Temporal Analysis of the Magnaporthe Oryzae Proteome During Conidial Germination and Cyclic AMP (cAMP)-mediated Appressorium Formation*

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Rice blast disease caused by Magnaporthe oryzae is one of the most serious threats to global rice production. During the earliest stages of rice infection, M. oryzae conidia germinate on the leaf surface and form a specialized infection structure termed the appressorium. The development of the appressorium represents the first critical stage of infectious development. A total of 3200 unique proteins were identified by nanoLC-MS/MS in a temporal study of conidial germination and cAMP-induced appressorium formation in M. oryzae. Using spectral counting based label free quantification, observed changes in relative protein abundance during the developmental process revealed changes in the cell wall biosynthetic machinery, transport functions, and production of extracellular proteins in developing appressoria. One hundred and sixty-six up-regulated and 208 down-regulated proteins were identified in response to cAMP treatment. Proteomic analysis of a cAMP-dependent protein kinase A mutant that is compromised in the ability to form appressoria identified proteins whose developmental regulation is dependent on cAMP signaling. Selected reaction monitoring was used for absolute quantification of four regulated proteins to validate the global proteomics data and confirmed the germination or appressorium specific regulation of these proteins. Finally, a comparison of the proteome and transcriptome was performed and revealed little correlation between transcript and protein regulation. A subset of regulated proteins were identified whose transcripts show similar regulation patterns and include many of the most strongly regulated proteins indicating a central role in appressorium formation. A temporal quantitative RT-PCR analysis confirmed a strong correlation between transcript and protein abundance for some but not all genes. Collectively, the data presented here provide the first comprehensive view of the M. oryzae proteome during early infection-related development and highlight biological processes important for pathogenicity. Molecular & Cellular Proteomics 12: 10.1074/mcp.M112.025874, 2249–2265, 2013.

The rice blast pathogen, Magnaporthe oryzae, is a filamentous fungus belonging to the Ascomycota. Rice blast is the most serious disease of rice worldwide and its presence has been documented in more than 85 countries including all major rice producing regions (1). Yield losses in cultivated rice associated with infection by M. oryzae can exceed 50% with typical losses ranging from 10 to 30% (1). M. oryzae also infects other grass hosts including wheat, barley and millet (2). The importance of rice as a staple food, the genetic tractability of M. oryzae, and the availability of genome sequences for both the fungus and the plant host have made the rice blast pathosystem a predominant model for the study of plant-fungal interactions.

Rice blast disease is initiated upon contact of an asexual conidium with the plant surface. Attachment to the leaf surface is mediated by spore tip mucilage produced at the conidial apex upon hydration of the conidium (3) followed by emergence of a germ tube from which development of a penetration structure known as an appressorium occurs in response to plant-derived cues. Appressoria of M. oryzae are characterized by a heavily melanized cell wall that facilitates, via accumulation of compatible solutes, generation of a large internal turgor pressure that provides the mechanical force required for penetration of the plant cuticle by a penetration peg formed at the appressorium pore (4). Proper development of the appressorium is essential for infection and understanding the biology of appressorium formation is critical for the development of control strategies for the rice blast disease.

Appressorium formation can be induced in vitro by germination of conidia on hard, hydrophobic surfaces that mimic the waxy outer cuticle of rice leaves (5). A number of chemical elicitors are also known to stimulate appressorium formation in vitro on hydrophilic surfaces that do not normally support the establishment of appressoria. The plant-derived cutin
monomers, cis-9,10-epoxy-18-hydroxyoctadecanoic acid, cis-9-octadecen-1-ol, and 1,16-hexadecadienol stimulate differentiation of appressoria (6). Furthermore, addition of the secondary messenger, cAMP, its analog 8-Bromo cAMP or the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine, to conidial solutions on noninductive hydrophilic surfaces is sufficient to trigger development of appressoria (7).

Previous studies established an essential role for cAMP signaling in the initiation and development of appressoria in M. oryzae. A loss-of-function mutation in the adenylyl cyclase, MAC1, results in the inability to form appressoria on inductive hydrophobic surfaces (8). Appressorium formation in the Δmac1 mutant can be restored by addition of exogenous cAMP to germinating conidia (8, 9). Deletion of the cAMP-dependent protein kinase catalytic subunit (CPKA) causes a significant delay in appressorium formation and production of nonfunctional appressoria resulting in a loss of pathogenicity (10, 11). A point mutation in the regulatory subunit of protein kinase A, SUM1, induces constitutive activation of CpkA leading to the formation of appressoria on non-inductive surfaces in absence of external stimulus (12). Furthermore, roles in the maintenance of intracellular cAMP levels during pathogenic development were recently demonstrated for the high and low-affinity phosphodiesterases, PdeH and Pdel, which catalyze the hydrolysis of cAMP (13, 14).

