3* is dispensable for appressorium formation and cuticle penetration but important for efficient tissue colonization and disease symptom development by the rice blast fungus.

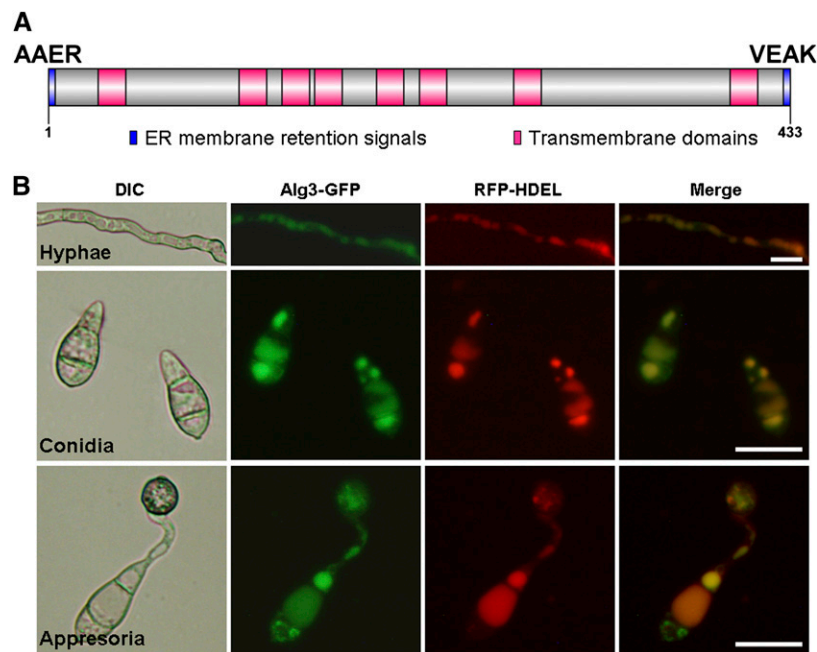
### *Alg3* Localizes to the ER

Orthologs of *Alg3* are well conserved from budding yeast to humans. In *S. cerevisiae*, for instance, *Alg3* is involved in the synthesis of dolichol-linked oligosaccharide donors for *N*-linked glycosylation of proteins and catalyzes the addition of the sixth Man moiety to the growing lipid-linked oligosaccharide, which is the first sugar added to the lipid-linked oligosaccharide in the ER lumen (Aebi et al., 1996). The predicted product of *M. oryzae ALG3* showed 49% identity to *S. cerevisiae ALG3* and 56% to human *Alg3* (Supplemental Figure 1). According to WoLF PSORT (<http://wolfsort.org/>), *Alg3* is predicted to localize to the ER. The *Alg3* protein has both an *N*-terminal XXRR-like motif and a KKXX-like motif at the C terminus (Figure 3A). These motifs have been shown to retrieve transmembrane proteins to the ER (Shin et al., 1991; Zerangue et al., 1999). Moreover, it has eight predicted transmembrane domains, suggesting that the *Alg3* protein may be anchored in the ER membrane (Figure 3A), consistent with the subcellular localization of *Alg3* in *S. cerevisiae* (Aebi et al., 1996).

To determine the localization of *Alg3* in *M. oryzae*, we generated *ALG3*-enhanced green fluorescent protein (eGFP) and red fluorescent protein (RFP)-HDEL fusion constructs and transformed them together into the  $\Delta alg3$  mutant. HDEL has been widely used as an ER marker (Pelham et al., 1988). In the resulting 20 transformants with detectable GFP signals, all showed wild-type colony growth, consistent with *ALG3*-eGFP encoding a functional protein. RFP fluorescence was detected in five of them, including transformant *ALG3DW-1*, which was used for microscopy observation. In vegetative hyphae, conidia, and appressoria, colocalization of *Alg3*-GFP with RFP-HDEL was observed (Figure 3B), consistent with *Alg3* being functional in the ER.

### *ALG3* Encodes an $\alpha$ -1,3-Mannosyltransferase Involved in the Biosynthesis of $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ for Protein *N*-Glycosylation

To determine its molecular function, we expressed a full-length cDNA of *ALG3* in pYES2 under the control of a Gal-inducible promoter, *GAL1* (Giniger et al., 1985). The resulting construct, pYES2-*ALG3*, was introduced into the *S. cerevisiae alg3 stt3* double mutant YG170, which is a conditional lethal strain that exhibits temperature sensitivity at 30°C (Aebi et al., 1996). The resulting transformants were grown on SC medium supplemented with Gal. The pYES2-*ALG3* transformants of YG170 grew as well as the control strain YG176 at 30°C. By contrast, the transformants carrying the empty vector control were



**Figure 3.** Alg3 Localizes to the ER in *M. oryzae*.

**(A)** Predicted ER membrane retaining signals and transmembrane domains of Alg3 are indicated by blue and red boxes, respectively. AAER and VEAQ motifs are predicted ER retaining signals.

**(B)** Dual-color imaging by confocal laser scanning microscopy of transformant ALG3DW-1 expressing both Alg3-GFP and RFP-HDEL. Top row, vegetative hyphae; middle row, conidia; bottom row, appressoria. DIC, differential interference contrast. Bars = 20  $\mu\text{m}$ .

temperature sensitive (Figure 4A). *M. oryzae* ALG3, therefore, rescued the *S. cerevisiae* *alg3* deficiency of YG170, indicating that ALG3 encodes a functional  $\alpha$ -1,3-mannosyltransferase.

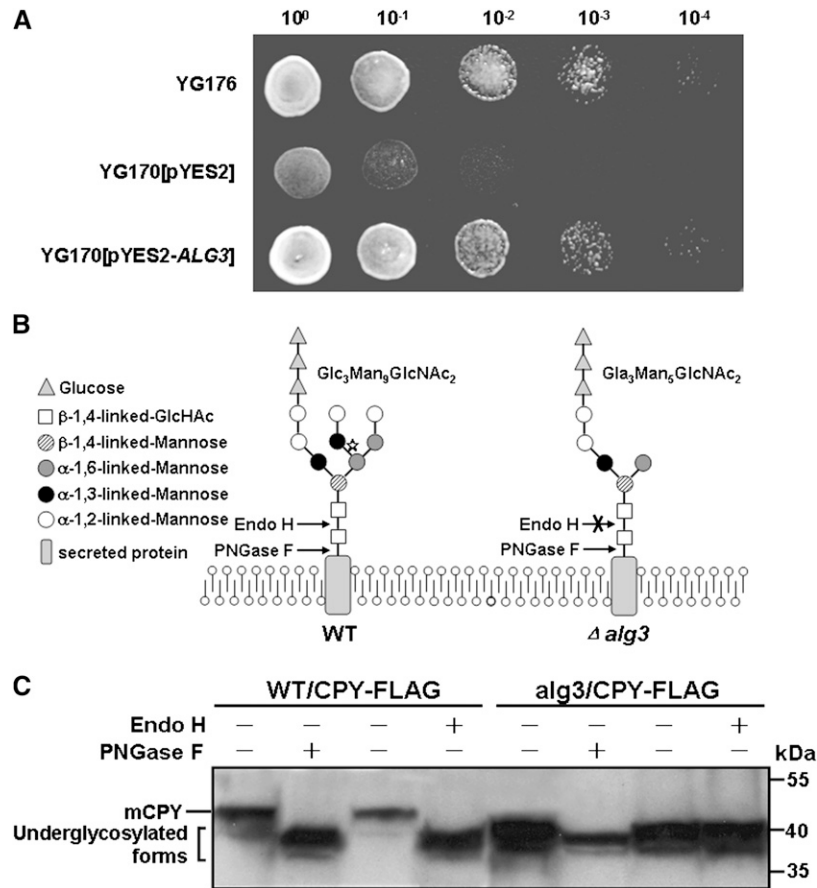
*N*-Glycans are synthesized in yeast through the ordered assembly of a lipid-linked core  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  structure that is subsequently transferred to a nascent protein in the ER. Once folded, glycoproteins are shuttled to the Golgi apparatus for secretion. Deletion of ALG3 results in the secretion of glycoproteins with  $\text{Man}_5\text{GlcNAc}_2$  instead of  $\text{Man}_9\text{GlcNAc}_2$  in *S. cerevisiae* (Figure 4B). Underglycosylated proteins are secreted in *alg3* mutants (Verostek et al., 1993; Zufferey et al., 1995; Aebi et al., 1996). Carboxypeptidase Y (CPY) is a vacuolar protein with *N*-glycosylation that has been widely used for assaying protein *N*-glycosylation (Kostova and Wolf, 2005). To determine whether *N*-glycosylation was affected in the  $\Delta\text{alg3}$  mutant, we assayed the *N*-glycosylation levels of CPY in the rice blast fungus. The CPY-3xFLAG fusion construct was generated and transformed into both the wild-type and  $\Delta\text{alg3}$  strains. Proteins isolated from the resulting transformants WT/CPY-FLAG and *alg3*/CPY-FLAG were used for immunoblot analysis with an anti-FLAG antibody. The WT/CPY-FLAG transformant exhibited fully *N*-glycosylated CPY (mature CPY), while the transformant *alg3*/CPY-FLAG produced an underglycosylated form of CPY (Figure 4C). Alg3, therefore, is required for full *N*-glycosylation of CPY in *M. oryzae*.

Deleting ALG3 in *S. cerevisiae* results in the production of  $\text{Man}_5$ -oligosaccharides that can be distinguished from  $\text{Man}_9$ -oligosaccharides by resistance to endoglycosidase H (Endo H),

which is a recombinant glycosidase cleaving within the chitobiose core of high Man and some hybrid oligosaccharides from *N*-linked glycoproteins.  $\text{Man}_5$ -oligosaccharides are resistant to Endo H, whereas  $\text{Man}_9$ -oligosaccharides are sensitive (Huffaker and Robbins, 1983; Verostek et al., 1993; Zufferey et al., 1995). We found that the glycosylated CPY protein from P131 could be successfully cleaved by Endo H. In contrast, Endo H failed to cleave CPY in the  $\Delta\text{alg3}$  mutant (Figure 4C). However, CPY glycoproteins isolated from both transformants of the wild-type P131 and the  $\Delta\text{alg3}$  mutant were successfully cleaved by the peptide:*N*-glycosidase F (PNGase F) (Figure 4C), an amidase that cleaves between the innermost GlcNAc and Asn residues of complex oligosaccharides from *N*-linked glycoproteins. Therefore, oligosaccharides linked to CPY were  $\text{Man}_5$ -oligosaccharides but not  $\text{Man}_9$ -oligosaccharides in the  $\Delta\text{alg3}$  mutant, which is consistent with the predicted function of ALG3. Thus, we conclude that the ALG3 gene of *M. oryzae* encodes an  $\alpha$ -1,3-mannosyltransferase involved in the formation of  $\text{Man}_9$ -oligosaccharides for protein *N*-glycosylation.

#### ALG3 Is Required for Suppressing the Production of ROS in Plant Cells

Plant immunity responses induced by fungal pathogens are often associated with rapid production of ROS. To test whether ALG3 is important for the induction of ROS in plant cells, ROS production was assayed with 3,3'-diaminobenzidine (DAB) staining in barley leaves inoculated with the wild-type and  $\Delta\text{alg3}$



**Figure 4.** ALG3 Encodes an α-1,3-Mannosyltransferase Required for Protein N-Glycosylation.

**(A)** Complementation of the *S. cerevisiae alg3 stt3* mutant by ALG3. Cells of YG176 (*stt3*) and transformants of YG170 (*alg3 stt3*) carrying pYES2 or pYES2-ALG3 were spotted in 10-fold dilutions on SC-Gal plates and incubated at 30°C for 2 d.

