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Phosphoproteome analysis links protein phosphorylation to cellular remodeling and metabolic adaptation during *Magnaporthe oryzae* appressorium development

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Abstract

The rice pathogen, *Magnaporthe oryzae*, undergoes a complex developmental process leading to formation of an appressorium prior to plant infection. In an effort to better understand phosphoregulation during appressorium development, a mass spectrometry based phosphoproteomics study was undertaken. A total of 2924 class I phosphosites were identified from 1514 phosphoproteins from mycelia, conidia, germlings and appressoria of the wild type and a protein kinase A (PKA) mutant. Phosphoregulation during appressorium development was observed for 448 phosphosites on 320 phosphoproteins. In addition, a set of candidate PKA targets was identified encompassing 253 phosphosites on 227 phosphoproteins. Network analysis incorporating regulation from transcriptomic, proteomic and phosphoproteomic data revealed new insights into the regulation of the metabolism of conidial storage reserves and phospholipids, autophagy, actin dynamics and cell wall metabolism during appressorium formation. In particular, protein phosphorylation appears to play a central role in the regulation of autophagic recycling and actin dynamics during appressorium formation. Changes in phosphorylation were observed in multiple components of the cell wall integrity pathway providing evidence that this pathway is highly active during appressorium development. Several transcription factors were phosphoregulated during appressorium formation including the bHLH domain transcription factor MGG_05709. Functional analysis of MGG_05709 provided further evidence for the role of protein phosphorylation in regulation of glycerol metabolism and the metabolic reprogramming characteristic of appressorium formation. The data presented here represent a comprehensive

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Associated Content

Supporting Information contains 10 Supplementary Tables and 4 Supplementary Figures. This material is available free of charge via the internet at <http://pubs.acs.org>

Author Contributions

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Notes

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investigation of the *M. oryzae* phosphoproteome and provide key insights on the role of protein phosphorylation during infection-related development.

Abstract Graphic

Keywords

Magnaporthe oryzae; quantitative phosphoproteomics; appressorium formation; network analysis; transcription factors

Introduction

Post translational modifications (PTMs) influence protein activity, stability, localization and interactions with other proteins and cellular components via the targeted addition of defined chemical groups to proteins¹. The ability to alter protein function via PTM(s) offers an additional level of cellular regulation beyond control of transcription and translation. The number of known amino acid modifications exceeds three hundred² with phosphorylation of serine, threonine or tyrosine being the most abundant PTM with respect to experimentally identified sites³. Recent evidence indicates that more than half of the *Saccharomyces cerevisiae* proteome can be phosphorylated⁴. Given the prevalence of protein phosphorylation, the central role it plays in the regulation of cellular signaling pathways, and its ability to influence protein-protein interactions, a thorough understanding of global protein phosphorylation dynamics is critical to understanding how PTMs impact protein function and cellular regulation.

Magnaporthe oryzae, a filamentous fungus of the phylum *Ascomycota*, is the casual agent of rice blast disease, a disease responsible for the destruction of millions of hectares of rice each year and found in all regions of the world where rice is grown^{5, 6}. The infection of rice by *M. oryzae* is characterized by a fungal developmental sequence that begins after contact of an asexual conidium with the leaf surface. Following germination of the conidium, perception of physical and chemical cues by the fungus triggers development of a dome-shaped penetration structure known as the appressorium⁷. Appressoria generate tremendous internal turgor pressure via the accumulation of compatible solutes which facilitates

mechanical penetration of the plant leaf surface ⁷. Subsequent to penetration, colonization of the first epidermal layers of the leaf occurs. Following invasion of the leaf, the fungus maintains a biotrophic relationship with the plant for a short period of time prior to entry into a necrotrophic phase characterized by the destruction of plant tissues and production of asexual conidia by the fungus leading to spread of the disease ⁸.

Pathogenic development of *M. oryzae* on the leaf surface requires the recognition of external cues followed by transmission of these signals to the nucleus triggering commitment to a morphogenetic sequence resulting in the production of an appressorium. Critical to this process are multiple phosphorylation dependent signaling pathways which are indispensable for pathogenicity and include the Pmk1 mitogen-activated protein (MAP) kinase, cyclic AMP dependent protein kinase A and Pkc1-Mps1 MAP kinase pathways ⁹. *MoPmk1* is homologous to the *S. cerevisiae* Fus3/Kss1 pheromone signaling MAP kinases and the Pmk1 MAP kinase pathway is required for appressorium formation as well as invasive hyphal growth in the plant ¹⁰. Orthologs of *MoPmk1* are required for pathogenicity in other phytopathogenic fungi ¹¹. It has been proposed that the Pmk1 pathway is involved in both cell cycle control and polarized cell growth ^{12, 13}. Additional components of the Pmk1 pathway have been identified ^{12, 14}. Downstream targets of the Pmk1 signaling pathway include three transcription factors, *MoMst12*, *MoMcm1* and *MoSfl1*, all of which are required for invasive hyphal growth and surface penetration in the case of *MoMst12* ¹⁵⁻¹⁸. Finally a number of studies have examined Pmk1 dependent transcriptional regulation and have concluded that the Pmk1 signaling pathway is central regulator of global gene expression during appressorium development ¹⁹⁻²¹.

The cyclic-AMP dependent protein kinase A (CpkA) is required for initial perception of the leaf surface, development of functional appressoria and timely mobilization of storage reserves in conidia including glycogen and lipid bodies ^{9, 22-24}. Constitutive activation of the regulatory subunit of protein kinase A, *MoSum1*, triggers appressorium formation in the absence of appropriate stimuli ²⁵. The adenylate cyclase, *MoMac1*, responsible for production of cAMP from ATP is indispensable for appressorium formation and pathogenicity ²⁶. The phosphodiesterase, *MoPdeH*, is a negative regulator of appressorium formation via its influence on cAMP levels and is essential for the development of invasive hyphae *in planta* ^{27, 28}. Recently, two transcriptional regulatory proteins, *MoSom1* and *MoCdtf1*, were identified as downstream targets of the PKA signaling pathway with loss of either gene resulting in defects in vegetative growth and loss of pathogenicity [29]. In addition, the APSES family transcriptional regulator, *MoMst1*, involved in the mobilization of lipid and glycogen reserves during appressorium formation ²⁹ was demonstrated to interact strongly with *MoSom1* ³⁰.

The Pkc1-Mps1 cell wall integrity pathway is analogous to the Pkc1-Slt2 pathway of *S. cerevisiae* and is conserved in filamentous fungi ¹¹. Components of the pathway including *MoMck1*, *MoMkk1*, and *MoMps1* are necessary for cell wall integrity, appressorial function and invasive growth in the plant ³¹⁻³³. Downstream of the pathway are two transcription factors, *MoMig1* and *MoSwi6*, that are required for invasive growth (*MoMig1*) and the response to cell wall and oxidative stress (*MoSwi6*) ^{34, 35}.

Although significant progress has been made identifying components of the Pmk1, CpkA and Pkc1-Mps1 signaling pathways and how leaf surface cues are transmitted into these pathways, their role in the regulation of global phosphorylation dynamics remains largely unexplored. In addition, the predicted *M. oryzae* genome encodes more than eighty protein kinases^{36, 37} most of which are uncharacterized. The field of mass spectrometry based phosphoproteomics has grown considerably in recent years with development and optimization of strategies for enrichment and detection of phosphopeptides. With regards to fungal species, multiple phosphoproteomics datasets are available for the yeast, *S. cerevisiae*^{4, 38-46}. In addition to *S. cerevisiae*, phosphoproteomics data sets are available for the single celled yeasts, *Schizosaccharomyces pombe*^{38, 47} and *Candida albicans*³⁸, the basidiomycete, *Cryptococcus neoformans*⁴⁸ and the filamentous ascomycetes, *Fusarium graminearum*^{49, 50}, *Aspergillus nidulans*⁵¹, *Alternaria brassicicola* and *Botrytis cinerea*⁵²⁻⁵⁵. Recently, phosphoproteome data from six life stages of the Oomycete *Phytophthora infestans* was published⁵⁶. However, studies in fungi other than *S. cerevisiae* primarily focus on phosphosite identification from a single experimental condition. In this study, we report a mass spectrometry-based identification of 1514 *M. oryzae* phosphoproteins from mycelia, conidia, germinated conidia and appressoria for wild type *M. oryzae* and a *cpkA* mutant. We also provide direct evidence that a key phosphoregulated transcription factor involved in regulating glycerol metabolism is required for appressorium function. The results of this study represent the first examination of the global phosphoproteome of *M. oryzae* and provide an overview of phosphoproteome dynamics during the initial stages of infection-related development.