Although a critical role for cAMP signaling and PKA activity in pathogenic development has been established for M. oryzae, the biological processes regulated by the pathway remain largely uncharacterized. A yeast two hybrid assay identified seven proteins that interact directly with CpkA (15). Recently, the expression of two transcriptional regulators, CDTF1 and SOM1, was reported to be reduced in mac1 and cpkA mutants with Som1 also being shown to interact weakly with CpkA (16). Mutational analysis of these two genes indicated that each has multiple pleiotrophic effects on growth and development in M. oryzae (16). Furthermore, a total of 110 cAMP responsive sequence tags were identified in a SAGE analysis of conidia following cAMP treatment of which 60 (50 up-regulated and 10 down-regulated) were assigned to a gene or expressed sequence tag (17). In a DNA microarray based analysis of conidia germinated for 9 h in the presence or absence of cAMP, a total of 1014 transcripts were differentially expressed (644 up-regulated and 370 down-regulated) and a set of 357 consensus appressorium genes regulated in both cAMP-induced and hydrophobic surface-induced appressoria was generated (18).

Although extensive analysis of the M. oryzae transcriptome has been performed (18–23), investigations of the M. oryzae proteome remain limited in number and scale. A total of four proteins were identified as induced during appressorium formation on an inductive wax surface in a two-dimensional gel-based approach (24). Two studies targeting secreted proteins identified 53 proteins from liquid cultures or appressoria formed on inductive surfaces (25) and 59 proteins differentially expressed in response to nitrogen starvation (26). Comparison of the conidial proteomes of wild-type M. oryzae and a Δcom1 mutant whose protein product is required for normal conidial morphogenesis revealed 31 proteins that changed in abundance (27). Finally, previous studies from our group (28, 29) reported a comprehensive characterization of the M. oryzae conidial proteome, the most recent having identified 2912 proteins from conidia using the filter aided sample preparation method (FASP) followed by stop and go extraction tip (StageTip) anion exchange fractionation in combination with nanoLC-MS/MS (30).

Here we extend our proteome analysis of M. oryzae through characterization of the proteome in a temporal analysis of conidial germination and cAMP-induced appressorium formation. In addition, label free quantification via spectral counting facilitated the identification of proteins whose relative abundance changes during conidial germination and appressorium formation. Additionally, comparison of the proteomes of wild-type M. oryzae and a ΔcpkA mutant strain offers further insight into the role of cAMP signaling during pathogenic development. A detailed examination of changes to the proteome is presented in the results and the major findings are synthesized in the discussion to provide an overview of the significant biological processes directing infection-related development.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions—Wild-type M. oryzae strain 70–15 cultures were maintained at 25 °C under constant illumination on a minimal medium agar consisting of the following components per liter: 10 g sucrose, 6 g NaN3, 0.52 g KCl, 0.52 g MgSO4-7H2O, 1.52 g KH2PO4, 5 μg biotin, 1 mg thiamine and 1 ml of 1000X trace element solution (2.2 g ZnSO4, 1.1 g H3BO3, 0.5 g MnCl2-4H2O, 0.5 g FeSO4-7H2O, 0.17 g CoCl2, 0.16 g CuSO4-5H2O, 0.15 g Na2MoO4-2H2O and 5 g disodium ethylenediaminetetraacetate per 100 ml). Escherichia coli strain DH5α was maintained on LB medium (31) and ampicillin was added at 100 μg/ml where appropriate.

Generation of a ΔcpkA Mutant—Since the original ΔcpkA mutant was constructed more than 20 years ago, it was recreated to ensure that it is isogenic with the wild type strain being used in this study. A gene replacement construct for deletion of CPKA (MGG_06638) was generated by PCR amplification of an ~6.7 kb fragment encompassing the insertion site of the hygromycin resistance cassette as well as upstream and downstream flanking sequence from the original ΔcpkA mutant described previously (10) using the primers cpkA mut F (5’-GTGCAAGCCTTCGCTTCGGA-3’) and cpkA mut R (5’-CAA-TCTGCACTCCTGAAATG-3’). The resulting PCR product was then cloned into the pGEMT-EASY vector (Promega, Madison, WI) and transformed into E. coli DH5α to generate pCPKAKO. M. oryzae protoplasts were generated as described previously (32). A PCR product (3 μg) derived from amplification of pCPKAKO with primers cpkA mut F and cpkA mut R was transformed into protoplasts as described previously (33). Candidate mutants were confirmed for the absence of the wild-type CpkA allele and the presence of a single copy of the hygromycin cassette by PCR and Southern blot analysis (data not shown).