**(B)** Lipid-linked or secreted protein-linked oligosaccharide structures of the wild-type and *alg3* strains of *S. cerevisiae*. The digestion sites of Endo H and PNGase F are labeled with arrows. The functional site of Alg3 is indicated by the star.

**(C)** Assays for CPY glycosylation by immunoblot analysis. Total proteins isolated from the transformants P131/CPY-FLAG and *alg3*/CPY-FLAG were treated with or without Endo H or PNGase F. The fully glycosylated form (mCPY) and underglycosylated forms of CPY were detected with an anti-FLAG antibody.

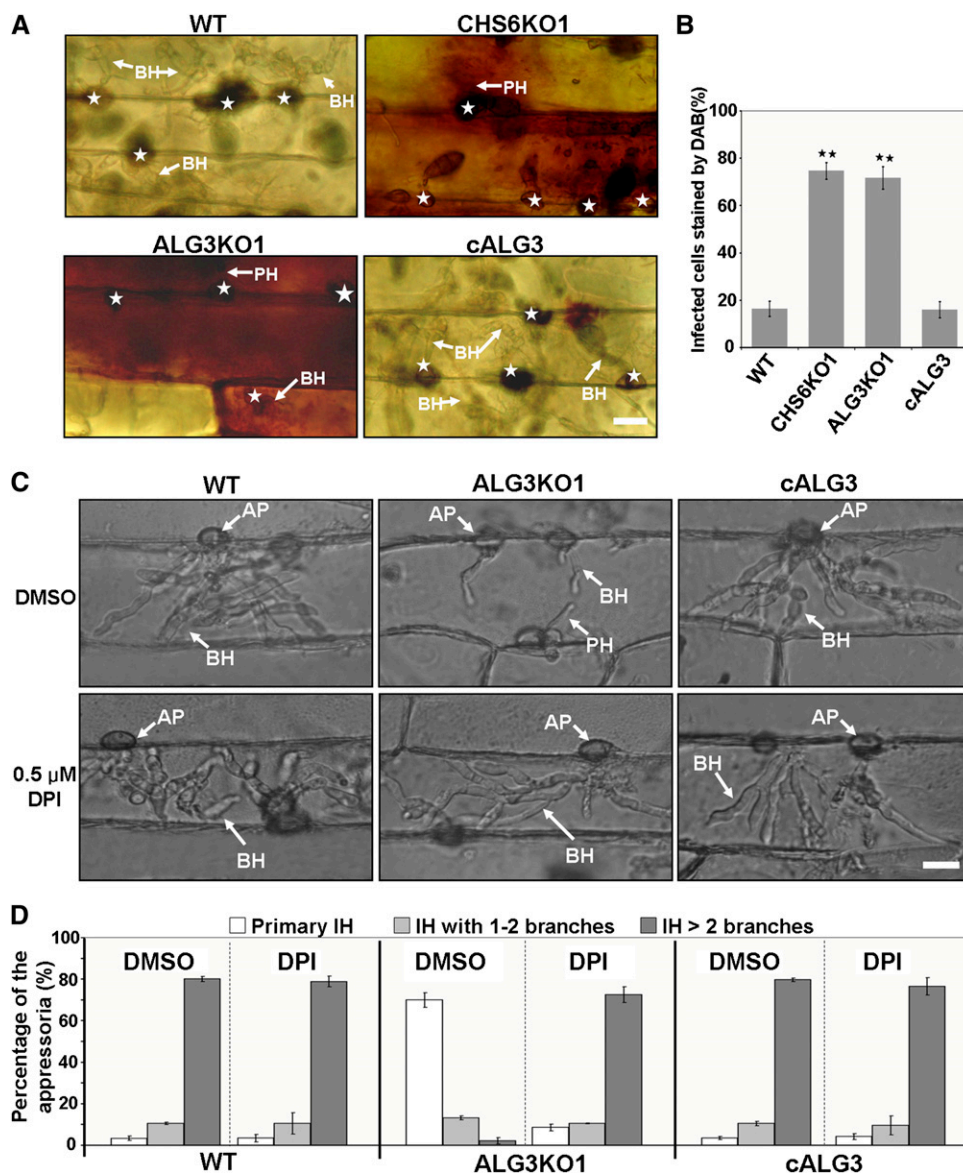
strains as described previously (Liu et al., 2007). Whereas ROS was barely detectable in barley epidermal cells penetrated by the wild-type P131 and complemented strain cALG3, strong induction of ROS was observed in cells infected by the *Δalg3* mutant (Figures 5A and 5B). These results suggest that the *Δalg3* mutant is unable to suppress or prevent ROS production in plant cells during infection.

To determine whether this characteristic is associated with an impairment in tissue colonization by the *Δalg3* mutant, we tested the effect of pretreatment with diphenyleneiodonium (DPI) on plant infection. DPI is a flavoenzyme inhibitor that prevents the activation of NADPH oxidases necessary for ROS generation in plants (Cross and Jones, 1986). In barley epidermal cells pretreated with DPI, the *Δalg3* mutant produced extensive bulbous IH as efficiently as the wild-type and complemented strains at 30 hpi (Figure 5C). Approximately 72.5% of appressoria of the mutant formed IH with more than two branches in the DPI-treated host cells, which was comparable to the invasive growth

of the wild-type P131 (78.8%) and the complemented strain cALG3 (76.4%) (Figures 5C and 5D). In samples treated with DMSO as the negative control, only 2.1% of the *Δalg3* appressoria formed IH with more than two branches, and ~75.8% of them were arrested at the primary IH stage (Figures 5C and 5D), indicating that suppression of ROS production in host cells rescued the defect of the *Δalg3* mutant in invasive growth. The *Δalg3* mutant, therefore, was able to recover its ability to proliferate in host cells following the suppression of ROS production, suggesting that reduced virulence of the *Δalg3* mutant is due to its inability to suppress plant immunity responses.

**Alg3 Is Required for N-Glycosylation of the Effector Proteins Slp1 and Bas4**

We reasoned that Alg3 might be involved in the N-glycosylation of secreted effector proteins produced by *M. oryzae* to suppress host immunity. This hypothesis was tested with Slp1,



**Figure 5.** A  $\Delta alg3$  Mutant Induces ROS Generation and Arrest of Invasive Growth during Plant Infection.

**(A)** DAB staining of penetrated plant cells. Barley leaves inoculated with conidium suspensions ( $1 \times 10^5$  spores/mL) of the wild-type P131, *CHS6* deletion mutant CHS6KO1, *ALG3* deletion mutant ALG3KO1, and complementation strain cALG3 were stained with DAB at 30 hpi. CHS6KO1 was used as a positive control strain (Kong et al., 2012). Stars and arrows indicate appressoria and IH, respectively. BH, branched IH; PH, primary IH. Bar = 20  $\mu$ m.

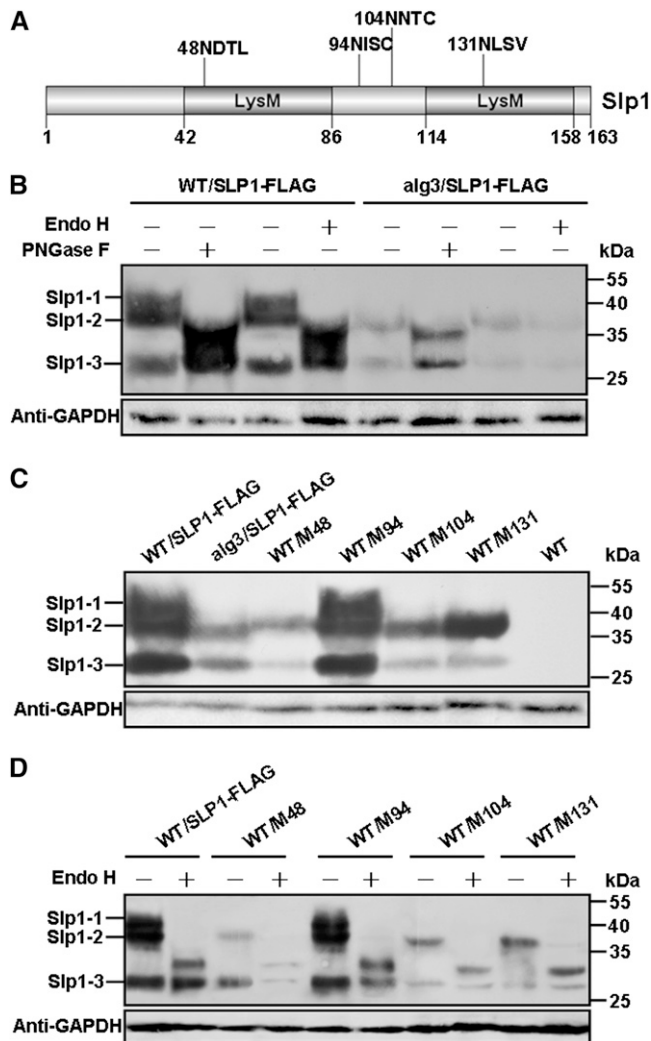
**(B)** Percentages of the DAB-stained cells. Means and SE were calculated from three independent replicates. Significant differences are indicated by stars ( $P = 0.01$ ;  $n > 100$ ).

**(C)** Invasive growth of IH restored by DPI treatment. Barley leaves were treated with or without DPI (0.5  $\mu$ M) dissolved in DMSO. Invasive growth was observed at 30 hpi. AP, appressoria. Bar = 20  $\mu$ m.

**(D)** Percentages of appressoria with distinct types of IH, primary IH, IH with one or two branches, and IH with more than two branches, formed at 30 hpi by P131, ALG3KO1, and cALG3 in barley leaves treated with or without DPI. DMSO treatment was used as a control. Means and SE were calculated from three independent replicates.

a secreted LysM domain protein that acts as an apoplastic effector to evade chitin-induced plant immune responses to *M. oryzae* (Mentlak et al., 2012). Using NetNGlyc 1.0 (<http://www.hiv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html>), Slp1 is predicted to be a glycoprotein with four putative

*N*-glycosylation sites that are located at Asn-48 (NDTL), Asn-94 (NISC), Asn-104 (NNTC), and Asn-131 (NLSV) (Figure 6A). To verify the *N*-glycosylation of Slp1, a *SLP1*-3xFLAG fusion construct was generated and transformed independently into the  $\Delta alg3$  mutant and the wild-type strain P131 to generate the



**Figure 6.** Alg3 Is Required for *N*-Glycosylation of the Effector Protein Slp1 in *M. oryzae*.

**(A)** The Slp1 effector has two LysM domains (dark boxes) and four predicted *N*-glycosylation sites.

**(B)** Assays for *N*-glycosylation of Slp1 in the wild type and the *ALG3* deletion mutant. Total proteins isolated from the transformants WT/SLP1-FLAG and *alg3*/SLP1-FLAG were treated with or without PNGase F or Endo H and detected with an anti-FLAG antibody by immunoblot analysis.

**(C)** Assays for the effects of point mutations at individual *N*-glycosylation sites of Slp1. Immunoblots of total proteins isolated from labeled strains were detected with an anti-FLAG antibody. Transformants WT/M48, WT/M94, WT/M104, and WT/M131 contain *SLP1*-3xFLAG fusion constructs carrying N48G, N94G, N104G, and N131G mutations, respectively. WT/SLP1-FLAG and *alg3*/SLP1-FLAG were used as controls.