Materials and Methods

Sample preparation for proteome analysis

M. oryzae wild type strain 70-15 and a *cpkA* mutant⁵⁷ were routinely maintained on minimal medium agar⁵⁷ at 25°C. Mycelial samples were harvested from 50 mL minimal medium broth cultures grown in 250 mL shake flasks for five days at 25°C and flash frozen in liquid nitrogen. Conidial samples were harvested from eight day old minimal medium plates by filtration through miracloth and aliquots of 1.8 million conidia were centrifuged for five minutes at 12,000 × g and 4°C after which the supernatants were removed and the pellets were frozen in liquid nitrogen. Additional aliquots of 1.8 million conidia were germinated on the hydrophilic surface of 205 × 110 mm GelBond® (Lonza, Rockland, ME) sheets in 16 mL of H₂O. Following eight hours of germination, germinated conidia were harvested from three sheets of GelBond® as described previously⁵⁷. Additional eight hour germinated samples were treated with 10 µM 1,16-hexadecanediol (wild type and *cpkA* mutant strains) or mock treated with ethanol (wild type only) via the addition of 4 mL of H₂O spiked with 20 µL of a 10 mM 1,16-hexadecanediol solution (in 100% ethanol) or 20 µL of 100% ethanol. A total of three biological replicates for each condition were produced with each replicate being a pool of samples from six sheets of GelBond® as required to generate sufficient material for phosphopeptide enrichment. Protein harvests were performed exactly as described previously^{57, 58} with the addition of a PhosSTOP phosphatase inhibitor cocktail (Roche, Mannheim, Germany) to the cell lysis buffer per the manufacturer's instructions. Protein concentrations were determined using a bicinchoninic acid assay. A

total of 250 µg of protein were trypsin digested using the FASP procedure as described previously⁵⁸.

Phosphopeptide enrichment

Phosphopeptides were enriched using an Iron-IMAC resin in combination with TiO₂ beads. The Iron-IMAC resin was prepared by washing NTA Agarose (Qiagen, Valencia, CA) once with 1% acetic acid followed by incubation with 100 mM FeCl₃ in 1% acetic acid for four hours with rotation. The iron-IMAC resin (100 µL) was loaded in a 200 µL gel-loading tip fitted with a 10 µm filter paper plug. All washes were performed by forcing the solution through the resin using a syringe fitted to the top of the gel-loader tip. The resin was first washed twice with 100 µL volumes of 2% acetic acid. Total tryptic peptides were then passed over the resin and the flow through collected and reloaded on the column after which the second flow through was collected and set aside for the TiO₂ enrichments described below. The iron-IMAC resin was then washed twice with 100 µL 2% acetic acid, twice with 100 µL of a solution of 74% 100 mM NaCl, 25% acetonitrile and 1% acetic acid, and twice with 100 µL of molecular biology grade H₂O. Phosphopeptides were eluted with two 100 µL washes of 5% NH₄OH and acidified with 30 µL of formic acid prior to storage at -80°C.

To perform the TiO₂ enrichments, 1.5 mg of TiO₂ beads (10 µM Titansphere, GL Sciences, Torrance, CA) were resuspended in a solution containing 2% acetic acid and 200 mg/mL lactic acid and transferred to a 200 µL gel-loading tip as described above. All washes of TiO₂ beads were performed by centrifugation at 1000 × g in pipette tip boxes fitted with a microcentrifuge rack to accommodate the insertion of 1.7 mL microcentrifuge tubes under each gel-loader tip. The TiO₂ beads were washed twice with 100 µL of 2% acetic acid and 200 mg/mL lactic acid and the flow through from the iron-IMAC enrichments was passed through the TiO₂ beads twice. The TiO₂ beads were washed twice with 100 µL 2% acetic acid and 200 mg/mL lactic acid, twice with 100 µL of a solution of 74% 100 mM NaCl, 25% acetonitrile and 1% acetic acid, and twice with 100 µL of molecular biology grade H₂O. The peptides were eluted and acidified as described above and then subjected to a second round of TiO₂ enrichment using the same protocol. Prior to MS/MS analysis the phosphopeptides from the iron-IMAC and TiO₂ enriched samples were pooled, dried to near completion and resuspended in 25 µL mobile phase A.

NanoLC MS/MS analysis

Samples were injected using a Thermo Scientific Easy nLC II (Thermo Scientific, San Jose, CA) integrated with an Eksigent cHiPLC-Nanoflex (Eksigent, Dublin, CA) operated in a trap and elute configuration. Nano cHiPLC columns were packed by the manufacturer (Eksigent) with ChromXP C18-CL (3 µm, 120 Å) to 15 cm × 75 µm i.d. for the analytical column and 0.5 mm × 200 µm i.d. for the trapping column. LC solvents were purchased from Burdick and Jackson (Muskegon, MI). Mobile phase A and mobile phase B were 98 % water, 2 % acetonitrile, 0.2 % formic acid and 98 % acetonitrile, 2 % water, and 0.2 % formic acid, respectively. The gradient was initiated at 5 % B and increased to 40 % B over the next 166 min before rising to 95 % B and re-equilibrating at 5 % B for a total elution time of 180 min.

Mass measurements were made using a quadrupole Orbitrap mass spectrometer (Q Exactive, Thermo Scientific) in data-dependent mode using a top-12 method. Resolving power was set at 70,000_{FWHM} at 200 *m/z* for MS acquisition and 17,500_{FWHM} at 200 *m/z* for MS/MS acquisition. AGC settings for MS and MS/MS were 1E6 and 2E4, respectively. Maximum ionization time was 60 ms and 250 ms for MS and MS/MS, respectively. Higher-energy collision dissociation (HCD) was employed using 27 normalized collision energy, and precursor ions were accumulated using an isolation width of 2 *m/z*. Charge state screening was utilized and unassigned and 1+ charge states were rejected for MS/MS. Dynamic exclusion was set to 30 s.

Data analysis

The .RAW files from the MS/MS analysis were searched against the *M. oryzae* genome version 8 (12,827 genes, *Magnaporthe* comparative Sequencing Project, Broad Institute of Harvard and MIT (<http://www.broadinstitute.org/>)) using the Andromeda search engine of MaxQuant version 1.3.05 [54,55] with the reverse database function enabled. Search parameters included a two missed trypsin cleavage tolerance, a precursor ion tolerance of 6 ppm, and an MS/MS tolerance of 0.02 Da. Carbamidomethylation of cysteine was set as a fixed modification and variable modifications allowed included methionine oxidation, N-terminal acetylation, deamidation of glutamine or asparagines and phosphorylation of serine, threonine and tyrosine. Protein, peptide and site false discovery rates were set to 1% in MaxQuant with the match between runs function enabled. Phosphorylation site information was obtained from the Phospho (STY) Site output file of MaxQuant. Class I sites were defined as having a localization probability greater than or equal to 0.75 and a score difference greater than or equal to five as described previously ^{59, 60}. For quantification, phosphosite intensities from MaxQuant were first log transformed (log base 2) and then normalized by sample median subtraction in the Perseus module of MaxQuant. Phosphosites were considered regulated at a 2 fold cutoff with a t-test p-value less than 0.05 and a requirement that the site be observed in at least two of three replicates for each of the conditions considered in a pairwise comparison. The total number of t-tests performed after filtering in the pairwise comparisons of selected samples as discussed below was 578. In addition, phosphosites were considered regulated if they were observed in all three replicates of one condition and absent in all replicates of a second condition for a given pairwise comparison. All mass spectrometry proteomics data obtained in this study have been deposited in the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository ⁶¹ with the dataset identifier PXD001389. [Reviewer access: username – reviewer55277@ebi.ac.uk; password – uwFUskEw] All mass spectrometry spectra are shown in Supplementary Figure 1.

Network Analysis

Generation of protein interaction networks was accomplished by first extracting *S. cerevisiae* orthologs of *M. oryzae* (phospho)proteins of interest from an OrthoMCL ⁶² analysis of 40 fungal genomes (Sailsbery and Dean, unpublished data). In instances where no *S. cerevisiae* orthologs for a given *M. oryzae* protein were identified, reciprocal best BLAST hits were manually identified and included as orthologs where appropriate. These orthologs were used to extract protein interaction networks from the STRING database (version 9.1) ⁶³.