Sample Preparation for Proteome Analysis—Conidia were harvested from 8-day-old minimal medium plates by filtration through...
Miracloth to separate conidia from mycelium. Aliquots of two million conidia in 20 ml of H2O (germination condition) or 50 mM cAMP (Sigma Aldrich, St. Louis, MO) (appressorium formation condition) were placed on the hydrophilic surface of 205 × 110 mm GelBond® (Lonza, Rockland, ME) sheets and incubated at room temperature in the dark. Aliquots of 500,000 conidia in 5 ml of H2O or 50 mM cAMP were used for absolute quantification studies. Samples were harvested by removal of the bulk liquid and subsequent scraping of the remaining liquid and sample from the surface with a razor blade into a 50 ml reagent reservoir. Samples were collected and then centrifuged at 13,000 × g and 4 °C for 5 min. Supernatants were removed from the pellets and discarded before freezing the pellets in liquid nitrogen followed by storage at −80 °C.

Total protein was collected from each sample by bead beating for 2 min in a buffer consisting of 1 × phosphate-buffered saline, 0.1% SDS and 2 mM urea using ~150 mg of 0.5 mm zirconia/silica beads (BioSpec Products, Inc., Bartlesville, OK). The samples were boiled for 5 min and centrifuged at 13,000 × g and 4 °C for 5 min to remove beads and cellular debris. A bicinchoninic acid assay was used to determine protein concentration. Protein samples were trypsin digested using the FASP methodology (30) as described previously (29). A total of six peptide fractions were generated by StageTip anion exchange fractionation (29) with successive peptide elutions in Britton Robinson buffers pH 11, 8, 6, 5, 4, and 3 followed by a cleanup on C18 StageTips as previously described.

NanoLC MS/MS Analysis—Separation was performed on a nanoLC-1D+ system from Eksigent (Dublin, CA) equipped with a continuous, vented column configuration, a 10 μl sample loop, 3 cm trap packed with Magic C18AQ packing material (Michrom Bio-Resources, Auburn, CA) and a 15 cm analytical column packed with the same packing material. The flow rate was set at 350 nL min−1 during separation and a 3.5 h linear gradient from 5% to 35% B was applied. A hybrid LTQ-Orbitrap XL MS (Thermo Fisher Scientific, Bremen, Germany) was used to perform MS/MS analysis. Optimal instrument parameters as reported in Gokce et al. were used (29).

In the second experiment comparing the wt and ΔcpxA mutant strains, a hybrid LTQ-FTICR MS (Thermo Fisher Scientific, Bremen, Germany) was used to perform MS/MS analysis. LC and MS instrument parameters were identical to those used for the LTQ-Orbitrap XL described above, except the source temperature was found to yield higher signal at 200 °C and a higher resolving power of 100,000 could be applied. The Lock mass function is not available for FTICR instruments.

Absolute Quantification—A nanoLC-2D system equipped with a cHiPLCnanoflex system (Eksigent, Dublin, CA), using a 5 cm trap (200 μm × 0.5 mm packed with ChromXP C18-CL 3 μm 120 Å), 15 cm column (75 μm × 15 cm packed with the same packing material) and a 20 μm i.d. SilicaTip ESI emitter (New Objective, Woburn, MA) was used to perform separation for SRM analysis. A 30 min gradient was applied and data was acquired on a TSQ Vantage triple stage quadrupole mass spectrometer (Thermo Scientific, San Jose, CA). Instrument parameters were same as reported by Shuford et al. (34).

To identify peptides suitable for SRM analysis, total peptides from four target proteins, assuming C peptide = C protein. Two fmol of IIAAP-K (MGG_05580), 10 fmol of VASDLVQLR (MGG_09359), 5 fmol of MMTGDAIAAK (MGG_04994), and 5 fmol of CFDYGVAK (MGG_08526) synthetic peptides (New England Peptides, Gardner, MA) were spiked into samples before trypsin digestion for absolute quantification. Samples for absolute quantification were generated exactly as described above for the global proteomics analysis with the addition of a 24 h time point. Peak areas for each transition were exported from Skyline and transition ratios were further inspected in Excel.

Data Analysis—RAW files obtained by LC-MS/MS were searched against a concatenated target-reverse database containing 12991 proteins from the M. oryzae genome version 8 (Magnaporthe comparative Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/)) using MASCOT Distiller version 2.3.01 (Matrix Science Inc., Boston, MA). MASCOT parameters were the same as reported in Gokce et al. (28, 29). Two missed trypsin cleavages were allowed. The precursor ion tolerance was set at ±5 ppm and the MS/MS fragment ion tolerance at ±0.6 Da. Cysteine carbamidomethylation was set as a fixed modification. Variable modifications allowed included oxidation of methionine and deamidation of glutamine or asparagine. ProteoIQ version 2.3.05 (Bio-Inquire, Athens, GA) was used to apply a 1% protein false discovery rate for confident protein identifications. Tandem mass spectra for proteins identified by a single peptide are presented in supplemental Fig. S1. For protein groups containing shared peptides, only the top protein as designated by ProteoIQ was retained for further analysis. Normalization based on total spectral counts was performed in ProteoIQ as described previously (28). Differential protein expression was determined using a pairwise Student’s t test (p value = 0.05) on normalized spectral counts. Proteins lacking spectral count data for one condition in a pairwise comparison were assigned a total of one spectral count for all three biological replicates to facilitate calculation of fold changes. Proteins of low abundance with fewer than 5 total spectral counts in at least one condition of a given pairwise comparison were removed from the analysis. Fold change cutoffs for determination of differential expression were applied based on total spectral count bin widths as determined previously (28, 29).