**(D)** Assays for *N*-glycosylation in WT/M48, WT/M94, WT/M104, and WT/M131 strains. Total proteins were digested by Endo H, and immunoblot analysis was performed as in **(C)**.

Three Slp1 forms (Slp1-1, Slp1-2, and Slp1-3) were detected and are marked on the left. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) levels were used as a loading control.

transformants *alg3*/SLP1-FLAG and WT/SLP1-FLAG, respectively. Total proteins isolated from these transformants were subjected to immunoblot analysis with an anti-FLAG antibody. As shown in Figures 6B to 6D, three forms of Slp1 were detected in the WT/SLP1-FLAG transformants, which were named Slp1-1, Slp1-2, and Slp1-3. By contrast, only two forms of Slp1 were detected in the *alg3*/SLP1-FLAG transformants, and notably, the Slp1-1 form was absent in the  $\Delta$ *alg3* mutant (Figures 6B and 6C). Significant reduction in protein levels was also observed for either Slp1-2 or Slp1-3 in the  $\Delta$ *alg3* mutant in comparison with the wild-type strain (Figures 6B and 6C). Both Slp1-1 and Slp1-2 from transformant WT/SLP1-FLAG were successfully cleaved by both PNGase F and Endo H, whereas Slp1-2 from the transformant *alg3*/SLP1-FLAG could only be cleaved by PNGase F and not by Endo H (Figure 6B). These results are consistent with *M. oryzae* having two *N*-glycosylated forms of Slp1 and strongly suggest that the *N*-glycosylation of Slp1 requires Alg3. It also suggests that the nonglycosylated form of Slp1 may be unstable. Based on these results, we conclude that the secreted effector protein Slp1 is *N*-glycosylated and *ALG3* is required for the full *N*-glycosylation of Slp1.

To further characterize the *N*-glycosylation sites of Slp1, point mutations were introduced into the four predicted *N*-glycosylation sites in the *SLP1*-3xFLAG construct. The resulting *SLP1*<sup>N48G</sup>, *SLP1*<sup>N94G</sup>, *SLP1*<sup>N104G</sup>, and *SLP1*<sup>N131G</sup> mutant alleles were transformed into the wild-type strain P131. Transformants expressing these constructs were identified and named WT/M48, WT/M94, WT/M104, and WT/M131, respectively. Immunoblot analysis was performed to detect changes of Slp1 glycosylation in these transformants. Interestingly, transformants WT/M48, WT/M104, and WT/M131 lacked the Slp1-1 band, compared with the WT/M94 and WT/SLP1-FLAG strains (Figures 6C and 6D). These results suggest that Slp1-1 is simultaneously *N*-glycosylated at Asn-48, Asn-104, and Asn-131. In the transformant WT/M94, protein levels of the three Slp1 forms, including Slp1-1, were comparable to those isolated from the wild-type strain, suggesting that Asn-94 of Slp1 is not an Alg3-mediated *N*-glycosylation site. However, in transformants WT/M48, WT/M104, and WT/M131, particularly in WT/M48, the protein levels of Slp1 were significantly reduced, further confirming that full *N*-glycosylation of Slp1 is required for its stability. Moreover, the larger form of Slp1 in the transformants WT/M48, WT/M104, and WT/M131 could be cleaved by Endo H (Figure 6D), indicating that they were proteins with two sites fully *N*-glycosylated. Taken together, these results revealed that Slp1 has three Alg3-mediated *N*-glycosylation sites (Asn-48, Asn-104, and Asn-131) and Slp1-1 is a glycoprotein that has the three sites simultaneously *N*-glycosylated.

To determine whether Alg3 mediates the *N*-glycosylation of other effector proteins, we analyzed the *N*-glycosylation status of Bas4 in the  $\Delta$ *alg3* mutant in comparison with the wild-type strain P131. Bas4 is an effector protein reported to locate between the extra invasive hyphal membrane and the IH cell wall (Mosquera et al., 2009). The protein was predicted to have one putative *N*-glycosylation site at Asn-36. A *BAS4*-3xFLAG fusion construct was generated and transformed independently into the  $\Delta$ *alg3* mutant and the wild-type strain P131 to generate the transformants *alg3*/BAS4-FLAG and WT/BAS4-FLAG,



respectively. As shown in Supplemental Figure 3, two forms of Bas4-3xFLAG protein were detected in the WT/BAS4-FLAG transformants. The larger Bas4 protein could be digested by both PNGase F and Endo H, indicating that it is an *N*-glycosylated protein. In contrast, only one form of Bas4 was detected in the alg3/BAS4-FLAG transformants, which could not be digested by either PNGase F or Endo H. These results indicate that Alg3 is also required for the *N*-glycosylation of other effector proteins, such as Bas4.

### ***N*-Glycosylation of Slp1 Is Required to Suppress Host Plant Innate Immunity**

We speculated that an underglycosylated Slp1 effector may be affected in its ability to suppress chitin-triggered immunity in host plants. To test this hypothesis, we transformed the  $\Delta slp1$  mutant with the *SLP1*<sup>N48G</sup>, *SLP1*<sup>N94G</sup>, *SLP1*<sup>N104G</sup>, and *SLP1*<sup>N131G</sup> alleles and compared the virulence of the corresponding transformants (named M48, M94, M104, and M131, respectively) with that of the wild-type strain and the  $\Delta slp1$  mutant. As shown in Figures 7A to 7C, the M94 strain formed similar numbers of disease lesions to the wild-type strain P131. By contrast, the number and size of lesions produced by transformants M48, M104, and M131 were significantly fewer and smaller than those by the wild-type strain (*t* test, *P* < 0.01). The Slp1<sup>N48G</sup>, Slp1<sup>N104G</sup>, and Slp1<sup>N131G</sup> forms of the Slp1 effector, therefore, were unable to rescue the virulence defect of the  $\Delta slp1$  mutant. We also generated an *SLP1* allele in which the N48G, N104G, and N131G sites were simultaneously mutated and expressed this in the  $\Delta slp1$  mutant. The resulting transformant M48/104/131 showed similarly reduced virulence to the  $\Delta slp1$  mutant. Thus, *N*-glycosylation at Asn-48, Asn-104, and Asn-131 is required for the effector function of Slp1 in the rice blast fungus. Consistent with this, it was found that transformants M48, M104, M131, and M48/104/131 induced ROS accumulation in plant cells at infection sites, while the wild-type strain P131 and complemented strains cSLP1 and M94 suppressed ROS production (Figure 7).

### ***N*-Glycosylation Does Not Affect Cellular Localization but Regulates the Chitin Binding Activity of the Slp1 Effector**

To understand how *N*-glycosylation is involved in regulating the function of Slp1, we investigated whether underglycosylated Slp1 has the same subcellular localization as glycosylated Slp1. The coding regions of the normal and the underglycosylated alleles of *SLP1* were independently fused with the *RFP* gene, and the two constructs were transformed into the  $\Delta slp1$  mutant. The wild-type *SLP1* construct without mutation was also introduced into the  $\Delta alg3$  mutant. Subsequently, the resulting three types of transformants were inoculated onto the barley leaves and compared microscopically at 28 hpi. In the three types of transformants, Slp1-RFP located at the plant–fungus interface as reported previously (Mentlak et al., 2012), and no change in the subcellular localization was observed between the underglycosylated and glycosylated Slp1-RFP proteins (Figure 8A). We also observed subcellular localization of three Slp1-RFPs, each of which had a single mutation of the three *N*-glycosylation sites. Again, they showed similar localization to

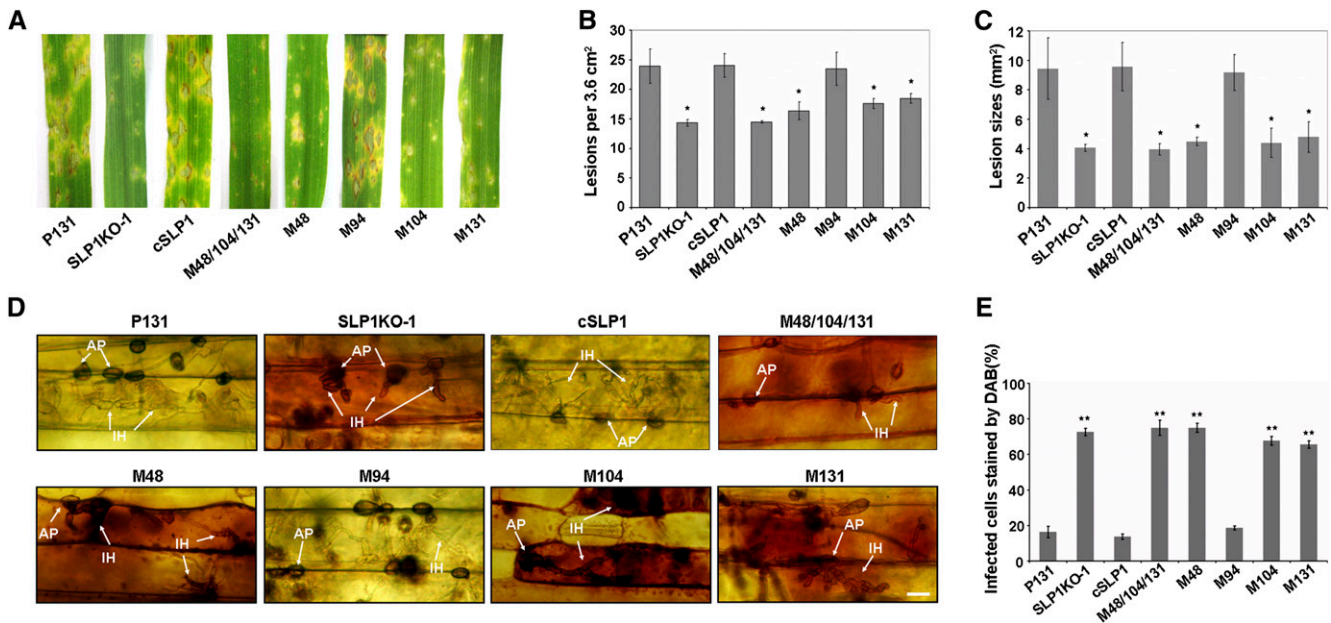
the wild-type Slp1 effector (Supplemental Figure 4). These results indicate that *N*-glycosylation is not required for the subcellular localization of Slp1.

We then investigated whether the chitin binding activity of Slp1 was affected by *N*-glycosylation. The wild-type *SLP1* allele and the *SLP1*<sup>N48G N104G N131G</sup> glycosylation-deficient allele were fused with the 6xHis tag and expressed in yeast. The Slp1:6xHis fusion protein (called ySlp1) was *N*-glycosylated but the Slp1<sup>N48G N104G N131G</sup>:6xHis fusion protein (called ySlp1M48/104/131) was not, following expression in yeast. The Slp1:6xHis fusion protein was also expressed in *Escherichia coli* (called bSlp1), which lacks the *N*-glycosylation system. When ySlp1, ySlp1M48/104/131, and bSlp1 were incubated with crab shell chitin beads, we observed that most *N*-glycosylated ySlp1 protein was coprecipitated with chitin beads, whereas only trace amounts of ySlp1M48/104/131 and bSlp1 were coprecipitated with chitin (Figure 8B), indicating that *N*-glycosylation is absolutely critical for the chitin binding activity of Slp1.