Experimental evidence was used as the sole prediction method at a confidence value of 0.7 for the initial phosphoprotein interaction network. Extended sub networks were generated in STRING via submission of selected sets of phosphoproteins and subsequent acquisition of the highest scoring interacting proteins using all evidence as the prediction method at a confidence value of 0.7. All networks were visualized in Cytoscape (version 3.0.1) ⁶⁴.

Targeted mutagenesis and complementation in *M. oryzae*

To generate a deletion mutant in MGG_05709, a gene replacement cassette was constructed as described previously ⁶⁵ using adaptamer mediated PCR to place a hygromycin resistance gene between sequences flanking MGG_05709. The primers MGG_05709 mut F (5'-CTGTCTACTTCACCGCCATCTT-3') and MGG_05709 mut LF_R (5'-CACGGCGCGCCTAGCAGCGGCTCAACTGTTTTGCGCCGAT-3') or MGG_05709 mut RF_F (5'-GCAGGGATGCGGCCGCTGACGACTTGAAGTCTAGCGTTG-3') and MGG_05709 mut R (5'-TTGTCGGATCTGTGAGTTTATG-3') were used to PCR amplify 5' and 3' regions, respectively, flanking MGG_05709 with the adaptamer sequences underlined. The two resulting PCR products were combined with a hygromycin resistance gene PCR amplified with the forward HPHF and reverse HPHR primers ⁶⁵ in a single PCR and amplified as a single cassette with the primers MGG_05709 mut F2 (5'-TGTGGTTGCAACGATCTTCAGC-3') and MGG_05709 mut R2 (5'-CCATCAACTACTCAGACCACATT-3'). This PCR product was cloned into the pGEMT-Easy vector (Promega, Madison, WI) and transformed into *E. coli* DH5 α . The gene replacement cassette was then transformed into *M. oryzae* protoplasts as previously described ⁶⁶. Mutants containing the correct replacement were identified by PCR and Southern blots (data not shown).

Complementation of the MGG_05709 mutant was performed by PCR amplification of the entire predicting coding sequence of MGG_05709 including 5' and 3' UTRs as well as 1.2 kb of promoter sequence using the primers MGG_05709 Comp F1 (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTATACAAAGGCCCTCCCACCA-3') and MGG_05709 Comp R1 (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTCACCTCACTTGAACGTCTTC-3') with *attB* sites underlined. The resulting PCR product was then cloned via a Gateway® BP recombination reaction into a pDONR221 vector containing the BAR resistance gene for selection in *M. oryzae*. Transformants containing the complementation construct were identified by PCR.

Appressorium, cytorrhysis and pathogenicity assays

To assess appressorium formation, conidia from eight day old minimal medium plates were germinated on the hydrophilic surface of GelBond® film in the presence of 10 μ M 1,16-hexadecanediol and incubated for 24 hr before checking for appressorium development. Cytorrhysis assays were performed by germinating conidia in 300 μ L of H₂O on the hydrophobic surface of GelBond® film and incubating for 48 hr to elicit appressorium formation. Mature appressoria were analyzed for internal glycerol content by gently removing the drop of water on the film and replacing it with 300 μ L of an appropriate glycerol solution and checking for collapse of appressoria fifteen minutes later.

Pathogenicity assays were performed using conidia collected from eight day old minimal medium plates and adjusted to the indicated conidial concentration with H₂O for barley inoculation or 0.1% Tween 20 for rice inoculation. Ten µL drops of the conidial solution were spotted onto detached seven day old leaves of the barley cultivar Robust or three week old leaves of the rice cultivar WIR1889 with mock inoculated controls included. Lesions were observed six days after inoculation.

Results and Discussion

Identification of *M. oryzae* phosphoproteins from distinct tissue types

As a first step in understanding how protein phosphorylation and other post translation modifications influence the pathogenic development of *M. oryzae* on host plants, a phosphoproteomics study was performed to characterize protein phosphorylation in multiple cell types, examine changes in phosphorylation dynamics during appressorium formation, and investigate the role of the cAMP-dependent protein kinase, *MoCpkA*, in appressorium formation. To achieve this, samples for phosphoproteome analysis were collected from liquid minimal medium grown mycelium, ungerminated conidia and a developmental time course encompassing both conidial germination and appressorium formation. Generation of germinated conidia and appressorial samples was performed by germinating conidia (1.8×10^6) on the hydrophilic surface of GelBond® film for a period of eight hours after which the germings were treated with 10 µM 1,16-hexadecanediol to trigger appressorium formation. The developmental progression of appressorium formation following treatment is shown in Figure 1A. At 60 minutes post treatment hooking and swelling of the germ tube is evident and within two hours an immature appressorium has formed which fully matures by 21 hours. Samples for phosphoproteome analysis were collected immediately prior to treatment (time = 0) and at 30 and 90 minutes post treatment. A mock treated control in which germ tube elongation continued through the time course analyzed was included for comparison. Samples were also collected for a *cpkA* mutant treated with 1, 16-hexadecanediol in a manner identical to the wild type as well as from *cpkA* mycelia and conidia for identification of the *MoCpkA* dependent phosphoproteome. As indicated in Figure 1A, the *cpkA* mutant does not generate an observable response to 1, 16-hexadecanediol within 90 minutes of treatment. After 24 hours, greater than 90% of wild type germinated conidia formed appressoria whereas fewer than 20% of *cpkA* conidia formed appressoria (Figure 1B).

Total protein was collected from three biological replicates for each sample using conditions optimized in previous studies of the *M. oryzae* proteome^{57, 58} and 250 µg of total protein was trypsin digested and subjected to dual phosphopeptide enrichment as illustrated in Figure 1C. Phosphopeptides were first enriched using an iron based immobilized metal affinity chromatography (Fe-IMAC) followed by two rounds of sequential enrichment using TiO₂ chromatography on the IMAC flow through, after which both the Fe-IMAC and TiO₂ enrichments were pooled into a single sample. The phosphopeptide enriched samples were analyzed by nanoLC-MS/MS on a Q-Exactive hybrid mass spectrometer and data was processed in MaxQuant.

A total of 1514 unique phosphoproteins were identified from 4894 phosphorylation sites including a subset of 2924 class I phosphorylation sites where the localization of the phosphorylation site was confidently assigned as defined previously [56,57]. The complete list of phosphosite identifications from the Phospho(STY) output file generated by MaxQuant is included here as Supplementary Table 1. The distribution of phosphosites by amino acid residue follows a pattern typical of eukaryotic organisms with 84.5% on serine, 15.1% on threonine and 0.3% on tyrosine (Supplementary Figure 2A). A total of 10 Class I phosphosites were observed on tyrosine residues of which six were observed on CMGC family kinases including phosphotyrosine in a TEY motif on PMK1. Interestingly, functional tyrosine kinases have not yet been identified in filamentous fungi although evidence for tyrosine phosphorylation has been reported in phosphoproteome analysis of other fungi in addition to that reported here⁵²⁻⁵⁵. Nearly one half of the phosphoproteins contained a single phosphorylation site and phosphoproteins containing one to three class I phosphosites accounted for the vast majority of phosphoproteins detected (Supplementary Figure 2B). The distribution of phosphorylation sites amongst the samples and two strains analyzed is presented in Figures 2A-B. A core set of 767 phosphosites were identified in all the wild type tissue types sampled (Figure 2A). An additional 550 wild type phosphosites were shared amongst conidia, germlings and 1,16-hexadecanediol treated germlings (Figure 2A). Fifty nine phosphosites were only observed in the wild type strain following 1,16-hexadecanediol treatment indicating a possible role in the development of the appressorium and will be discussed further below (Figure 2A). In a comparison of the two strains analyzed, 376 and 109 phosphosites were identified in only the wild type and *cpkA* strains respectively (Figure 2B). Included in the 1514 phosphoproteins identified here are 571 proteins not previously identified in recent global proteome analyses of *M. oryzae* [52,53] bringing the total number of protein identifications for the *M. oryzae* proteome to 4111 proteins which represents 31.6% of the predicted protein coding genes in *M. oryzae* (*Magnaporthe* comparative Sequencing Project, Broad Institute of Harvard and MIT (<http://www.broadinstitute.org>)).