Quantitative RT-PCRs—Samples for quantitative RT-PCR (qRT-PCR) analysis were prepared exactly as described above. Frozen tissues were suspended in 500 μl TRI Reagent (Sigma Aldrich) and RNA was extracted by bead beating with ~300 mg of 0.5 mm zirconia/silica beads (BioSpec Products, Inc., Bartlesville, OK) for two 25 s intervals separated by a 30 s incubation on ice. The remainder of the RNA extraction was completed following the standard TRI reagent protocol. RNA samples were further purified using RNeasy minicolumns (Qiagen, Valencia, CA) per the manufacturer’s instructions. Synthesis of cDNA from 800 ng of RNA was performed in a 15 μl reaction using M-MLV Reverse Transcriptase (Promega Madison, WI) and 0.5 μg oligo(dT)16 primer (Promega, Madison, WI) per reaction according to the manufacturer’s instructions. Primers used for qRT-PCR are listed in supplemental Table S1. The qRT-PCR was performed in 10 μl reactions using SYBR Green I Master Mix (Roche, Indianapolis, IN) with 0.5 μl of cDNA as template and a final primer concentration of 0.5 μM for each primer. A DNA Engine Opticon2 (Bio-Rad, Hercules, CA) was used with the following cycle profile: 95 °C for 2 min, 40 cycles of 95 °C for 10 s, 60 °C for 30 s and 72 °C for 30 s, and 72 °C for 5 min. The qRT-PCR data was analyzed as described previously (36) with data normalized to actin (MGG_03982).

RESULTS

Identification and Label Free Quantification of Proteins During Germination and Appressorium Formation—To investigate proteome changes occurring during M. oryzae conidial germination and appressorium formation following cAMP treatment, a label free quantitative proteomics study was undertaken. Conidia were germinated on the hydrophilic surface of GelBond® film in the presence or absence of 50 mM cAMP. In the first experiment total protein was collected from triplicate samples at 4, 8, 12, and 18 h as well as from conidia. In a

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second experiment, wild-type *M. oryzae* and ΔcpkA mutant conidia were germinated in the presence of 50 mM cAMP and total protein was collected from triplicate samples at 8 and 18 h. Wild-type fungal morphology at each time point as well as germination and appressorium formation rates are depicted in Figs. 1A–1C. At 4 h ~80% of wild-type conidia have germinated and swelling of the germ tube tips is evident but appressoria are not yet visible (Figs. 1A and 1B). By 8 h, ~60% of germinated conidia have produced appressoria, which start to melanize by 12 h and are fully melanized by 18 h (Figs. 1A and 1C). Germination of ΔcpkA mutant conidia is slightly delayed at 4 h but returns to wild-type levels by 8 h and appressoria are first observed after 12 h with fewer than 20% of germinated conidia producing appressoria by 18 h (Fig. 1C). Those appressoria that form in the ΔcpkA mutant in response to cAMP treatment are not observed before 12 h and appressoria produced by 18 h are small and nonfunctional, consistent with previous reports (10, 11). In addition, appressorium formation by the ΔcpkA mutant on hydrophobic surfaces is also delayed but the final number of germinated conidia that form appressoria approaches wild-type levels as reported previously (11).

Collectively, between the two experiments a total of 841899 tandem mass spectra were collected and assigned to peptides. Peptide assignments are presented in supplemental Table S2. The distribution of total mass spectra, unique peptide identifications and protein identifications collected from three biological replicates for each experimental condition is shown supplemental Table S3. In the first study analyzing wild-type conidial germination and appressorium formation at multiple time points, a total of 3170 proteins were identified at a 1% protein false discovery rate (FDR). A list of the identified proteins and their accompanying raw and normalized spectral count data for each sample is presented in supplemental Table S4. In this experiment, 193 proteins, representing six percent of the proteins identified, were specific to germination or appressorium formation and not identified in conidia (Fig. 2A).

In the second experiment comparing cAMP-mediated appressorium development in the wild-type and ΔcpkA mutant strains, 1651 proteins were identified (supplemental Table S3) including 30 proteins not identified in the first experiment bringing the total number of protein identifications reported in this study to 3200. Proteins identified in the second experiment and their spectral count data is presented in supplemental Table S5. Collectively, the number of unique proteins reported here (3200) and by Gokce et al. (29) (2912) totals 3540 proteins (Fig. 2B), representing 27.6% of the predicted *M. oryzae* proteome.