Finally, we tested whether exogenous ySlp1, ySlp1M48/104/131, and bSlp1 are able to rescue infection defects of the  $\Delta slp1$  mutant. As shown in Figures 8C and 8D, addition of 1  $\mu$ M ySlp1 was able to almost recover the growth of wild-type IH of the  $\Delta slp1$  mutant in barley leaves. However, addition of 1  $\mu$ M ySlp1M48/104/131 and bSlp1 was only able to partially restore the growth of IH of the  $\Delta slp1$  mutant when compared with ySlp1. When considered together, these results indicate that *N*-glycosylation affects the biological function of Slp1 in driving the capacity of IH to proliferate in plant cells.

### **ALG3 Is Also Important for Maintaining Cell Wall Integrity**

The outer layer of the fungal cell wall is important for protecting the inner chitins or glucans from recognition by host plants (Fujikawa et al., 2012). Glycoproteins are known to be an important component of the fungal cell wall (Bowman and Free, 2006). Therefore, we hypothesized that *ALG3* may also mediate the *N*-glycosylation of glycoproteins in the cell wall and be involved in the cell wall integrity of the rice blast fungus. To test this hypothesis, we assayed the sensitivity of the  $\Delta alg3$  mutant to 0.01 mg/mL Calcofluor white (CFW), 0.2 mg/mL Congo red (CR), and 0.005% SDS, which are cell wall-perturbing agents widely used to assay defects in fungal cell wall integrity (Elorza et al., 1983; Wood and Fulcher, 1983; Roncero and Durán, 1985). When the wild-type strain P131 was cultured on CM plates with CFW, CR, or SDS, vegetative growth was reduced 37.9, 39.5, and 25.0%, respectively, in comparison with growth in CM. By contrast, the  $\Delta alg3$  mutant was reduced 57.7, 52.5, and 34.5%, respectively, in CM cultures supplemented with CFW, CR, and SDS (Supplemental Figure 5), showing increased sensitivity of the  $\Delta alg3$  mutant to cell wall-perturbing reagents. These results suggest that cell wall integrity is affected in the  $\Delta alg3$  mutant. The  $\Delta slp1$  mutant grew in a similar way to the wild-type strain on CM with CFW, CR, or SDS (Supplemental Figure 6), suggesting that the Slp1 effector, which is expressed only during infection (Mentlak et al., 2012), does not play a significant role in cell wall integrity; thus, the defects in cell wall integrity of the  $\Delta alg3$  mutant are unrelated to the under-*N*-glycosylation of Slp1.



**Figure 7.** N-Glycosylation of Slp1 Is Required for Virulence and Evasion of Chitin-Triggered Immunity.

**(A)** Barley leaves sprayed with the wild-type strain P131, *SLP1* deletion mutant SLP1KO1, and complement strains cSLP1, M48/104/131, M48, M94, M104, and M131 were photographed at 5 d post inoculation. M48, M94, M104, and M131 contain the *SLP1*-3xFLAG fusion construct carrying the N48G, N94G, N104G, and N131G mutations, respectively. M48/104/131 contains the *SLP1*-3xFLAG fusion construct with the N48G, N104G, and N131G triple mutation.

**(B)** Lesion numbers on barley leaves caused by the same set of strains described in **(A)**.

**(C)** Average lesion size of barley leaves inoculated with the same set of strains described in **(A)**. Lesion size was measured as reported by Mentlak et al. (2012).

For **(B)** and **(C)**, means and SE were calculated from three independent replicates. Significant differences are indicated by stars ( $P = 0.05$ ).

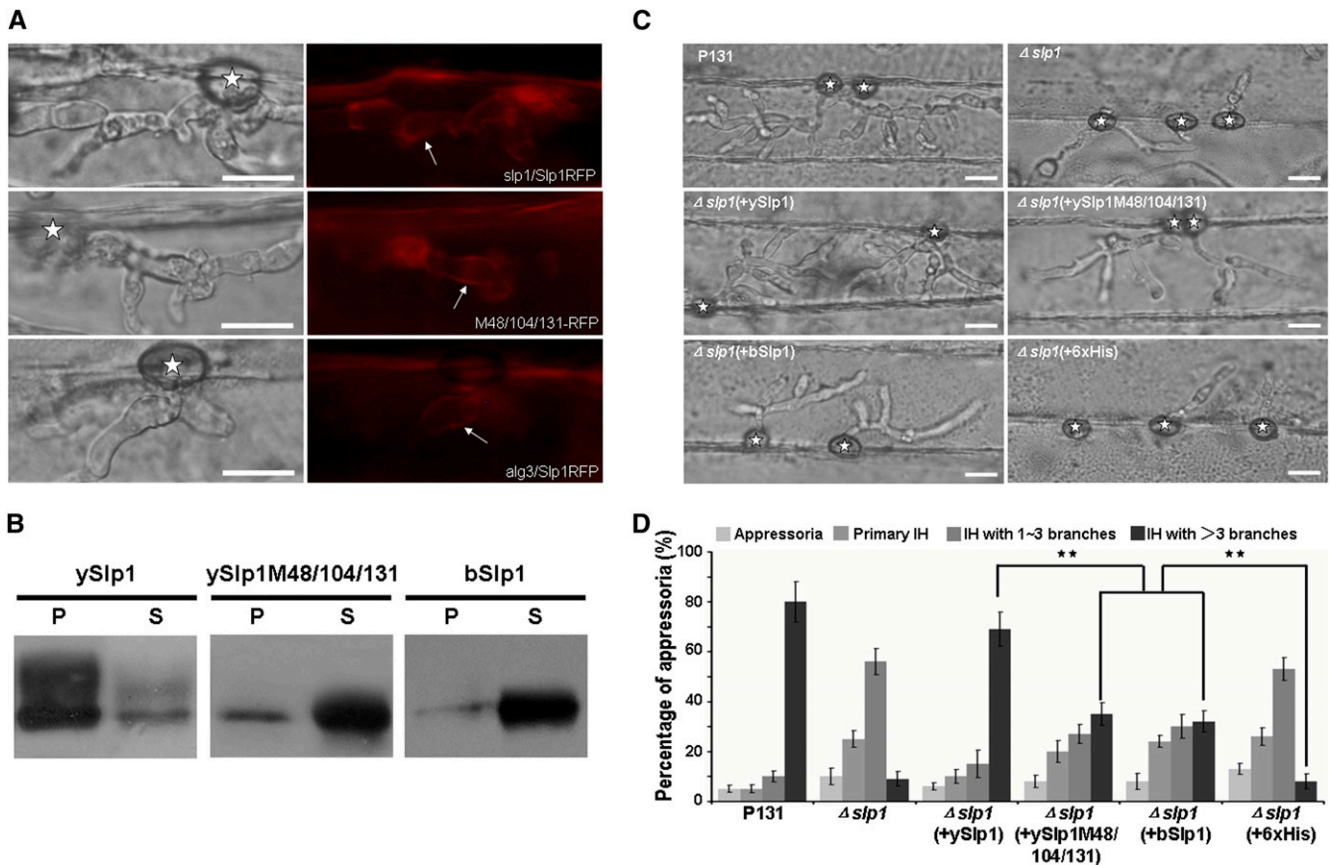
**(D)** DAB staining assays of penetrated plant cells. Barley leaves were sprayed with the same set of strains described in **(A)**, and the epidermis was stained with DAB at 30 hpi and observed with a Nikon microscope. AP, appressoria. Bar = 20  $\mu$ m.

**(E)** Percentages of DAB-stained cells. Means and SE were calculated from three independent replicates. Significant differences are indicated by double stars ( $P = 0.01$ ;  $n > 100$ ).

## DISCUSSION

The *N*-glycosylation pathway of *S. cerevisiae* is widely conserved (Deshpande et al., 2008), but the biological functions of *N*-glycosylation are not well characterized in filamentous fungi, particularly in fungal plant pathogens. In this study, the *ALG3* gene was identified by insertional mutagenesis as an important virulence factor in *M. oryzae*. *ALG3* encodes an ER protein orthologous to yeast Alg3. When expressed in *S. cerevisiae*, the *M. oryzae* *ALG3* functionally complemented the temperature sensitivity of an *alg3 stt3* double mutant (Aebi et al., 1996). We demonstrated that *ALG3* is required for full *N*-glycosylation of two effector proteins, Slp1 and Bas4, and the vacuolar protein CYP. Furthermore, we showed that oligosaccharides linked to Slp1 or CPY in the *ALG3* deletion mutant could not be digested by the enzyme Endo H; thus, it can be postulated that they are Man<sub>5</sub>-oligosaccharides but not Man<sub>9</sub>-oligosaccharides in the wild type. Therefore, in the same way as its yeast ortholog, *ALG3* encodes an  $\alpha$ -1,3-mannosyltransferase that is involved in the biosynthesis of *N*-glycan for the *N*-glycosylation of proteins in *M. oryzae*.

In *M. oryzae*, we found that *ALG3* was required for the suppression of ROS production in plant cells, and the disruption or deletion of *ALG3* resulted in a severe defect in pathogenesis. Three earlier reports have shown that *N*-glycosylation plays an important role in the pathogenesis of plant pathogenic fungi. In *U. maydis*, the ER glucosidase I gene, *Gls1*, is required for the initial stages of infection following appressorium penetration (Fernández-Álvarez et al., 2013) and deletion of the ER glucosidase II gene, *GAS1*, resulted in arrested growth of IH in the epidermal cell layer of maize (*Zea mays*) (Schirawski et al., 2005), and in *M. graminicola*, the  $\alpha$ -1,2-mannosyltransferase Alg2 was shown to be important for switching from the yeast-like phase to the hyphal form (Motteram et al., 2011). These studies suggested the importance of *N*-glycosylation of proteins in the pathogenesis of plant fungal pathogens but did not offer mechanistic insight into why *N*-glycosylation is important for pathogenesis in plant pathogens. In this study, we demonstrated that *ALG3* mediates the *N*-glycosylation of Slp1, which is a virulence factor and effector protein playing an important role in suppressing chitin-triggered ROS production and other immune responses in rice cells (Mentlak et al., 2012). Three



**Figure 8.** *N*-Glycosylation of Slp1 Is Critical for Its Chitin Binding Activity.

(A) The *N*-glycosylated and underglycosylated Slp1 proteins were similarly located at the plant–fungus interface. *slp1*/Slp1RFP, the  $\Delta slp1$  mutant transformed with the *SLP1*-RFP fusion construct; M48/104/131-RFP, the  $\Delta slp1$  mutant transformed with the *SLP1*<sup>N48G N104G N131G</sup>-RFP fusion construct; *alg3*/Slp1RFP, the  $\Delta alg3$  mutant transformed with the *SLP1*-RFP fusion construct. Stars indicate appressoria, and arrows point to the plant–fungus interface. Bars = 20  $\mu$ m.

(B) Affinity precipitation of ySlp1, ySlp1M48/104/131, and bSlp1 with chitin beads detected by immunoblot analysis with an anti-His antibody. Most ySlp1 coprecipitated with chitin beads, but only trace amounts of ySlp1M48/104/131 and bSlp1 coprecipitated with chitin beads. P, pellet; S, supernatant.