Gene Ontology analysis of identified phosphoproteins

The 1514 identified phosphoproteins were subjected to Gene Ontology (GO) analysis using the BLAST2GO application⁶⁷ and those GO categories overrepresented in the phosphoproteome relative to the genome are presented in Figure 2C. Overrepresented in the phosphoproteome are cellular components related to the nucleus (nucleoplasm, GO: 0005654; chromosome, GO: 0005694) and cellular transport (Golgi apparatus, GO:0005794; endosome, GO:0005768; microtubule organizing center, GO:0005815) as well as biological processes and molecular functions related to signal transduction and regulation (e.g. protein kinase activity, GO:0004672; transcription regulator activity, GO:0030528), cellular structure (e.g. cytoskeleton organization, GO:0007010; actin binding, GO:0003779) and the cell cycle (cell cycle, GO:0007049). These results indicate that the phosphoproteins identified in *M. oryzae* represent elements of signaling pathways, cellular trafficking pathways, and cytoskeletal structure. As such, many of the identified phosphosites will be involved in the regulation and function of these processes and have important roles in the developmental biology of *M. oryzae* as discussed below.

Conservation of phosphorylation sites with *S. cerevisiae*

To explore the level of phosphosite conservation between *M. oryzae* and *S. cerevisiae*, a BLAST search of the 2924 *M. oryzae* Class I phosphosites against the *S. cerevisiae* proteome was performed. A 21 amino acid sequence window centered on the identified phosphosite was used as a query and matches were retained at an E-value threshold of 1.0E-04 with a requirement that the best *S. cerevisiae* BLAST hit belong to an ortholog of the *M. oryzae* phosphoprotein in question. In cases where multiple matches met the E-value threshold, the ortholog with the lowest E-value was reported. Based on these criteria, a total of 76 *M. oryzae* phosphosites were retained (Supplementary Table 2). For 60 of these *M. oryzae* phosphosites, there was conservation of the *M. oryzae* phosphosite residue between the two species. In the remaining 16 instances, the residue phosphorylated in *M. oryzae* was not strictly conserved in *S. cerevisiae*. However, six of these 16 sites contain a different amino acid residue in *S. cerevisiae* that is phosphorylatable and in the correct location. Therefore, 66 *M. oryzae* phosphosites on 54 phosphoproteins were either conserved or retained a phosphorylatable residue at the correct location in *S. cerevisiae*. The Phosphogrid database ⁶⁸ provides evidence for phosphorylation of 49 of these *S. cerevisiae* sites. An examination of these 49 conserved phosphosites revealed that 14 sites reside on protein kinases of which 13 are located in the predicted activation loop of the kinase in both species. These phosphosites reside on highly conserved protein kinases (*MoPmk1*, *MoCpkA*, *MoOsm1* etc.) involved in important cellular processes and as such, the phosphosites critical for protein function have been retained. The remaining conserved protein kinase phosphorylation site is Y15 on MGG_01362, an ortholog of *S. cerevisiae* cyclin dependent kinase, CDC28. Phosphorylation of the corresponding Y19 site on *S. cerevisiae* CDC28 inhibits its kinase activity and serves as one means of regulating CDK activity and progression through the cell cycle [65]. The remaining conserved phosphosites predominantly reside on conserved metabolic enzymes, ribosomal components and chaperone proteins. The results of this analysis indicates that strict phosphosite conservation is limited between these two species or that sufficient evolutionary distance exists between the two species to preclude identification of phosphosite conservation at the level of primary amino acid sequence.

Phosphorylation motif analysis of identified phosphosites

The identification of protein kinases active in *M. oryzae* under the conditions investigated in this study is of particular interest as a means of further elucidating the roles of phosphorylation-dependent signaling pathways in the development of appressoria. As a first step towards this objective, an identification of phosphorylation motifs from the set of 2924 class I phosphosites was performed using the Motif-X algorithm ⁶⁹. A search for motifs was performed on a thirteen amino acid window centered on each phosphosite using the *M. oryzae* version 8 proteome as a background with a significance threshold set at 1E-06 and requiring that each motif be found in at least one percent of the total sites identified for a given phosphorylated residue. The list of motifs identified in entire set of phosphosites is presented in Supplementary Table 3. In total, 21 phosphoserine and six phosphothreonine motifs were identified and encompass 2280 of the identified 2924 class I phosphosites. To examine the biological roles of these phosphorylation motifs, the phosphoproteins associated with each of the most abundant motifs (present in at least five percent of the total sites for a

given phosphorylated residue) were subjected to GO enrichment analysis and the results for each enriched motif are presented in Table 1. The GO analysis revealed distinct but overlapping sets of GO terms associated with each of these motifs. As expected, some GO terms were associated with many motifs (e.g. protein kinase activity, GO:0004672; signal transduction, GO:0007165; enzyme regulator activity, GO:0030234). However, some GO terms are specifically associated with individual motifs. For example, seven phosphoproteins containing the motif RxxSP have GO terms associated with the cytoskeleton (cytoskeleton, GO:0005856; cytoskeletal protein binding, GO:0008092). This motif was previously identified in fly, mouse, human and *Arabidopsis* although information regarding a protein kinase targeting this motif is lacking^{70,71}. Five of these phosphoproteins (MGG_11773, Bud6 ortholog; MGG_04116, Bbc1 ortholog; MGG_12839, Pan1 ortholog; MGG_05315, gelosin-like and MGG_06726, *MoSep4*) are predicted to be involved in regulation of the actin cytoskeleton and one more (MGG_02875, Kel1-like) is likely involved in establishing cell morphology via regulation of mitosis. Interestingly, *MoSep4*, is one of five core septins demonstrated to play a role in organization of the F-actin network at the appressorium pore prior to penetration of the leaf surface⁷². MGG_05315 and three additional phosphoproteins (MGG_06180, End3 ortholog; MGG_06358, Abp1 ortholog; and MGG_06389, Crn1 ortholog) contain a PxSP motif identified as potential MAPK target⁷⁰ and are also predicted to be involved in regulation and organization of the actin cytoskeleton. The identification of two distinct phosphorylation motifs targeting multiple proteins involved in regulation of the actin cytoskeleton will aid in identification of protein kinases important for controlling actin dynamics during both appressorium formation and penetration of the host plant.

Of particular interest to this study are 195 phosphosites that may be *MoCpkA* substrates including 146 RRxS sites, 41 RKxS sites and 8 RRxT sites which contain a PKA consensus phosphorylation motif⁷³ and are listed in Supplementary Table 4. Of these 195 sites, 26 were identified solely in the wild type. Overlapping target site specificity is a common feature of AGC family protein kinases⁷⁴ and therefore detection of a phosphosite in the *cpkA* mutant does not preclude it from being a target of protein kinase A. A number of GO terms were associated with the 195 RRXS sites including the vacuole GO term (GO:0005773), protein kinase activity (GO:0004672), and signal transduction (GO:0007165). Proteins associated with the vacuole GO term include two putative vacuolar amino acid transporters (MGG_08827 and MGG_04698). Included in the latter categories are proteins involved in cellular signaling including twelve protein kinases (ten serine/threonine kinases and two histidine kinases), the regulatory subunit of protein kinase A, *MoSum1* (MGG_07335), a regulator of G protein signaling, *MoRgs5* (MGG_08735), two putative guanine nucleotide exchange factors (MGG_01695 and a Sec7 ortholog, MGG_14173) and an ortholog of the *Neurospora crassa* putative transcription factor, White collar-1 (MGG_03538). The ten serine/threonine protein kinases include orthologs of the *S. cerevisiae* protein kinases *ScRim15* (MGG_00345), *ScPsk2* (MGG_05220), *ScPrr1* (MGG_06760) and *ScArk1/Prk1* (MGG_11326). *ScRim15* is negatively regulated by PKA phosphorylation and is involved in regulation of the cell cycle in a nutrient dependent fashion⁷⁵. *ScPrr1* is a negative regulator of the *S. cerevisiae* pheromone responsive MAPK signaling pathway functioning downstream of the MAPK, *ScFus3*⁷⁶. This pathway has been co-opted in *M. oryzae* for regulation of appressorium formation pointing towards a possible

role for MGG_06760 in regulation of appressorium formation. The protein kinase, *ScPsk2*, phosphorylates both glycogen synthase and UDP-glucose pyrophosphorylase in *S. cerevisiae*⁷⁷. Phosphorylation of UDP-glucose pyrophosphorylase results in a change in its localization and directs glucose towards synthesis of glucans at the cell wall⁷⁸. Finally, *ScArk1* and *ScPrk1*, are *S. cerevisiae* protein kinases that target multiple components of the yeast endocytotic machinery and regulate actin dynamics in relation to the endocytotic process⁷⁹. The *ScArk1* and *ScPrk1* kinases have a target specificity characterized by the motif L(I)XXQXTG⁷⁹. A QXTG motif was observed 11 times on nine proteins in the *M. oryzae* phosphoproteome (Supplementary Table 3) and in seven instances, L or I was observed at the -5 position. Five of the nine QXTG motif containing proteins are predicted to associate with the actin skeleton and three are predicted to be involved in endocytosis.