In addition to expanding the coverage of the *M. oryzae* proteome, we identified proteins whose relative abundance changed during germination and appressorium formation by spectral counting based label free quantification. Protein regulation during conidial germination was assessed by compar-

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**Fig. 1.** cAMP-induced appressorium formation in *M. oryzae*. A, Light micrographs of *M. oryzae* conidia germinated in 50 mM cAMP on a hydrophilic surface showing the development of appressoria at the time points used in the proteomics study. Scale bar = 10 μm. B, Conidial germination of the wild type (black and white bars) and ΔcpkA mutant (dark gray and light gray bars) strains in the presence (black and dark gray bars) and absence (white and light gray bars) of cAMP under the conditions described in panel A. Percentages are the average with standard error shown from three biological replicates with 100 conidia counted per replicate. C, Appressorium formation of the wild type (black bars) and ΔcpkA mutant (dark gray bars) strains following cAMP treatment as described in panel A. Percentages are the average with standard error shown from three biological replicates with 100 conidia counted per replicate.
The four germination time points directly to conidia and regulation in response to cAMP was determined by comparing cAMP treated and untreated samples at each respective time point. Likewise, in the second experiment, the impact of the \( \text{H9004} \) \( \text{cpkA} \) mutation was determined by direct comparison of wild-type and mutant cAMP treated samples at early (8 h) and late (18 h) time points. In a previous study encompassing independent analyses of a single sample, a false positive rate for detection of biological change was established (28). Proteins were divided into bin widths based on their number of spectral counts. A smaller fold change (1.5-fold) can be applied to proteins with larger numbers of spectral counts (average greater than 10 spectral counts per replicate) while maintaining a false positive rate below 10% (28). However, proteins at the lower end of the spectral count distribution require a higher fold change (twofold) to maintain a similar false positive rate (28). Table I presents the number of differentially expressed proteins for each comparison and the total number of unique proteins regulated in at least one time point based on criteria described in the experimental procedures. A total of 33 proteins were up-regulated (Table II) and 26 down-regulated (Table III) by both cAMP treatment and in the wild type versus \( \text{H9004} \) \( \text{cpkA} \) mutant comparison. These comprise a subset of proteins regulated during appressorium formation that are both responsive to cAMP treatment and also require a functional CpkA for normal regulation.

**Gene Ontology Analysis of Regulated Proteins**—The regulated proteins from each comparison were subjected to Gene Ontology (GO) analysis using the BLAST2GO program (37). Over and underrepresented GO categories for each comparison were identified using Fisher’s exact test in the BLAST2GO program with the 3170 and 1651 proteins from experiments 1 and 2 as reference data sets (supplemental Table S6). Two specific trends involving regulation of mitochondrial and transport proteins were observed. Proteins up-regulated during conidial germination were underrepresented in mitochondrial proteins (GO:0005739) and conversely, the down-regulated protein set was enriched for mitochondrial proteins as well as ion transport proteins (GO:0006811). Additionally, proteins associated with transporter activity (GO:0005215) were overrepresented in proteins up-regulated by cAMP treatment. Proteins from these GO categories will be addressed below in the context of germination and appressorium formation.

**Table I**

| Number of differentially expressed proteins for each comparison | 4 hr | 8 hr | 12 hr | 18 hr | Totals (unique) |
|---------------------------------------------------------------|------|------|-------|-------|-----------------|
| +/− cAMP                                                     |      |      |       |       |                 |
| Up                                                           | 37   | 53   | 51    | 49    | 166             |
| Down                                                         | 19   | 102  | 106   | 145   | 327             |
| − cAMP/conidia                                               |      |      |       |       |                 |
| Up                                                           | 66   | 75   | 63    | 101   | 208             |
| Down                                                         | 163  | 158  | 206   | 167   | 383             |
| wt/ΔcpkA                                                     |      |      |       |       |                 |
| Up                                                           | 16   | 134  | 148   |       | 148             |
| Down                                                         | 98   | 28   | 125   |       | 125             |

**Fig. 2. Overview of protein identifications from multiple proteome analyses.** A, Venn diagram displaying the number and overlap of proteins identified from conidia (2977 proteins), germinating conidia (3057 proteins) and appressoria (3071 proteins) samples in the wild type time course study (experiment 1). Germination and appressorium proteins were identified in at least one time point (4, 8, 12, or 18 h). B, Venn diagram displaying the number and overlap of proteins from multiple independent analyses of the *M. oryzae* proteome including 3170 proteins identified in the wild type time course study (experiment 1), 1651 proteins identified in the wild type versus ΔcpkA mutant study (experiment 2), and 2912 proteins from the conidial proteome reported by Gokce et al. (29).