(C) Effect of exogenous addition of ySlp1, ySlp1M48/104/131, and bSlp1 proteins on the infection hyphal growth of  $\Delta slp1$ . Purified ySlp1, ySlp1M48/104/131, and bSlp1 proteins were added to the inoculation sites at 16 hpi, and the growth of IH was photographed at 28 hpi. Appressoria are indicated by stars. Purified 6xHis peptide was used as a control. Bars = 20  $\mu$ m.

(D) Percentages of appressoria that formed primary IH, IH with one to three branches, and IH with more than three branches at 28 hpi in (C). Means and SE were calculated from three independent replicates, and double stars indicate statistically significant differences ( $P < 0.01$ ).

For (B) to (D), ySlp1 and bSlp1 were 6xHis-tagged Slp1 expressed in *P. pastoris* and *E. coli*, respectively. ySlp1M48/104/131 was 6xHis-tagged Slp1<sup>N48G N104G N131G</sup> expressed in *P. pastoris*.

*N*-glycosylation sites were identified in Slp1. Our data showed that simultaneous *N*-glycosylation of these three sites is required for the function of Slp1 in suppressing chitin-elicited ROS production in plant cells. Thus, we conclude that Alg3-mediated *N*-glycosylation of Slp1 is an important mechanism by which the rice blast fungus evades chitin-triggered host immunity.

Slp1 was previously demonstrated to sequester chitin oligosaccharides to avoid their recognition by the rice pattern recognition receptor CEBiP (Mentlak et al., 2012). The  $\Delta alg3$  mutant lacks fully *N*-glycosylated Slp1 and accumulates a significantly reduced level of underglycosylated Slp1 (Figure 6B) during

infection. However, the underglycosylated Slp1 had the same subcellular localization as the wild-type Slp1 (Figure 8; Supplemental Figure 4) and was able to partially rescue invasive growth of IH. These results indicate that nonglycosylated Slp1 in the  $\Delta alg3$  mutant is unable to provide enough capacity for binding chitin oligosaccharides, which results in greater accumulation of free chitin oligosaccharides that can be recognized by CEBiP and, in turn, trigger plant innate immunity responses, including ROS generation. In other words, Alg3-mediated *N*-glycosylation of Slp1 is important as a means of maintaining a suitable level of the protein to evade plant innate immunity. In eukaryotic cells,

there is an ER quality control system to monitor the folding status of client proteins and eliminate misfolded proteins through the ER-associated degradation mechanism (Anelli and Sitia, 2008). Studies in mammals and yeast have revealed that *N*-glycosylation plays important roles in ER quality control (Helenius and Aebi, 2004). The underglycosylated Slp1, therefore, is possibly misfolded so that it is monitored and degraded by the ER quality control system, thus leading to lowered accumulation of aberrant protein in the  $\Delta alg3$  mutant.

We also showed that the non-*N*-glycosylated Slp1 had dramatically lowered chitin binding activity when compared with the wild-type protein, indicating that *N*-glycosylation is necessary for the chitin binding activity of Slp1 (Figure 8B). Interestingly, among the three *N*-glycosylation sites identified in this study, two of them, Asn-48 and Asn-131, lie within the two LysM motifs of Slp1 (Figure 6A). A recent study revealed that intermolecular dimerization of *Arabidopsis thaliana* CERK1, the chitin elicitor receptor kinase with three LysM motifs, is crucial for binding chitin oligosaccharides and its signaling function (Liu et al., 2012). Ecp6, the ortholog of Slp1 in the fungal pathogen *Cladosporium fulvum* (de Jonge et al., 2010), also has three LysM motifs that form intrachain LysM dimers to provide a groove for chitin binding (Sánchez-Vallet et al., 2013). To further determine how *N*-glycosylation affects the chitin binding activity of Slp1, it will be important to compare differences in the crystal structure of Slp1 between *N*-glycosylated and nonglycosylated Slp1. Such a comparison will reveal whether Slp1 undergoes intramolecular or intermolecular dimerization and whether *N*-glycosylation affects the dimerization and its effect on chitin binding activity. Preliminary analysis suggests that Slp1 undergoes dimerization (Mentlak et al., 2012). Of the three *N*-glycosylation sites identified in Slp1 in this study, the *N*-glycosylation site Asn-131 is widely conserved in Slp1 orthologous proteins of filamentous fungi (Supplemental Figure 7), suggesting a critical role for this *N*-glycosylation site in LysM protein function.

Our study also showed that the  $\Delta alg3$  mutant was inhibited during development of the invasive hyphae and allowed the induction of ROS in penetrated barley leaf cells. When plant cells were pretreated with DPI, an inhibitor of NADPH oxidase, to suppress ROS generation, the  $\Delta alg3$  mutant could proliferate in host cells almost as efficiently as the wild-type strain. The impairment in the growth of invasive hyphae, therefore, can be attributed to ROS accumulation within plant cells due to the absence of Alg3. In addition to the  $\Delta slp1$  and  $\Delta alg3$  mutants, several other *M. oryzae* mutants have been reported to induce ROS accumulation in penetrated plant cells, including mutants disrupted in *DES1* (Chi et al., 2009), *ATF1* (Guo et al., 2010), *AP1* (Guo et al., 2011), *HYR1* (Huang et al., 2011), *CHS6* (Kong et al., 2012), and *CRC1* (Yang et al., 2012) genes. *DES1*, *AP1*, *ATF1*, and *HYR* may be involved in ROS detoxification, because expression levels of ROS-detoxifying genes were significantly reduced in these mutants (Chi et al., 2009; Guo et al., 2010, 2011; Huang et al., 2011; Adam et al., 2012). *ALG3*, however, is involved in preventing host plants from producing ROS. Deletion of *ALG3* may also result in enhanced adaptability to oxidative conditions and increased laccase activity; the expression levels of genes encoding putative laccases, peroxidases, and some ROS detoxification-related genes were comparable or even

higher than those in the wild-type strain under 5 mM H<sub>2</sub>O<sub>2</sub> (Supplemental Figure 8). It is likely, therefore, that the rice blast fungus has two distinct systems to prevent ROS accumulation in plant cells, one for scavenging ROS and the other for avoiding ROS induction by the action of secreted effector proteins such as Slp1.

In addition to *N*-glycosylation of the Slp1 effector, the Alg3  $\alpha$ -1,3-mannosyltransferase may be involved in the glycosylation of enzymes involved in cell wall synthesis or other cell wall components. For example, *M. oryzae* *CHS6* encodes a chitin synthase with 10 predicted *N*-glycosylation sites that is essential for pathogenicity (Kong et al., 2012). It is tempting to speculate that *ALG3* may also mediate the *N*-glycosylation of Chs6 and, therefore, that Chs6 may not be properly glycosylated and folded in the absence of Alg3. The rice blast fungus likely has a sophisticated system for synthesizing and protecting chitin in its cell wall to avoid PAMP-triggered immunity in rice plants. It is possible that some cell wall proteins glycosylated by *ALG3* also are involved in protecting or masking cell wall components being recognized by the host, such that *ALG3* is likely to play a wider role in functions associated with evading chitin-triggered immunity, in addition to its role in regulating Slp1 activity.

The fungal cell wall consists of multiple polysaccharides and cross-linked glycoproteins; therefore, dysfunction of the major cell wall components may disrupt cell wall integrity (Bowman and Free, 2006). Cell integrity defects observed in  $\Delta alg3$  mutants are likely to be due to under-*N*-glycosylation of glycoproteins other than Slp1, because deletion of Slp1 did not result in any such defects in colony growth and cell wall integrity. *ALG3*, therefore, may mediate the *N*-glycosylation of multiple glycoproteins in addition to Slp1. Further investigation is required to identify cell wall glycoproteins glycosylated by *ALG3* that are important for cell wall integrity.

In addition to Slp1, more than a dozen effector proteins have been identified in *M. oryzae*, such as Pwl2 (for pathogenicity toward weeping lovegrass protein2) (Sweigard et al., 1995), AVR-Pita1 (Jia et al., 2000), AVR-Pia (Miki et al., 2009; Yoshida et al., 2009), AVR-Pik/km/kp, AVR-Pii (Yoshida et al., 2009), AVR-CO39 (Farman et al., 2002), Bas1 to Bas4 (Mosquera et al., 2009), and AvrPiz-t (Li et al., 2009). *N*-Glycosylation sites are predicted in some of these effector proteins, including Bas2, Bas4, and AvrPiz-t, suggesting that Alg3 may mediate the *N*-glycosylation of several effector proteins in *M. oryzae* during plant infection. In this study, we showed that Alg3 mediates the *N*-glycosylation of Bas4, for example (Supplemental Figure 6). Putative *N*-glycosylation sites also exist in various effector proteins that have been identified in other plant pathogenic fungi, including Pep1 (Doehlemann et al., 2009; Hemetsberger et al., 2012) and Pit1 (Doehlemann et al., 2011) in *U. maydis* and Ecp6 in *C. fulvum* (de Jonge et al., 2010). As components of the yeast *N*-glycosylation pathway are conserved in filamentous fungi (Deshpande et al., 2008), the *N*-glycosylation of effector proteins may be widely deployed by plant fungal pathogens as a common mechanism to regulate their function in evading host innate immunity. Therefore, it will be important in the future to use Alg3 as a means of identifying further effectors that are glycosylated in order to help determine their precise effector functions.

## METHODS

### Strains and Culture Conditions

Two wild-type strains of *Magnaporthe oryzae* were used in this study. One was the field isolate P131 (Peng and Shishiyama, 1988; Xue et al., 2012), which was used to generate all mutant strains. The other was S1528, which was used to perform genetic crosses with P131. The wild-type strains and corresponding transformants generated in this study were maintained on OTA plates at 28°C. For extraction of genomic DNA, RNA, and protein and isolation of protoplasts, mycelium was collected from liquid CM cultured at 180 rpm for 36 h. For measuring colony sizes, mycelial blocks ( $\Phi = 5$  mm) were inoculated into the center of OTA plates and cultured for 120 h. Conidiation was performed as described previously (Peng and Shishiyama, 1988). Conidia harvested from 10-d-old OTA cultures were used for the virulence test, infection process observation, and cell wall integrity assay. For the cell wall integrity test, the mycelial plugs of diverse strains were cultured on CM agar added in advance with different agents, 0.01 mg/mL CFW (Sigma-Aldrich), 0.2 mg/mL CR (Sigma-Aldrich), and 0.005% SDS, and the colony sizes were measured at 5 d after inoculation.

### Molecular Manipulations with Nucleic Acids

Genomic DNA was extracted from mycelia using the cetyltrimethylammonium bromide protocol as described by Xu and Hamer (1996). Total RNA was extracted with the Trizol kit (Invitrogen). Standard molecular manipulation procedures were followed for plasmid isolation, DNA gel blot analysis, and enzymatic digestion with DNA (Sambrook and Russell, 2001). Probes used for DNA gel blots were labeled with the Random Primer DNA Labeling Kit (Takara). Plasmid rescue was performed as described previously (Sweigard et al., 1998).

### Gene Disruption and Complementation of *ALG3*

For constructing the *ALG3* gene replacement construct, 1.5-kb upstream and downstream flanking sequence segments were amplified from genomic DNA of P131, with primer pairs 08010LF/08010LR and 08010RF/08010RR, respectively. The two flanking sequences were cloned into pKNH (Yang et al., 2010) as the deletion vector pKNH-*ALG3*, which was linearized by *NotI* digestion and transformed into protoplasts of P131. Protoplasts were isolated and transformed with the polyethylene glycol/CaCl<sub>2</sub> approach as described (Yang et al., 2010). For selecting hygromycin-resistant or neomycin-resistant transformants, CM plates were supplemented with 250  $\mu$ g/mL hygromycin B (Roche) or 400  $\mu$ g/mL neomycin (Ameresco).