An additional 376 phosphosites were identified only in the wild type strain as indicated in Figure 2B. A motif analysis of these 376 wild type specific phosphosites identified 5 pS (RXXS, SP, SDD, SXSE, SXSE) motifs and 1 pT motif (TP), all of which were identified in the analysis of entire global phosphoproteome presented in Supplementary Table 3. However, the motif RXXS is overrepresented in the 376 wild type specific phosphosites relative to the global phosphoproteome (Fisher's exact test, $p < 0.0001$). Protein kinase A is also known to phosphorylate substrates with a RXXS/T motif⁷³. Combining the 195 PKA consensus sites with the RXXS/T sites from the 376 wild type specific sites results in a set of 253 phosphosites on 227 proteins which are candidate *MoCpkA* targets (Supplementary Table 4). Forty eight of these phosphosites demonstrated a change in abundance during appressorium formation as described below. The AGC subfamily of protein kinases including PKA, show a phosphosite target preference for basic amino acids in the -3 or -2 position. Although a consensus motif for PKA has been identified as R-R/K-X-S/T, phosphorylation of sites with deviations from this motif is well documented. In addition, the *M. oryzae* genome is predicted to encode at least 21 members of the AGC protein kinase subfamily and an overlap in substrate specificity is expected due to the preference of similar target motifs for the family. However, the set of candidate PKA target sites identified in this study presents an excellent point from which to start a detailed examination of *MoCpkA*-dependent phosphorylation. In particular, the candidate PKA targets provide connections to cAMP signaling, MAPK signaling, Ca^{2+} signaling, phosphoinositide signaling, actin and septin dynamics, autophagy, phospholipid metabolism, glycogen metabolism, trehalose metabolism and cell wall biosynthesis. Each of these processes plays a role in *M. oryzae* appressorium development and is therefore critical to its success as a pathogen. Discerning which of these phosphosites are direct targets of *MoCpkA* represents the next step in the study of cAMP signaling during infection-related development in *M. oryzae*.

Phosphosite regulation during appressorium formation

To assess protein phosphorylation dynamics during appressorium development we sought to identify those phosphosites unique to or changing in abundance during wild type appressorium development as well as those that are *MoCpkA* responsive. To accomplish this, five sets of phosphosites were generated. The first set is 59 phosphosites that were only observed in the wild type strain following 1,16-hexadecanediol treatment as described above (Figure 2A, Supplementary Table 5). The second and third sets involve comparison of the

1,16-hexadecanediol treated and untreated wild type samples. The second set includes those sites that were two fold up- or down-regulated (t-test p-value < 0.05) at either 30 or 90 minutes post treatment as described in the materials and methods (Supplementary Table 6). The third set includes sites that were identified in all three biological replicates of one condition for either time point but were unidentified in the other condition at the same time point (Supplementary Table 7). Those identified in three replicates of one condition were considered to be up-regulated relative to the condition in which the phosphosite was not identified. The fourth and fifth sets are identical to the second and third sets but involve comparison of 1,16-hexadecanediol treated wild type and *cpkA* samples (Supplementary Tables 8 and 9). Cumulatively from these five datasets, a total of 448 phosphosites on 320 phosphoproteins showed a change in abundance in response to 1,16-hexadecanediol treatment or the *cpkA* mutation during appressorium development. The biological impact of the observed phosphoregulation is addressed in the following sections.

Multi-omics network analysis—The phosphoproteomics data presented here extends the available ‘omics’ data encompassing appressorium development previously generated by our group at the transcriptome and proteome levels^{57, 58, 65}. As a first step towards a systems level understanding of appressorium development in *M. oryzae*, we sought to incorporate regulation at the transcriptome, proteome and phosphoproteome levels into a series of protein interaction networks that highlight important aspects of appressorium biology. As limited data is available for the generation of protein interaction networks in *M. oryzae*, the wealth of data available for *S. cerevisiae* was utilized to generate these networks. *S. cerevisiae* orthologs for 184 of the 320 phosphoproteins with appressorium regulated phosphosites were identified from which a total of 146 interactions amongst 114 proteins were extracted from the STRING database. The resulting protein interaction network is presented in Supplementary Figure 3. Examination of this network revealed multiple sub networks of phosphoproteins involved in glycerophospholipid metabolism, glycogen and trehalose metabolism, autophagy, actin cytoskeleton dynamics, and components of the cell wall integrity pathway. Using phosphoproteins from Supplementary Figure 3 corresponding to each biological process of interest as input, extended protein interaction networks for *S. cerevisiae* were extracted from the STRING database. The resulting interaction networks including regulation at the transcript, protein and phosphosite levels are presented in Figure 3. Examination of these networks indicates that in some instances regulation is only observed at the level of protein phosphorylation with no or few changes observed at the transcript or protein level as is the case for the networks associated with the actin cytoskeleton, autophagy, and the cell wall integrity pathway. On the other hand, regulation of phospholipid metabolism as well as glycogen and trehalose metabolism occurs at all three levels examined. Each network will be discussed in more detail in the following sections.

Metabolism of conidial storage reserves—The development of an appressorium requires the mobilization of conidial storage reserves, including glycogen, trehalose and lipid bodies, during the development of appressoria, presumably to fuel biosynthetic processes and turgor generation in the developing appressorium²³. Glycogen present as granules in the conidium is degraded during germination and glycogen granules reappear for a short period in immature appressoria²³. This process is delayed in the *cpkA* mutant and

glycogen accumulating in the *cpkA* strain fails to degrade during maturation⁸⁰. *M. oryzae* contains a single glycogen synthase, *MoGSN1* (MGG_07289), which is required for the accumulation of intracellular glycogen but is dispensable for appressorium function and pathogenicity⁸⁰. *MoGsn1* is a highly abundant protein and six class I phosphosites were observed. Two sites S633 and S637, which are conserved in *S. cerevisiae*, were more abundant in 1,16-hexadecandiol treated samples at the thirty minute time point. In *S. cerevisiae* phosphorylation at these sites inactivates the enzyme and mutations in either site increases intracellular glycogen levels^{81, 82}. Also up-regulated at the 30 minute time point were two phosphosites (S634 and T638) on a putative glycogenin glucosyltransferase, MGG_07439, predicted to be involved in initiation of glycogen synthesis. *MoGsn1* and MGG_07439 also both contain multiple phosphosites that are more abundant in the *cpkA* mutant than the wild type during appressorium formation. A possible conclusion from these observations is that *MoGsn1* and potentially MGG_07439 are inactivated by phosphorylation during the early stages of appressorium formation following glycogen degradation during germination. Subsequently, *MoCpkA* regulates directly or indirectly, an unknown phosphatase(s) that targets *MoGsn1* and MGG_07439 for activation by dephosphorylation during appressorium maturation which results in the transient accumulation of glycogen granules observed in immature appressorium. Furthermore, two phosphorylation sites on MGG_09466, an ortholog of *ScGlc8*, were up-regulated at the early time point during appressorium formation. In *S. cerevisiae*, *ScGlc8* can both activate and inhibit the protein phosphatase-1, *ScGlc7*, which is responsible for regulation of multiple cellular processes including glycogen metabolism⁸³. *ScGlc8* activation of *ScGlc7* is dependent upon *ScGlc8*'s phosphorylation by the cyclin dependent kinase, *ScPho85*, which links glycogen metabolism to the cell cycle⁸⁴.