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The abbreviations used are: GO, Gene Ontology; cAMP, cyclic adenosine monophosphate; FASP, filter aided sample preparation; SAGE, serial analysis of gene expression.
Fungal chitin is composed of repeating
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microfibrils that are then covalently attached to cell wall
&9252
glucosamine units, which form chains that assemble into
/H9252
a major role in both the germination and appressorium formation
microfibril
and is extruded into the extracellular space on the surface of the plasma membrane and plays a
critical role in both the germination and appressorium formation processes. Cell walls of filamentous fungi are composed pri-
arily of β- and α-glucans, chitin, and glycoproteins (38–40). Fungal chitin is composed of repeating β-1,4-linked N-acetyl-
olism of N-acetylglucosamine, catalyzing the conversion of
Chs1 (MGG_01802), was previously
abundant in conidia, Chs1 (MGG_01802), was previously
shown to play a role in pathogenesis. A chs1 mutant was demonstrated to be impaired in
appressorium development and virulence (42). Two chitin synthase proteins, Chs5 (MGG_13014) and Chs7 (MGG_06064), were significantly up-
regulated at the 4 h germination time point (supplemental Table S4). A chs7 mutant was demonstrated to be impaired in
Cell Wall Biosynthesis and Remodeling—The process of conidal germination in M. oryzae is characterized by the
emergence of a germ tube that elongates in a polarized fashion and develops into an appressorium in the presence of
appropriate stimuli. De novo cell wall biosynthesis and remodeling of existing cell wall structures occurs in the extracellular space on the surface of the plasma membrane and plays a critical role in both the germination and appressorium formation processes. Cell walls of filamentous fungi are composed primarily of β- and α-glucans, chitin, and glycoproteins (38–40). Fungal chitin is composed of repeating β-1,4-linked N-acetyl-glucosamine units, which form chains that assemble into microfibrils that are then covalently attached to cell wall β-1,3-Glucans. Chitin is synthesized at the fungal plasma membrane in regions of active cell wall growth or remodeling and is extruded into the extracellular space where microfibril formation and cross-linking to cell wall glucans occurs. Fungal chitin synthases are grouped into seven distinct classes with three specific to filamentous fungi (41) and are frequently found in multiple copies in a single organism (38). The M. oryzae genome encodes seven chitin synthase proteins with a single representative of each of the seven classes (42). Six of
the seven M. oryzae chitin synthase proteins were identified in conidia and all time points during germination. The most abundant in conidia, Chs1 (MGG_01802), was previously shown to play a role in pathogenesis. A chs1 mutant was reduced in conidal germination, appressorium formation and virulence (42, 43). Interestingly, Chs7 and Chs5 were down-regulated by cAMP treatment at 12 and 18 h respectively (supplemental Table S4). Finally, Chs6 (MGG_13013), the only chitin synthase required for plant infection (42), was identified but not regulated.
In addition to chitin synthases, a number of proteins involved in chitin metabolism were also regulated. Two of the most strongly up-regulated proteins during conidal germination are a putative glucosamine-6-phosphate isomerase (MGG_00625) and a putative N-acetylglucosamine-6-phosphate deacetylase (MGG_00620) (supplemental Table S4). These two proteins are predicted to be involved in the catabolism of N-acetylglucosamine, catalyzing the conversion of