For complementation assays, the *ALG3* gene containing a 1.5-kb native promoter fragment and a 0.5-kb terminator region was amplified with primers ALG3CF/ALG3CR and cloned into pKN (Yang et al., 2010). The resulting construct pKN-*ALG3* was digested with *NotI* and transformed into the  $\Delta$ *alg3* mutant. Neomycin-resistant transformants were obtained and verified by PCR.

### Generation of Different *SLP1* Mutant Alleles

To complement the  $\Delta$ *slp1* mutant with the mutated *SLP1* alleles, the vector pKNFLAG-*SLP1* was mutated at different *N*-glycosylation sites by the PCR-mediated approach (Horton, 1995). Total genomic DNA was extracted and amplicons of *SLP1* were ligated into vector pKNFLAG, which contains the constitutive promoter in the 5' terminus and the 3xFLAG sequence in the 3' terminus, by the primer pair SLP1-F/SLP1-R. This vector, pKNFLAG-*SLP1*, was used to mutate putative *N*-glycosylation sites. Four pairs of primers, SLP1M1-F/SLP1M1-R, SLP1M2-F/SLP1M2-R, SLP1M3-F/SLP1M3-R, and SLP1M4-F/SLP1M4-R, were

used to perform the ligation PCR to generate the mutation for Asn to Gly at Asn-48, Asn-94, Asn-104, and Asn-131, respectively. Subsequently, vectors pKNFLAG-M48, pKNFLAG-M94, pKNFLAG-M104, and pKNFLAG-M131 were obtained and used to transform the wild-type strain P131 to obtain strains WT/M48, WT/M94, WT/M104, and WT/M131 or to transform the  $\Delta$ *slp1* mutant to obtain strains M48, M94, M104, and M131. A similar approach was used to obtain vector pKNFLAG-M48/104/131, expressing the Slp1-3xFLAG fusion protein with simultaneous mutations N48G, N104G, and N131G, which was also used to transform the  $\Delta$ *slp1* mutant to obtain strain M48/104/131.

### Subcellular Localization Analysis

For colocalization analysis of Alg3 and ER, the *ALG3*-eGFP and *RFP*-HDEL fusion constructs were generated by overlapping PCR and separately cloned into pKN. The resulting constructs, pKN-*ALG3*GFP and pKN-*RFP*HDEL, were digested with *NotI* and cotransformed into the  $\Delta$ *alg3* mutant. Colocalization of Alg3-GFP and RFP-HDEL fusion proteins were analyzed with a Nikon A1 laser scanning confocal microscope.

To analyze the subcellular localization pattern of Slp1, coding regions of wild-type *SLP1* and *SLP1*<sup>N48G N104G N131G</sup> were separately fused with *RFP* and cloned into pKNRP27. The resulting constructs, pKN*SLP1**RFP* and pKN*SLP1*M48/104/131*RFP*, were digested with *NotI* and transformed into the wild-type strain. Similar approaches were performed to obtain the *SLP1*<sup>N48G</sup>:*RFP*, *SLP1*<sup>N104G</sup>:*RFP*, and *SLP1*<sup>N131G</sup>:*RFP* fusion constructs. The vector pKN*SLP1**RFP* was also transformed into the  $\Delta$ *alg3* mutant. Subsequent transformants were observed with an epifluorescence microscope (Nikon).

### Virulence Test and Infection Process Observation

To investigate the infection process, a conidial suspension with a concentration of  $5 \times 10^5$  conidia/mL in 0.025% Tween 20 was spotted onto the lower epidermis of barley (*Hordeum vulgare*) leaves and then incubated in a moist, dark chamber at 28°C. After inoculation, microscopy observations were performed at 2, 12, 18, 24, 30, and 36 hpi. Rice (*Oryza sativa* cv LTH) seedlings at the fifth leaf stage and 8-d-old barley seedlings (cv E9) were sprayed with conidia suspension of  $10^5$  conidia/mL and incubated as described previously (Yang et al., 2010). Lesion formation was examined at 5 d after inoculation. To test the effect of exogenous Slp1 proteins on infection of the  $\Delta$ *slp1* and  $\Delta$ *alg3* mutants, 1  $\mu$ M purified ySlp1 (Slp1:6xHis tag fusion protein expressed and purified from yeast [*Pichia pastoris*]), ySlp1M48/104/131 (Slp1<sup>N48G N104G N131G</sup>:6xHis tag fusion protein expressed and purified from yeast), or bSlp1 (Slp1:6xHis tag fusion protein expressed and purified from *Escherichia coli*) was separately added onto the  $\Delta$ *slp1* mutant inoculation site at 16 hpi, and subsequent microscopy observations were performed at 28 hpi.

Host-derived ROS was observed by staining with DAB (Sigma-Aldrich) as described (Ding et al., 2010). Barley leaves inoculated with the mutant and wild-type strains for 30 hpi were incubated in 1 mg/mL DAB solution, pH 3.8, at room temperature for 8 h and destained with clearing solution (ethanol:acetic acid = 94:4, v/v) for 1 h.

For evaluating the growth of IH in ROS-suppressed barley cells, a conidial suspension supplemented with 0.5  $\mu$ M DPI was dropped on barley leaves (Chi et al., 2009). At 30 hpi, the growth of IH was evaluated by microscopy. Routinely, the infection process was observed by differential interference contrast microscopy imaging using a Nikon Ni90 epifluorescence microscope.

### *N*-Glycosylation Analysis

To generate the *CPY*-3xFLAG and *SLP1*-3xFLAG constructs, cDNAs of *CPY* and *SLP1* were amplified with primer pairs *CPY*-F/*CPY*-R and *SLP1*-F/*SLP1*-R, respectively, and cloned into pKNFLAG. In the resulting

vectors, pKNFLAG-CPY and pKNFLAG-SLP1, the 3xFLAG fusion constructs were expressed under the control of the RP27 promoter (Bourett et al., 2002). These vectors were transformed to protoplasts of P131 and the  $\Delta alg3$  mutant to obtain the FLAG-tagged strains.

For the extraction of total protein, a 0.2-g aliquot of mycelium collected from 2-d cultures in liquid CM was ground into powder in liquid nitrogen and then buffered with 50 mM HEPES buffer, pH 7.4, and agitated three times before being centrifuged at 13,500 rpm for 30 min at 4°C. The supernatant was quantified using the Bradford assay (Bio-Rad), and ~20  $\mu$ g of total proteins was immediately mixed with SDS-PAGE loading buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, and 1%  $\beta$ -mercaptoethanol), denatured at 95°C for 5 min, and subjected to 12% SDS-PAGE. The gel blotting was performed using polyvinylidene difluoride membranes blocked with 5% (w/v) nonfat dry milk in Tris-buffered saline (20 mM Tris-HCl, pH 7.6, and 137 mM NaCl) with 0.05% Tween 20. The membranes were probed with an anti-FLAG antibody (1:2,000; Abmart). Detection of bound primary antibodies was performed with Lumi-Light and Lumi-Imager (Roche Diagnostics) after incubation with goat anti-rabbit antibodies (1:10,000; Abmart). PNGase F and Endo H from New England Biolabs were used for glycosylation analysis of CPY, Slp1, and Bas4 following instructions from the manufacturer. For glycosylation analysis, total protein extracted with HEPES buffer was digested with PNGase F or Endo H for 1 h before being mixed with the loading buffer.

#### Yeast Complementation

The *ALG3* cDNA was amplified using primers ALG3F and ALG3R and cloned between the *Hind*III and *Sph*I sites of pYES2. The resulting pYES2-*ALG3* vector was confirmed by DNA sequence analysis and introduced into the yeast *alg3 stt3* mutant YG170. The transformants were selected on SC medium containing 2% Glc at 25°C. A series of 1:10 dilutions of transformants suspended in distilled water were spotted onto SC medium containing 2% Gal and incubated at 30°C for 2 d.

#### Purification of Recombinant Slp1 Protein

To obtain eukaryotic cell-expressed Slp1 recombinant protein, *SLP1* cDNAs with or without the three *N*-glycosylation sites mutated were amplified using the primers SLP1-EcoRI and SLP1-KpnI and cloned into the vector pPICZB (Invitrogen). The resulting constructs, pPICZB-*SLP1* and pPICZB-*SLP1M*, were transformed into competent cells of *P. pastoris* X33 to express 6xHis-tagged Slp1 and 6xHis-tagged Slp1<sup>N48G N104G N131G</sup>.

To obtain prokaryotic cell-expressed Slp1 recombinant protein, *SLP1* cDNA was amplified using the primers SLP1-NcoI and SLP1-XhoI and cloned into the vector pETM20 (Novagen). The resulting construct, pETM20-*SLP1*, was transformed into competent cells of *E. coli* strain BL21 to express the 6xHis-tagged Slp1 without *N*-glycosylation. All proteins were subsequently purified with nickel-chelating Sepharose Fast Flow (GE Healthcare Biosciences) according to the manufacturer's instructions.

#### Chitin Binding Assay

The chitin binding activity of glycosylated and nonglycosylated Slp1 was assayed as described previously (de Jonge et al., 2010). Each purified protein was incubated with crab shell chitin beads (Sigma-Aldrich). For each treatment, 1 mL of a mixture containing 50  $\mu$ g/mL protein and 5 mg of chitin beads was incubated at 4°C on a rocking platform for 16 h. The mixtures were then centrifuged for 5 min at 13,000g. The supernatant was collected and concentrated to dryness. The pellet fractions were rinsed three times with distilled sterile water. Both the pellet and supernatant fractions were boiled in 200  $\mu$ L of SDS solution (1%) for 10 min, and then 20  $\mu$ L of the solutions was used to perform SDS-PAGE and immunoblot analysis with anti-His antibody (Abmart).

#### Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *M. oryzae* *ALG3* (KC236413), *S. cerevisiae* *ALG3* (AA75352), *Arabidopsis* *ALG3* (AAL16193), *Homo sapiens* *ALG3* (AAH04313), *M. oryzae* *SLP1* (EHA51101), *M. oryzae* *CPY* (EHA57949), and *M. oryzae* *CHS6* (JF912409).

#### Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Alignment of Alg3 Amino Acid Sequences from *Arabidopsis*, *S. cerevisiae*, *M. oryzae*, and *H. sapiens*.

**Supplemental Figure 2.** Infection Hyphae Growth of the Wild-Type P131, *ALG3* Deletion Mutant ALG3KO1, and Complemented Transformant cALG3 at 36 h Postinoculation.

**Supplemental Figure 3.** *N*-Glycosylation of Bas4 in the Wild-Type P131 and *ALG3* Deletion Mutant ALG3KO1 Detected by Immunoblot Analysis.

**Supplemental Figure 4.** Subcellular Localization of Slp1 Proteins in Which Each of the *N*-Glycosylation Sites Was Mutated.

**Supplemental Figure 5.** *ALG3* Is Important for Maintaining Cell Wall Integrity.

**Supplemental Figure 6.** *SLP1* Is Not Relevant to Cell Wall Integrity.

**Supplemental Figure 7.** Alignment of Amino Acid Sequences of Slp1 and Its Orthologous Proteins from Filamentous Fungi Showing *N*-Glycosite Conservation.