Two proteins involved in the breakdown of glycogen, a glycogen phosphorylase, *MoGph1*, and an amyloglucosidase, *MoAlg1*, are required for mobilization of glycogen and full virulence⁸⁰. Multiple phosphorylation sites were detected on both proteins but no appressorium dependent regulation was observed. *MoGph1* and *MoAlg1* both influence expression of trehalose-6-phosphate synthase, *MoTps1*⁸⁰. *MoTps1* is involved in trehalose biosynthesis and is indispensable for appressorium function and pathogenicity⁸⁵. Phosphorylation of *MoTps1* was detected at a single site at its N terminus with no observed regulation. However, two other proteins of the trehalose-synthase complex *MoTps2*, MGG_03441, and *MoTps3*, MGG_14118, are dephosphorylated during appressorium formation. *MoTps2* and *MoTps3* are orthologs of *ScTps2* and *ScTsl1*, a trehalose-6-phosphate phosphatase and a regulatory subunit of the trehalose-synthase complex. Evidence in *S. cerevisiae* indicates that *Tps3* is phosphorylated by PKA resulting in an inhibition of trehalose biosynthesis⁸⁶ and that components of trehalose-synthase complex are negatively regulated by the RAS-cAMP pathway at the transcriptional level in response to nutrient status⁸⁷. Finally, a role for *MoTps1* in integration of carbon metabolism and nitrogen source utilization via a mechanism involving perception of intracellular NADPH levels distinct from its catalytic activity has been established^{88, 89}. The catalytic activity, but not the NADPH sensor activity of *MoTps1*, is dispensable for pathogenicity⁸⁹. Although neither metabolism of glycogen or trehalose appears to be essential for appressorium formation, the

influence of each may be critical to regulation of carbon and nitrogen metabolism via the activities of *MoTps1*.

Lipid bodies present in *M. oryzae* conidia are translocated in a *MoPmk1* dependent manner out of the conidium during the germination process and are trafficked to the developing appressorium where they coalesce into larger bodies which are taken up by the appressorial vacuole where lipolysis occurs²³. Triacylglycerol (TAG) lipase activity is induced during appressorium formation and maximal activity is dependent upon the presence of *MoCpkA*²³. Degradation of triacylglycerols also represents one potential source of glycerol production in the appressorium. Functional analysis of seven predicted intracellular TAG lipases revealed none are essential for appressorium function⁹⁰. One putative TAG lipase, MGG_12849, showed *MoCpkA*-dependent dephosphorylation during appressorium formation. MGG_12849 has homology to the lipid particle-localized bifunctional lipase, *ScTgl5*, and contains the patatin domain lipase motif, GxSxG, but not the HxxxxD domain associated with the acyltransferase activity of *ScTgl5*^{91, 92}. β -oxidation of fatty acids derived from appressorial triacylglycerol lipase activity is critical for appressorium function as a source of acetyl-CoA whose metabolism plays a central role in appressorium biology.

Regulation of phospholipid metabolism—Diacylglycerol liberated by TAG lipase activity can also be utilized in the Kennedy pathway for phospholipid biosynthesis and network analysis revealed regulation of this pathway during appressorium formation. Up-regulation at the transcript and protein levels was observed for *M. oryzae* orthologs of *ScEpt1* and *ScCpt1*, respectively. *Ept1* and *Cpt1* catalyze the final step in the synthesis of phosphatidylethanolamine and phosphatidylcholine, respectively. Phosphosite regulation was also observed in four proteins connecting phosphatidylcholine to fatty acid, glycerol and phosphatidic acid metabolism. An ortholog of *ScNte1*, MGG_06143, encoding a phosphatidylcholine phospholipase b which catalyzes the conversion of phosphatidylcholine to glycerophosphocholine and fatty acids had two phosphosites up-regulated at 30 minutes, one of which was then down-regulated at 90 minutes. Glycerophosphocholine derived from *Nte1* activity is converted to choline and glycerol phosphate via the activity of a glycerophosphocholine phosphodiesterase, *Gde1*. The *M. oryzae* ortholog of *ScGde1*, MGG_06704, was transcriptionally up-regulated and dephosphorylated. Glycerol phosphate can be converted to glycerol in a single step catalyzed by the *Hor2* protein and an ortholog of *ScHor2* was detected in appressoria. Glycerolphosphate can also be converted to phosphatidic acid via the combined activity of a glycerol 3-phosphate acyltransferase, *Sct1*, and a 1-acyl-sn-glycerol-3-phosphate acyltransferase, *Slc1*. Orthologs of *ScSct1*, MGG_01291 and *ScSlc1*, MGG_11040, were up-regulated at the phosphorylation and transcriptional levels respectively. MGG_00960, an ortholog of the phospholipase D *ScSpo14*, catalyzing the conversion of phosphatidylcholine to choline and phosphatidic acid, also had two phosphosites increasing in abundance during appressorium formation. Finally, an ortholog of *ScPah1*, MGG_01311, had two sites showing a *MoCpkA* dependent-dephosphorylation at the 90 minute time point. *ScPah1* is a phosphatidate phosphatase that converts phosphatidic acid to diacylglycerol. In *S. cerevisiae*, phosphatidic acid signaling plays a central role in the transcriptional regulation of phospholipid metabolism and control of *Pah1* activity is a primary means of regulating phosphatidic acid^{93, 94}. In *M. oryzae*,

phosphatidic acid signaling has not been investigated although it has been reported that diacylglycerol can stimulate appressorium formation ⁹⁵.

Combining multiple ‘omics’ data sets provide evidence for regulation of phospholipid metabolism at multiple points in the biosynthetic pathway. Interestingly, two aminophospholipid translocases, *MoPde1* and *MoApt2*, were shown to play a role in appressorium function and development of invasive hyphae ^{96, 97}. Aminophospholipid translocases are involved in establishing or maintaining membrane phospholipid asymmetry which is required for proper vesicle fusion during intracellular trafficking, endocytosis and exocytosis. This observation, when combined with the regulation of phospholipid metabolism observed here suggests a possible alteration in phospholipid membrane dynamics during appressorium development and penetration of the host plant. This may be a consequence of a reliance on triacylglycerols as a source of phospholipids for appressorium formation and the need to balance membrane biogenesis with energy production from β -oxidation of triacylglycerol-derived fatty acids and production of glycerol for turgor generation. However, metabolic flux through the phospholipid biosynthetic pathways remains unexplored in *M. oryzae* although it appears changes in the metabolic flux through these pathways may play a vital role in appressorium biology.

Autophagy—Autophagic recycling of cellular components provides biosynthetic precursors to the appressorium and was shown to play a critical role in conidial cell death and cellular differentiation prior to formation of the appressorium ^{98, 99}. Deletion of any of the *M. oryzae* genes involved in nonselective macroautophagy resulted in strains that were nonpathogenic or dramatically reduced in virulence ⁹⁸. An increase in phosphorylation during appressorium formation was observed for six sites on five proteins involved in autophagy including *MoAtg1*, *MoAtg2*, *MoAtg3*, *MoAtg17* and *MoAtg18*. A *MoCpkA*-dependent decrease in phosphorylation was observed for a single site on *MoAtg13*. The *S. cerevisiae* serine/threonine protein kinase, *ScAtg1*, is part of a multiprotein complex including *ScAtg13* and *ScAtg17* that is involved in the initiation of autophagy ¹⁰⁰. The phosphorylation status of *ScAtg13* is controlled by the TOR signaling pathway and dephosphorylation of *ScAtg13* is required for maximal induction of *ScAtg1* ¹⁰¹. Evidence indicates that *ScAtg1* may itself be a target of PKA signaling ¹⁰². *ScAtg2* and *ScAtg18* are peripheral membrane proteins whose role is thought to involve proper localization of the membrane protein *ScAtg9* which is required for initiation of autophagy ¹⁰³. *ScAtg3* is an E2 like ubiquitin-conjugating enzyme responsible for conjugating the ubiquitin-like protein *ScAtg8* to phosphatidylethanolamine ¹⁰⁴. The *ScAtg8*-PE conjugate plays a role in autophagosome formation and expansion ¹⁰⁵. Although it is well established that autophagy is critical to pathogenic development in *M. oryzae*, little is known about its regulation. Changes in phosphosite abundance during appressorium development observed on *M. oryzae* proteins whose *S. cerevisiae* orthologs play central roles in regulation of the localization and initiation of autophagosomes offers a potential avenue to begin looking at the regulation of autophagy in *M. oryzae*.