### Table II

| Gene          | Annotation                                      | +cAMP vs. Untreated* | Wild type vs. ΔcpkA* |
|---------------|-------------------------------------------------|----------------------|----------------------|
| MGG_08526     | Hypothetical protein                            | 1.1 31.3 106.0 296.2* | 3.4 146.9*          |
| MGG_10036     | Phenylalanine and histidine ammonia-lyase       | 1.1 10.8* 15.3 60.5* | 2.0 21.2*           |
| MGG_13253     | Hypothetical protein                            | 1.1 18.2 12.8 56.8*  | 2.0 6.1*            |
| MGG_03436     | Hypothetical protein                            | 1.1 42.2 75.7 31.8*  | 2.0 35.6*           |
| MGG_00559     | Glucan 1,3-beta-glucosidase                     | 3.5 38.0* 77.7* 19.7 | 7.3 21.0*           |
| MGG_05366     | Furuloyl esterase B                             | 1.0 8.3 19.6 7.9*   | 1.9 9.5*            |
| MGG_17528     | Hypothetical protein                            | 2.9 4.6* 7.7 5.2*   | 5.6 9.2*            |
| MGG_08975     | 3-hydroxy-3-methylglutaryl-coenzyme A reductase | 1.7 4.1 4.1 4.2*   | 14.1 31.4*          |
| MGG_07790     | Ligninase H2                                     | 1.1 7.4* 4.9 4.1   | 2.0 7.2*            |
| MGG_13669     | Peptide transporter PTR2                        | 0.4 1.3 19.1* 3.8   | 1.5 5*              |
| MGG_01029     | Acetyl-hydrolase                                 | 1.6 0.7 1.4 3.8*   | 1.0 7.5*            |
| MGG_09372     | Hypothetical protein                            | 1.2 2.1 1.4 3.4*   | 1.4 3.8*            |
| MGG_11475     | Hypothetical protein                            | 1.5 1.5 1.7 2.9*   | 2.0 7.2*            |
| MGG_15950     | Hypothetical protein                            | 1.4 6.7 6.1* 2.7   | 1.7 25.7*           |
| MGG_05433     | Solute carrier family 6 protein                 | 1.8 1.5 3.9 2.6*   | 2.0 10.4*           |
| MGG_00141     | Leptomycin B resistance protein pmd1            | 2.3* 3.3 5.0 2.5   | 1.7 10.3*           |
| MGG_05989     | Seprase                                         | 1.9 2.0 3.0 2.5*   | 1.0 6.3*            |
| MGG_00871     | Phosphatidylinositol transfer protein CSR1      | 0.7 2.3 2.1 2.4*   | 1.6 7.2*            |
| MGG_01026     | Hydroxymethylglutaryl-CoA synthase              | 1.1 3.4* 2.4 2.3*   | 21.2 28.9*          |
| MGG_07219     | Polyetide synthase Alb1                         | 4.0* 3.9* 2.0 2.0  | 1.0 29.2*           |
| MGG_00878     | Hypothetical protein                            | 1.7 1.7* 1.3 2.0   | 1.8 3.7*            |
| MGG_03554     | 60S ribosomal protein L36                       | 1.1 1.9 2.2* 2.0*  | 2.0* 1.2            |
| MGG_16203     | SMC1A protein                                   | 3.6* 2.3 1.9 1.7   | 0.6 5.5*            |
| MGG_01210     | Mitochondrial hypoxia responsive domain-containing protein | 2.0 2.1* 1.0 1.6   | 2.3* 22.1*          |
| MGG_00819     | Niemann-Pick C1 protein                         | 1.2 2.1 2.9* 1.6   | 2.4 17.1*           |
| MGG_01557     | Phosphatidyglycerol/phosphatidylinositol transfer protein | 1.1 1.3 1.1 1.6*   | 1.3 2.4*            |
| MGG_09272     | Beta-glucosidase 1                              | 1.2 1.4 1.7* 1.5   | 1.9* 1.4            |
| MGG_00755     | Indoleamine 2,3-dioxgenase                      | 1.4 1.3 1.8* 1.4   | 1.2 2.4*            |
| MGG_01674     | Multidrug resistance-associated protein 1       | 1.3 1.6* 1.4 1.4   | 0.5 5.3*            |
| MGG_04378     | Integral membrane protein                       | 2.5* 1.7 1.2 1.3   | 1.9 7.2*            |
| MGG_15308     | Ubiquitin-protein ligase Sel1/Ubx2              | 0.9 3.6* 3.4 1.1   | 0.2 2.1*            |
| MGG_19610     | Hydrolase                                       | 0.9 1.1 1.8* 1.0   | 1.0 2.5*            |

*Fold changes marked by an asterisk were determined to be significantly differentially expressed (p value < 0.05)
N-acetylglucosamine-6-phosphate to fructose-6-phosphate. Homologous proteins in C. albicans, Nag1 and Nag2, are required for use of N-acetylglucosamine as a carbon source (44) suggesting M. oryzae may be recycling intermediates of chitin biosynthesis as a source of carbon during conidial germination. A putative β-N-acetyl-β-glucosaminidase, MGG_09922, containing a glycosyl hydrolase family 20 domain and predicted to catalyze the removal of N-acetylglucosamine residues from oligosaccharides was up-regulated during both germination and appressorium formation (supplemental Table S4). MGG_09922 is homologous to the Exc1Y Fks1 homolog, MGG_00865, which was detected in all bio-