**Supplemental Figure 8.** Gene Activation of ROS Detoxification Enzymes in the  $\Delta alg3$  Mutant Compared with the Wild-Type Strain.

**Supplemental Table 1.** Phenotype Characterization of Some *M. oryzae* Strains.

**Supplemental Table 2.** Comparison of the Growth of Infection Hyphae.

**Supplemental Table 3.** Fungal Strains Used in This Study.

**Supplemental Table 4.** Plasmids Used in This Study.

**Supplemental Table 5.** Primers Used in This Study.

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#### AUTHOR CONTRIBUTIONS

Y.-L.P., X.-L.C., and T.S. designed the project. X.-L.C., T.S., J.Y., W.S., X.G., D.C., and X.X. performed the experiments. All authors contributed to the analysis and interpretation of the results. Y.-L.P., X.-L.C., J.-R.X., and N.J.T. wrote the article with contributions from J.Y.

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## REFERENCES

- Adam, T., Bouhidel, K., Der, C., Robert, F., Najid, A., Simon-Plas, F., and Leborgne-Castel, N. (2012). Constitutive expression of clathrin hub hinders elicitor-induced clathrin-mediated endocytosis and defense gene expression in plant cells. *FEBS Lett.* **586**: 3293–3298.
- Aebi, M., Gassenhuber, J., Domdey, H., and te Heesen, S. (1996). Cloning and characterization of the *ALG3* gene of *Saccharomyces cerevisiae*. *Glycobiology* **6**: 439–444.
- Anelli, T., and Sitia, R. (2008). Protein quality control in the early secretory pathway. *EMBO J.* **27**: 315–327.
- Bourett, T.M., Sweigard, J.A., Czymmek, K.J., Carroll, A., and Howard, R.J. (2002). Reef coral fluorescent proteins for visualizing fungal pathogens. *Fungal Genet. Biol.* **37**: 211–220.
- Bowman, S.M., and Free, S.J. (2006). The structure and synthesis of the fungal cell wall. *Bioessays* **28**: 799–808.
- Burda, P., and Aebi, M. (1999). The dolichol pathway of *N*-linked glycosylation. *Biochim. Biophys. Acta* **1426**: 239–257.
- Chi, M.-H., Park, S.-Y., Kim, S., and Lee, Y.H. (2009). A novel pathogenicity gene is required in the rice blast fungus to suppress the basal defenses of the host. *PLoS Pathog.* **5**: e1000401.
- Chisholm, S.T., Coaker, G., Day, B., and Staskawicz, B.J. (2006). Host-microbe interactions: Shaping the evolution of the plant immune response. *Cell* **124**: 803–814.
- Cross, A.R., and Jones, O.T.G. (1986). The effect of the inhibitor diphenylene iodonium on the superoxide-generating system of neutrophils: Specific labelling of a component polypeptide of the oxidase. *Biochem. J.* **237**: 111–116.
- Dean, N. (1999). Asparagine-linked glycosylation in the yeast Golgi. *Biochim. Biophys. Acta* **1426**: 309–322.
- Dean, R., Van Kan, J.A., Pretorius, Z.A., Hammond-Kosack, K.E., Di Pietro, A., Spanu, P.D., Rudd, J.J., Dickman, M., Kahmann, R., Ellis, J., and Foster, G.D. (2012). The top 10 fungal pathogens in molecular plant pathology. *Mol. Plant Pathol.* **13**: 414–430.
- de Jong, J.C., McCormack, B.J., Smirnov, N., and Talbot, N.J. (1997). Glycerol generates turgor in rice blast. *Nature* **389**: 244–245.
- de Jonge, R., van Esse, H.P., Kombrink, A., Shinya, T., Desaki, Y., Bours, R., van der Krol, S., Shibuya, N., Joosten, M.H., and Thomma, B.P. (2010). Conserved fungal LysM effector Ecp6 prevents chitin-triggered immunity in plants. *Science* **329**: 953–955.
- Deshpande, N., Wilkins, M.R., Packer, N., and Nevalainen, H. (2008). Protein glycosylation pathways in filamentous fungi. *Glycobiology* **18**: 626–637.
- Ding, S.L., Liu, W., Iliuk, A., Ribot, C., Vallet, J., Tao, A., Wang, Y., Lebrun, M.H., and Xu, J.R. (2010). The *tig1* histone deacetylase complex regulates infectious growth in the rice blast fungus *Magnaporthe oryzae*. *Plant Cell* **22**: 2495–2508.
- Doehlemann, G., Reissmann, S., Assmann, D., Fleckenstein, M., and Kahmann, R. (2011). Two linked genes encoding a secreted effector and a membrane protein are essential for *Ustilago maydis*-induced tumour formation. *Mol. Microbiol.* **81**: 751–766.
- Doehlemann, G., van der Linde, K., Assmann, D., Schwambach, D., Hof, A., Mohanty, A., Jackson, D., and Kahmann, R. (2009). Pep1, a secreted effector protein of *Ustilago maydis*, is required for successful invasion of plant cells. *PLoS Pathog.* **5**: e1000290.
- Elorza, M.V., Rico, H., and Sentandreu, R. (1983). Calcofluor white alters the assembly of chitin fibrils in *Saccharomyces cerevisiae* and *Candida albicans* cells. *J. Gen. Microbiol.* **129**: 1577–1582.
- Farman, M.L., Eto, Y., Nakao, T., Tosa, Y., Nakayashiki, H., Mayama, S., and Leong, S.A. (2002). Analysis of the structure of the AVR1-CO39 avirulence locus in virulent rice-infecting isolates of *Magnaporthe grisea*. *Mol. Plant Microbe Interact.* **15**: 6–16.
- Fernández-Álvarez, A., Elías-Villalobos, A., Jiménez-Martín, A., Marín-Menguiano, M., and Ibeas, J.I. (2013). Endoplasmic reticulum glucosidases and protein quality control factors cooperate to establish biotrophy in *Ustilago maydis*. *Plant Cell* **25**: 4676–4690.
- Fujikawa, T., Sakaguchi, A., Nishizawa, Y., Kouzai, Y., Minami, E., Yano, S., Koga, H., Meshi, T., and Nishimura, M. (2012). Surface  $\alpha$ -1,3-glucan facilitates fungal stealth infection by interfering with innate immunity in plants. *PLoS Pathog.* **8**: e1002882.
- Giniger, E., Varnum, S.M., and Ptashne, M. (1985). Specific DNA binding of GAL4, a positive regulatory protein of yeast. *Cell* **40**: 767–774.
- Giraldo, M.C., Dagdas, Y.F., Gupta, Y.K., Mentlak, T.A., Yi, M., Martinez-Rocha, A.L., Saitoh, H., Terauchi, R., Talbot, N.J., and Valent, B. (2013). Two distinct secretion systems facilitate tissue invasion by the rice blast fungus *Magnaporthe oryzae*. *Nat. Commun.* **4**: 1996.
- Guo, M., Chen, Y., Du, Y., Dong, Y., Guo, W., Zhai, S., Zhang, H., Dong, S., Zhang, Z., Wang, Y., Wang, P., and Zheng, X. (2011). The bZIP transcription factor MoAP1 mediates the oxidative stress response and is critical for pathogenicity of the rice blast fungus *Magnaporthe oryzae*. *PLoS Pathog.* **7**: e1001302.
- Guo, M., et al. (2010). The basic leucine zipper transcription factor Moatf1 mediates oxidative stress responses and is necessary for full virulence of the rice blast fungus *Magnaporthe oryzae*. *Mol. Plant Microbe Interact.* **23**: 1053–1068.
- Hamer, J.E., Howard, R.J., Chumley, F.G., and Valent, B. (1988). A mechanism for surface attachment in spores of a plant pathogenic fungus. *Science* **239**: 288–290.
- Häweker, H., Rips, S., Koiwa, H., Salomon, S., Saijo, Y., Chinchilla, D., Robatzek, S., and von Schaewen, A. (2010). Pattern recognition receptors require *N*-glycosylation to mediate plant immunity. *J. Biol. Chem.* **285**: 4629–4636.
- Helenius, A., and Aebi, M. (2001). Intracellular functions of *N*-linked glycans. *Science* **291**: 2364–2369.
- Helenius, A., and Aebi, M. (2004). Roles of *N*-linked glycans in the endoplasmic reticulum. *Annu. Rev. Biochem.* **73**: 1019–1049.
- Helenius, J., and Aebi, M. (2002). Transmembrane movement of dolichol linked carbohydrates during *N*-glycoprotein biosynthesis in the endoplasmic reticulum. *Semin. Cell Dev. Biol.* **13**: 171–178.
- Hemetsberger, C., Herrberger, C., Zechmann, B., Hillmer, M., and Doehlemann, G. (2012). The *Ustilago maydis* effector Pep1 suppresses plant immunity by inhibition of host peroxidase activity. *PLoS Pathog.* **8**: e1002684.
- Hiller, E., Zavrel, M., Hauser, N., Sohn, K., Burger-Kentischer, A., Lemuth, K., and Rupp, S. (2011). Adaptation, adhesion and invasion during interaction of *Candida albicans* with the host—Focus on the function of cell wall proteins. *Int. J. Med. Microbiol.* **301**: 384–389.
- Horton, R.M. (1995). PCR-mediated recombination and mutagenesis: SOEing together tailor-made genes. *Mol. Biotechnol.* **3**: 93–99.
- Howard, R.J., Ferrari, M.A., Roach, D.H., and Money, N.P. (1991). Penetration of hard substrates by a fungus employing enormous turgor pressures. *Proc. Natl. Acad. Sci. USA* **88**: 11281–11284.
- Huang, K., Czymmek, K.J., Caplan, J.L., Sweigard, J.A., and Donofrio, N.M. (2011). HYR1-mediated detoxification of reactive oxygen species is required for full virulence in the rice blast fungus. *PLoS Pathog.* **7**: e1001335.
- Hubbard, S.C., and Ivatt, R.J. (1981). Synthesis and processing of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* **50**: 555–583.
- Huffaker, T.C., and Robbins, P.W. (1983). Yeast mutants deficient in protein glycosylation. *Proc. Natl. Acad. Sci. USA* **80**: 7466–7470.
- Jia, Y., McAdams, S.A., Bryan, G.T., Hershey, H.P., and Valent, B. (2000). Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. *EMBO J.* **19**: 4004–4014.