Actin cytoskeleton dynamics and the cell wall integrity pathway—The fungal actin cytoskeletal network is involved in the regulation of a number of cellular processes

including but not limited to morphogenesis, cytokinesis, establishment of cell polarity, endocytosis, and exocytosis^{106, 107}. Actin network dynamics are coordinated by the activity of a set of highly conserved proteins and changes in phosphoregulation during appressorium development were observed for ten phosphoproteins involved in coordinating actin dynamics (Figure 3). Five are orthologs of *S. cerevisiae* actin binding proteins including *ScAbp1* (MGG_06358), *ScCrn1* (MGG_06389), *ScVrp1* (MGG_11243), *ScScp1* (MGG_14713), and *ScSla2* (MGG_02949), all of which localize to actin patches in *S. cerevisiae*. *ScAbp1* binds filamentous actin (F-Actin), is a component of cortical actin patches involved in endocytosis and is important for activation of the Arp2/3 actin nucleating complex¹⁰⁷. *ScAbp1* is a target of the Pho85 and Cdc28 kinases and its phosphorylation status influences its susceptibility to proteolytic degradation¹⁰⁸. The coronin, *ScCrn1*, and transgelin-like protein, *ScScp1*, produce F-actin bundles via a cross linking of filaments^{109, 110}. *ScSla2* is an actin binding protein that secures actin to membranes via an interaction with the lipid binding protein Ent1¹¹¹. Orthologs of the *S. cerevisiae* type II and type III phosphatidylinositol 4-kinases, *Lsb6* (MGG_07151) and *Stt4/Pik1* (MGG_12862) also showed phosphoregulation during appressorium formation (Figure 3). *ScStt4* and *ScPik1* are the primary phosphatidylinositol 4-kinases in *S. cerevisiae* with *ScStt4*, a component of the Pkc1 pathway, influencing actin organization and *ScPik1* being required for endocytosis¹¹². Finally, *ScLsb6* is a regulator of the Arp2/3 activating protein Las17¹¹³.

The observed phosphosite regulation for numerous proteins with predicted roles in actin patch assembly points, not unexpectedly, towards a key role for reorganization of the actin cytoskeleton during appressorium development. Prior to initiation of appressorium formation, conidia produce germ tubes that extend in a polarized manner. Proper organization of the actin cytoskeleton is critical to establishment and maintenance of both the apical Spitzenkörper and subapical regions of endocytosis that support polar growth. Initiation of appressorium formation in *M. oryzae* involves a cessation of polar growth in the germ tube and commitment to a new morphogenetic program which will require reorganization of the actin network. Interestingly, pore formation at the base of the appressorium also involves reorganization of the F-actin mediated by a septin ring⁷². Collectively, the phosphoregulation of actin network proteins observed here likely reflects the contribution of protein phosphorylation to reorganization of the actin network during infectious development in *M. oryzae*.

The Pkc1-Mps1 MAP kinase cell wall integrity (CWI) signaling pathway is known to play a role in the production and function of *M. oryzae* appressoria^{32, 33}. Deletion of *MoMPS1*, an ortholog of *ScSLT2* and terminal MAP kinase of cell wall integrity pathway, results in a loss of pathogenicity and production of appressoria that are unable to penetrate the host plant³³. Likewise, deletion of *MoMCK1*, a MAP kinase kinase kinase positioned upstream of *MoMps1* in the CWI pathway and orthologous to *ScBck1*, results in a phenotype similar to a *mps1* mutant³². In this study, a change in phosphorylation status was observed for *MoMck1*, *MoPkc1*, a protein kinase C ortholog that is predicted to function upstream of the Mps1 pathway and *MoSmi1*, an ortholog of *ScSmi1*, a yeast protein involved in coordinating cell wall synthesis¹¹⁴ (Figure 3). Proteins involved in the synthesis of β -1,3-glucans, *MoFks1*, and chitin, *MoChs4*, as well as a transcription factor, *MoCrz1*, were also phosphoregulated during appressorium formation. *MoCrz1* is responsive to calcium

signaling and regulates expression of both *MoFsk1* and *MoChs4*¹¹⁵. Changes in the phosphorylation state of proteins in the cell wall integrity pathway provide insight and preliminary data for further examination of the role of the CWI pathway in *M. oryzae*.

A putative transcription factor involved in appressorium function

Network analysis identified multiple aspects of appressorium biology that appear to be influenced by protein phosphorylation in addition to transcriptional and translational regulation. We sought to identify differentially phosphorylated transcription factors that could be linked to these biological processes important to appressorium formation. A survey of transcription factors regulated during appressorium development revealed four proteins with phosphosites whose abundance increased following 1,16-hexadecanediol treatment. One of these proteins, MGG_05709, was previously identified as a member of fungal bHLH protein group F2¹¹⁶ and transcripts of the gene were shown to be up-regulated following cAMP treatment⁶⁵. In this study, five class I phosphosites were identified on MGG_05709 at positions T114, S122, S206, S335 and S343. Three sites (T114, S122, and S343) increased in abundance during appressorium formation. Four of the five sites including all three regulated sites are proline-directed sites indicating a possible connection to cyclin dependent kinase or MAPK signaling. This protein is highly conserved in filamentous fungi and a homolog, *AnGlcD*, was demonstrated to be required for utilization of glycerol as a carbon source in *Aspergillus nidulans*^{117, 118}.

As the production of glycerol in the appressorium is an important endpoint in the metabolism of conidial storage reserves, the role of MGG_05709 in appressorium biology and pathogenicity was investigated via construction of a deletion mutant in the *M. oryzae* 70-15 background. Appressoria of the MGG_05709 mutant and a mutant strain complemented via reintroduction of the wild type allele expressed from its native promoter form at rates consistent with the wild type and appear normal in shape and melanization (Figure 4A). However, pathogenicity assays on detached leaves of susceptible barley and rice leaves revealed that the mutant is reduced in virulence relative to the wild type strain (Figure 4B). Prior to penetration of the plant leaf surface, appressoria accumulate high concentrations of glycerol to create an influx of water resulting in the generation of large internal turgor pressure which facilitates mechanical penetration of the leaf cuticle. Previous reports indicate that the internal concentration of glycerol in appressoria ranges from 2-4 M based upon the results of cytorrhysis assays in which mature appressoria are exposed to increasing concentrations of external glycerol and collapse of the appressorial wall is observed when the concentration of external glycerol exceeds that of internal glycerol¹¹⁹. Cytorrhysis assays revealed collapse of appressoria in the MGG_05709 mutant beginning at glycerol concentrations as low as 0.5 M (Figure 4C). Collapse of the wild type and complemented mutant strains occurred at glycerol concentrations greater than 2M, consistent with previous reports.

Loss of MGG_05709 resulted in no defects in vegetative growth on minimal medium (Figure 4D). However, a dramatic reduction in growth of the mutant was observed when cultured on minimal medium in which a high concentration of glycerol (500 mM) was provided as a sole carbon source (Figure 4D). Complementation of the mutant restored

growth to wild type levels (Figure 4D). At lower glycerol concentrations (10 mM), the reduction in growth of the mutant was not observed indicating that unlike *GlcD* in *A. nidulans*, MGG_05709 is not required for utilization of glycerol as a carbon source but rather loss of MGG_05709 confers sensitivity to high concentrations of glycerol. However, in minimal medium containing sucrose as a carbon source, growth of the mutant was restored to near wild type levels in the presence of up to 500 mM glycerol. To investigate the role of MGG_05709 in the general osmotic stress response, the mutant was cultured in minimal medium supplemented with 1M sorbitol or 0.4M NaCl which represent conditions previously established for studying the osmotic stress response of *M. oryzae*^{119, 120}. Under these conditions, growth of the mutant was only slightly reduced relative to the wild type level indicating that MGG_05709 is not required for the general osmotic stress response (Supplementary Figure 4). Cumulatively, these results suggest that the transcription factor, MGG_05709, is important for appressorium function presumably affecting the ability of *M. oryzae* to accumulate or retain glycerol in the appressorium. Phosphorylation of MGG_05709 during appressorium formation further suggests that its regulation may be important for transcriptional control of the metabolism of conidial reserves leading to the production of glycerol and is a topic for future investigation.

Conclusions

Advances in phosphopeptide isolation and mass spectrometry based phosphopeptide identification have facilitated investigation of protein phosphorylation at a global level. However, few global phosphoproteomic data sets have been generated to date for filamentous fungi. Moreover, opportunities for comparative analyses of phosphoproteome dynamics during pathogenic development of fungi are not possible at the current time due to lack of data. A global study of six stages of development in the Oomycete pathogen *P. infestans* identified 2,922 phosphopeptides. Although a number of sites were differentially regulated, only 13 phosphosites were regulated specifically during appressorium formation. Six were from different transport proteins, a protein kinase and five other proteins. Although orthologs can be identified between Stamenopiles and true fungi in a number of instances, none could be clearly identified for these proteins in *M. oryzae* precluding further comparative interrogation.

In this study, we have identified 1514 phosphoproteins and confidently localized 2924 phosphorylation sites from mycelia, asexual conidia, conidial germlings and chemically induced appressoria for the wild type *M. oryzae* 70-15 and a protein kinase A mutant, *cpkA*. Analysis of phosphosite motifs in the phosphoproteomics data generated here revealed an abundance of phosphorylation motifs consistent in nature with those observed in large scale phosphoproteome analyses of other eukaryotic organisms. In particular, a collection of 253 phosphosites from 227 phosphoproteins was assembled that contain candidate *MoCpkA* target sites and this information will aid in experimental identification and validation of *MoCpkA* phosphorylation substrates.

Changes in phosphorylation dynamics during appressorium formation were examined by collecting samples from 1,16-hexadecandiol treated germlings at 30 and 90 minutes post treatment revealing changes in abundance for 448 phosphosites from 320 phosphoproteins.

Previous studies of phosphorylation signaling pathways in *M. oryzae* have identified roles for the Pmk1 MAPK, cAMP-dependent, Ca²⁺ and cell wall integrity signaling pathways in appressorium biology. The primary components of each pathway were identified in forward genetic screens and via homology to analogous pathways in other organisms. However, knowledge of the downstream targets of each of these pathways is scarce. The data presented here, in particular, those phosphosites regulated during appressorium formation will aid in the identification of phosphorylation targets for each of these pathways.

A protein interaction network analysis of regulated phosphosites revealed a number of biological processes in which changes in protein phosphorylation are abundant during appressorium formation. Generation of expanded subnetworks for each of these processes and incorporation of transcript and protein regulation from existing 'omics' data demonstrated the importance of changes in phosphorylation status for apparent regulation of these networks as in multiple instances transcript or protein level regulation was not observed. The insights gained from the incorporation of three 'omics' datasets clearly illustrate the need to look beyond transcript abundance to understand complex developmental processes. In particular, of primary interest is connecting the transcriptional, translational and post-translational regulation emerging from these large scale datasets to the cellular signaling pathways previously identified as critical to infectious development in *M. oryzae* and the data presented here offers a numbers of interesting testable hypotheses for future studies.

Finally, functional characterization of a putative transcription factor undergoing phosphorylation during appressorium formation identified a role for this protein in appressorium function and virulence. This protein is required for normal accumulation of glycerol in mature appressoria and tolerance of high levels of glycerol in culture. Future studies directed at elucidating the exact role of this protein in gene regulation as it relates to glycerol metabolism should further the understanding of the molecular mechanisms of turgor generation in *M. oryzae*.

Supplementary Material

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Figure 1. Experimental setup and phosphoproteomic workflow

A. Microscopic examination of appressorium formation following 1,16-hexadecanediol treatment of 8 h germlings of the wild type and $\Delta cpkA$ strains. Scale bar equals 10 microns.

B. Percent of wild type (white bar) and $\Delta cpkA$ (black bar) germlings that form appressoria 24 h after treatment with 1,16-hexadecanediol. Data shown is an average of three replicates (100 germlings counted per replicate) with standard deviations shown.

C. Flow chart illustrating phosphopeptide enrichment and mass spectrometric analysis.

Figure 2. Phosphosite distribution and Gene ontology analysis

A. Venn diagram illustrating the distribution of wild type class I phosphosites by sample type.

B. Venn diagram illustrating the distribution of class I phosphosites between the wild type and $\Delta cpkA$ strains for all sample types.

C. GO terms overrepresented in the observed *M. oryzae* phosphoproteome (black bars) relative to the *M. oryzae* genome (white bars)

Figure 3. Multi-omics network analysis

Protein interaction networks incorporating transcriptomic, proteomic and phosphoproteomic data for proteins related to the actin cytoskeleton (A), phospholipid metabolism (B), autophagy (C), glycogen and trehalose metabolism (D) and the cell wall integrity pathway (E). Node border lines indicate the presence of a transcript (solid line, transcript detected; dashed line, no transcript observed). Node border color represents regulation at the transcript level (green, transcript up-regulated; red, transcript down-regulated). Node color represents regulation at the protein level (white, no protein detected; grey, protein detected; green, protein up-regulated; and red, protein down-regulated). The color of inset circles represents phosphosite regulation (white; phosphosite observed; green, phosphosite up-regulated; red, phosphosite down-regulated; and yellow, both up- and down-regulation of phosphosite(s) observed). See text for details regarding network generation. Ortholog information for each node can be found in Supplementary table 10.

Figure 4. Phenotypic characterization a MGG_05709 mutant

A. Appressoria of the wild type (W), MGG_05709 (M), and complemented (C) strains at 24 h post treatment with 10 μ M 1,16-hexadecanediol.

B. Pathogenicity assays were performed via spot inoculation of water (U) or conidia of the wild type (W), MGG_05709 (M), and complemented (C) strains onto barley or rice at indicated concentrations as described in the materials and methods. Lesions were photographed at six days post inoculation.

C. Cytorrhysis assays of wild type (blue bars), MGG_05709 (red bars), and complemented (green bars) strains. Data presented is an average of three replicates with 100 appressoria observed per replicate and standard deviations shown.

D. Growth of wild type, MGG_05709, and complemented strains on minimal medium plates with sucrose, 10 mM glycerol, 500 mM glycerol or sucrose plus 500 mM glycerol as carbon sources. Plates were seeded in the center of the plate with 10 μ l of water containing 1,000 conidia and photographed 18-20 days after inoculation. Each plate was photographed on both white and black backgrounds for visualization.

Table 1

Gene Ontology enrichments associated with each of the most abundant phosphorylation motifs.

Motif	GO Term	Name	FDR	# of Phosphoproteins	# of Phosphosites
.....SP.....	GO:0004672	protein kinase activity	1.40E-03	15	20
	GO:0030234	enzyme regulator activity	2.20E-03	10	10
	GO:0007165	signal transduction	1.20E-02	15	19
	GO:0006464	cellular protein modification process	2.00E-02	20	27
...R..SP.....	GO:0007165	signal transduction	8.80E-03	12	13
	GO:0003676	nucleic acid binding	1.80E-02	31	33
	GO:0008092	cytoskeletal protein binding	2.80E-02	4	10
	GO:0009653	anatomical structure morphogenesis	2.80E-02	6	6
	GO:0005856	cytoskeleton	3.20E-02	6	7
	GO:0000003	reproduction	3.20E-02	6	6
	GO:0016049	cell growth	3.80E-02	2	2
...R..S.....	GO:0004672	protein kinase activity	2.20E-02	10	12
	GO:0006464	cellular protein modification process	3.90E-02	14	16
....P.SP.....	GO:0005768	endosome	3.70E-03	4	4
	GO:0003779	actin binding	3.90E-03	4	6
	GO:0007165	signal transduction	3.90E-03	10	13
	GO:0030234	enzyme regulator activity	4.30E-03	6	8
	GO:0008289	lipid binding	8.70E-03	4	4
	GO:0005886	plasma membrane	5.00E-02	4	4
...RR.S.....	GO:0004672	protein kinase activity	8.50E-05	11	12
	GO:0006464	cellular protein modification process	1.30E-02	12	13
	GO:0007165	signal transduction	1.30E-02	9	9
	GO:0005773	vacuole	1.30E-02	4	5
	GO:0004871	signal transducer activity	1.90E-02	4	4
.....TP.....	GO:1901363	heterocyclic compound binding	3.40E-02	35	41
	GO:0030234	enzyme regulator activity	4.90E-02	5	6
	GO:0016301	kinase activity	4.90E-02	9	9
...G..TP.....	GO:0043234	protein complex	2.90E-02	12	12
	GO:0009653	anatomical structure morphogenesis	4.50E-02	4	4
...R..TP.....	None	N/A	N/A	N/A	N/A
...R..T.....	None	N/A	N/A	N/A	N/A
.....TPP....	None	N/A	N/A	N/A	N/A