- Jones, J.D., and Dangl, J.L. (2006). The plant immune system. *Nature* **444**: 323–329.
- Kaku, H., Nishizawa, Y., Ishii-Minami, N., Akimoto-Tomiyama, C., Dohmae, N., Takio, K., Minami, E., and Shibuya, N. (2006). Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. *Proc. Natl. Acad. Sci. USA* **103**: 11086–11091.
- Kang, J.S., et al. (2008). Salt tolerance of *Arabidopsis thaliana* requires maturation of N-glycosylated proteins in the Golgi apparatus. *Proc. Natl. Acad. Sci. USA* **105**: 5933–5938.
- Kankanala, P., Czymmek, K., and Valent, B. (2007). Roles for rice membrane dynamics and plasmodesmata during biotrophic invasion by the blast fungus. *Plant Cell* **19**: 706–724.
- Kelleher, D.J., and Gilmore, R. (2006). An evolving view of the eukaryotic oligosaccharyltransferase. *Glycobiology* **16**: 47R–62R.
- Kong, L.A., Yang, J., Li, G.T., Qi, L.L., Zhang, Y.J., Wang, C.F., Zhao, W.S., Xu, J.R., and Peng, Y.L. (2012). Different chitin synthase genes are required for various developmental and plant infection processes in the rice blast fungus *Magnaporthe oryzae*. *PLoS Pathog.* **8**: e1002526.
- Kostova, Z., and Wolf, D.H. (2005). Importance of carbohydrate positioning in the recognition of mutated CPY for ER-associated degradation. *J. Cell Sci.* **118**: 1485–1492.
- Lehle, L., Strahl, S., and Tanner, W. (2006). Protein glycosylation, conserved from yeast to man: A model organism helps elucidate congenital human diseases. *Angew. Chem. Int. Ed. Engl.* **45**: 6802–6818.
- Lerouge, P., Cabanes-Macheteau, M., Rayon, C., Fischette-Lainé, A.C., Gomord, V., and Faye, L. (1998). N-Glycoprotein biosynthesis in plants: Recent developments and future trends. *Plant Mol. Biol.* **38**: 31–48.
- Li, W., et al. (2009). The *Magnaporthe oryzae* avirulence gene *AvrPiz-t* encodes a predicted secreted protein that triggers the immunity in rice mediated by the blast resistance gene *Piz-t*. *Mol. Plant Microbe Interact.* **22**: 411–420.
- Liebinger, E., Hüttner, S., Vavra, U., Fischl, R., Schoberer, J., Grass, J., Blaukopf, C., Seifert, G.J., Altmann, F., Mach, L., and Strasser, R. (2009). Class I  $\alpha$ -mannosidases are required for N-glycan processing and root development in *Arabidopsis thaliana*. *Plant Cell* **21**: 3850–3867.
- Liu, G., Greenshields, D.L., Samyanaiken, R., Hirji, R.N., Selvaraj, G., and Wei, Y. (2007). Targeted alterations in iron homeostasis underlie plant defense responses. *J. Cell Sci.* **120**: 596–605.
- Liu, J.X., and Howell, S.H. (2010). Endoplasmic reticulum protein quality control and its relationship to environmental stress responses in plants. *Plant Cell* **22**: 2930–2942.
- Liu, T., Liu, Z., Song, C., Hu, Y., Han, Z., She, J., Fan, F., Wang, J., Jin, C., Chang, J., Zhou, J.M., and Chai, J. (2012). Chitin-induced dimerization activates a plant immune receptor. *Science* **336**: 1160–1164.
- Mentlak, T.A., Kombrink, A., Shinya, T., Ryder, L.S., Otomo, I., Saitoh, H., Terauchi, R., Nishizawa, Y., Shibuya, N., Thomma, B. P., and Talbot, N.J. (2012). Effector-mediated suppression of chitin-triggered immunity by *Magnaporthe oryzae* is necessary for rice blast disease. *Plant Cell* **24**: 322–335.
- Miki, S., et al. (2009). Molecular cloning and characterization of the AVR-Pia locus from a Japanese field isolate of *Magnaporthe oryzae*. *Mol. Plant Pathol.* **10**: 361–374.
- Mitchell, T.K., and Dean, R.A. (1995). The cAMP-dependent protein kinase catalytic subunit is required for appressorium formation and pathogenesis by the rice blast pathogen *Magnaporthe grisea*. *Plant Cell* **7**: 1869–1878.
- Mora-Montes, H.M., Bates, S., Netea, M.G., Castillo, L., Brand, A., Buurman, E.T., Díaz-Jiménez, D.F., Jan Kullberg, B., Brown, A.J., Odds, F.C., and Gow, N.A. (2010). A multifunctional mannosyltransferase family in *Candida albicans* determines cell wall mannan structure and host-fungus interactions. *J. Biol. Chem.* **285**: 12087–12095.
- Mosquera, G., Giraldo, M.C., Khang, C.H., Coughlan, S., and Valent, B. (2009). Interaction transcriptome analysis identifies *Magnaporthe oryzae* BAS1-4 as biotrophy-associated secreted proteins in rice blast disease. *Plant Cell* **21**: 1273–1290.
- Motteram, J., Lovegrove, A., Pirie, E., Marsh, J., Devonshire, J., van de Meene, A., Hammond-Kosack, K., and Rudd, J.J. (2011). Aberrant protein N-glycosylation impacts upon infection-related growth transitions of the haploid plant-pathogenic fungus *Mycosphaerella graminicola*. *Mol. Microbiol.* **81**: 415–433.
- Ohtsubo, K., and Marth, J.D. (2006). Glycosylation in cellular mechanisms of health and disease. *Cell* **126**: 855–867.
- Pelham, H.R., Hardwick, K.G., and Lewis, M.J. (1988). Sorting of soluble ER proteins in yeast. *EMBO J.* **7**: 1757–1762.
- Peng, Y.L., and Shishiyama, J. (1988). Temporal sequence of cytological events in rice leaves infected with *Pyricularia oryzae*. *Can. J. Bot.* **66**: 730–735.
- Poulain, D., and Jouault, T. (2004). *Candida albicans* cell wall glycans, host receptors and responses: Elements for a decisive crosstalk. *Curr. Opin. Microbiol.* **7**: 342–349.
- Qin, C., Qian, W., Wang, W., Wu, Y., Yu, C., Jiang, X., Wang, D., and Wu, P. (2008). GDP-mannose pyrophosphorylase is a genetic determinant of ammonium sensitivity in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* **105**: 18308–18313.
- Roncero, C., and Durán, A. (1985). Effect of Calcofluor white and Congo red on fungal cell wall morphogenesis: *In vivo* activation of chitin polymerization. *J. Bacteriol.* **163**: 1180–1185.
- Roth, J. (2002). Protein N-glycosylation along the secretory pathway: Relationship to organelle topography and function, protein quality control, and cell interactions. *Chem. Rev.* **102**: 285–303.
- Ruiz-May, E., Kim, S.J., Brandizzi, F., and Rose, J.K. (2012). The secreted plant N-glycoproteome and associated secretory pathways. *Front. Plant Sci.* **3**: 117.
- Saijo, Y., Tintor, N., Lu, X., Rauf, P., Pajeroska-Mukhtar, K., Häweker, H., Dong, X., Robatzek, S., and Schulze-Lefert, P. (2009). Receptor quality control in the endoplasmic reticulum for plant innate immunity. *EMBO J.* **28**: 3439–3449.
- Sambrook, J., and Russell, D.W. (2001). *Molecular Cloning: A Laboratory Manual*. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).
- Sánchez-Vallet, A., Saleem-Batcha, R., Kombrink, A., Hansen, G., Valkenburg, D.J., Thomma, B.P., and Mesters, J.R. (2013). Fungal effector Ecp6 outcompetes host immune receptor for chitin binding through intrachain LysM dimerization. *Elife* **2**: e00790.
- Schirawski, J., Böhnert, H.U., Steinberg, G., Snetselaar, K., Adamikowa, L., and Kahmann, R. (2005). Endoplasmic reticulum glucosidase II is required for pathogenicity of *Ustilago maydis*. *Plant Cell* **17**: 3532–3543.
- Shimizu, T., Nakano, T., Takamizawa, D., Desaki, Y., Ishii-Minami, N., Nishizawa, Y., Minami, E., Okada, K., Yamane, H., Kaku, H., and Shibuya, N. (2010). Two LysM receptor molecules, CEBIP and OsCERK1, cooperatively regulate chitin elicitor signaling in rice. *Plant J.* **64**: 204–214.
- Shin, J., Dunbrack, R.L., Jr., Lee, S., and Strominger, J.L. (1991). Signals for retention of transmembrane proteins in the endoplasmic reticulum studied with CD4 truncation mutants. *Proc. Natl. Acad. Sci. USA* **88**: 1918–1922.
- Snider, M.D., Sultzman, L.A., and Robbins, P.W. (1980). Transmembrane location of oligosaccharide-lipid synthesis in microsomal vesicles. *Cell* **21**: 385–392.



- Sweigard, J.A., Carroll, A.M., Farrall, L., Chumley, F.G., and Valent, B.** (1998). *Magnaporthe grisea* pathogenicity genes obtained through insertional mutagenesis. *Mol. Plant Microbe Interact.* **11**: 404–412.
- Sweigard, J.A., Carroll, A.M., Kang, S., Farrall, L., Chumley, F.G., and Valent, B.** (1995). Identification, cloning, and characterization of *PWL2*, a gene for host species specificity in the rice blast fungus. *Plant Cell* **7**: 1221–1233.
- Thines, E., Weber, R.W., and Talbot, N.J.** (2000). MAP kinase and protein kinase A-dependent mobilization of triacylglycerol and glycogen during appressorium turgor generation by *Magnaporthe grisea*. *Plant Cell* **12**: 1703–1718.
- Verostek, M.F., Atkinson, P.H., and Trimble, R.B.** (1993). Glycoprotein biosynthesis in the *alg3 Saccharomyces cerevisiae* mutant. II. Structure of novel Man<sub>6-10</sub>GlcNAc<sub>2</sub> processing intermediates on secreted invertase. *J. Biol. Chem.* **268**: 12104–12115.
- Wood, P.J., and Fulcher, R.G.** (1983). Dye interactions: A basis for specific detection and histochemistry of polysaccharides. *J. Histochem. Cytochem.* **31**: 823–826.
- Xu, J.R., and Hamer, J.E.** (1996). MAP kinase and cAMP signaling regulate infection structure formation and pathogenic growth in the rice blast fungus *Magnaporthe grisea*. *Genes Dev.* **10**: 2696–2706.
- Xu, J.R., Staiger, C.J., and Hamer, J.E.** (1998). Inactivation of the mitogen-activated protein kinase Mps1 from the rice blast fungus prevents penetration of host cells but allows activation of plant defense responses. *Proc. Natl. Acad. Sci. USA* **95**: 12713–12718.
- Xue, M., et al.** (2012). Comparative analysis of the genomes of two field isolates of the rice blast fungus *Magnaporthe oryzae*. *PLoS Genet.* **8**: e1002869.
- Yang, J., Kong, L., Chen, X., Wang, D., Qi, L., Zhao, W., Zhang, Y., Liu, X., and Peng, Y.L.** (2012). A carnitine-acylcarnitine carrier protein, MoCrc1, is essential for pathogenicity in *Magnaporthe oryzae*. *Curr. Genet.* **58**: 139–148.
- Yang, J., Zhao, X., Sun, J., Kang, Z., Ding, S., Xu, J.R., and Peng, Y. L.** (2010). A novel protein Com1 is required for normal conidium morphology and full virulence in *Magnaporthe oryzae*. *Mol. Plant Microbe Interact.* **23**: 112–123.
- Yoshida, K., Saitoh, H., Fujisawa, S., Kanzaki, H., Matsumura, H., Yoshida, K., Tosa, Y., Chuma, I., Takano, Y., Win, J., Kamoun, S., and Terauchi, R.** (2009). Association genetics reveals three novel avirulence genes from the rice blast fungal pathogen *Magnaporthe oryzae*. *Plant Cell* **21**: 1573–1591.
- Zerangue, N., Schwappach, B., Jan, Y.N., and Jan, L.Y.** (1999). A new ER trafficking signal regulates the subunit stoichiometry of plasma membrane K(ATP) channels. *Neuron* **22**: 537–548.
- Zufferey, R., Knauer, R., Burda, P., Stagljar, I., te Heesen, S., Lehle, L., and Aebi, M.** (1995). STT3, a highly conserved protein required for yeast oligosaccharyl transferase activity *in vivo*. *EMBO J.* **14**: 4949–4960.