| Gene       | Annotation                      | +cAMP vs. Untreated | Wild type vs. ΔcpkA |
|------------|---------------------------------|----------------------|----------------------|
|            |                                  | 4 hr | 8 hr | 12 hr | 18 hr | 8 hr | 18 hr |
| MGG_00432  | Hypothetical protein             | 0.71 | 0.56 | 0.68 | 0.45* | 0.46* | 0.28 |
| MGG_00522  | Hypothetical protein             | 0.57 | 0.40 | 0.28* | 0.13* | 0.20* | 0.57* |
| MGG_00719  | Aldehyde dehydrogenase 3H1       | 0.93 | 0.14* | 0.30 | 2.68 | 0.37* | 4.29 |
| MGG_00968  | Mitochondrial outer membrane protein porin | 1.04 | 0.95 | 0.62* | 0.84* | 1.19 | 0.67** |
| MGG_01153  | Proteasome subunit alpha type-6  | 0.83 | 0.76 | 0.91 | 0.52* | 0.66 | 0.41* |
| MGG_01622  | Uroporphyrinogen decarboxylase    | 0.82 | 0.61* | 0.57 | 0.68 | 0.20* | 0.65 |
| MGG_02570  | Dienealactone hydrolase          | 0.52 | 0.38 | 0.29 | 1.14* | 0.20* | 1.08 |
| MGG_02713  | Nascent polypeptide-associated complex subunit beta | 1.07 | 0.65* | 0.56 | 0.89 | 0.69 | 0.45* |
| MGG_02833  | GTPase-activating protein GYP2    | 0.69 | 0.60 | 0.46* | 0.76 | 0.27* | 0.26 |
| MGG_04311  | 3’,5’-bisphosphate nucleotidase   | 1.19 | 0.46* | 0.58 | 0.41 | 0.21* | 0.54 |
| MGG_04425  | Phenylalanine-5'-ribonucleotide synthase subunit alpha | 0.66 | 0.56 | 0.62 | 0.59* | 0.09* | 1.09 |
| MGG_04584  | HNRP arginine N-methyltransferase| 1.06 | 0.60 | 0.33* | 0.66 | 0.50* | 1.16 |
| MGG_05250  | Hypothetical protein             | 0.88 | 0.39 | 0.51 | 0.32* | 0.38* | 0.95 |
| MGG_05956  | Glutamyl-5'-ribonucleotide synthase| 0.97 | 0.62* | 0.94 | 0.85 | 0.61* | 0.84 |
| MGG_06751  | Hypothetical protein             | 0.84 | 0.56* | 0.26* | 0.30* | 0.26* | 0.24* |
| MGG_08622  | Nucleoside diphosphate kinase    | 0.70 | 0.77 | 0.64 | 0.61* | 1.31 | 0.67* |
| MGG_09288  | Hexokinase                       | 1.06 | 0.45 | 0.67 | 0.28 | 2.07 | 0.13* |
| MGG_09355  | NACHT domain-containing protein  | 0.46 | 0.25* | 0.19* | 0.12* | 0.35* | 0.30 |
| MGG_09902  | F-actin-capping protein subunit beta | 0.63 | 0.48* | 0.45* | 0.52 | 0.22* | 0.51 |
| MGG_10111  | Glucose and ribitol dehydrogenase| 0.83 | 0.43* | 0.40 | 0.13 | 0.36* | 0.65 |
| MGG_11196  | CAP20                            | 1.04 | 0.86 | 0.58* | 0.50 | 1.40 | 0.56* |
| MGG_12822  | Glucose-6-phosphate isomerase    | 0.98 | 0.42* | 0.36 | 0.43* | 0.79 | 0.47* |
| MGG_13020  | Glucose-repressible alcohol dehydrogenase transcriptional effector | 0.91 | 0.59 | 0.28* | 0.92 | 0.43* | 2.91 |
| MGG_13068  | Venom protein 2                  | 0.72 | 0.52 | 0.27* | 1.04 | 0.16* | 1.62 |
| MGG_1527   | Hypothetical protein             | 1.09 | 0.42* | 0.54 | 0.71 | 0.26* | 1.12 |
| MGG_17278  | Arginosuccinate lyase            | 0.93 | 0.56* | 0.56 | 0.67 | 0.22* | 0.76 |

Expression of MGG_06771 transcripts was documented in infected rice leaves but not mycelium cultured in vitro, however, its role in the infection process is currently unknown (47).

β-1,3-Glucans, the most abundant component of fungal cell walls, are synthesized in a vectorial manner by a glucan synthase complex located at the plasma membrane (39). In S. cerevisiae, two of three FKS genes encoding β-1,3-Glucan catalytic subunits are involved in synthesis of β-1,3-Glucan from UDP-Glucose. The M. oryzae genome encodes a single Fks1 homolog, MGG_00865, which was detected in all biological conditions but was not differentially expressed. β-1,3-Glucan chains extruded into the periplasmic space by the glucan synthase complex require elongation and covalent linkage to other cell wall components. Elongation of β-1,3-Glucans is catalyzed by β-1,3-glucanotransferases of the GH72 family of glycoside hydrolases (49), which cleave the β-1,3 linkage of an existing β-1,3-Glucan chain and transfer the new reducing end to the nonreducing end of another β-1,3-Glucan chain producing a new β-1,3 linkage. The first member of the family was described in A. fumigatus whose genome encodes seven members of the GH72 family (49). Four members of the GH72 family are present in M. oryzae (MGG_03208, MGG_06722, MGG_07331, and MGG_08370) with a fifth, MGG_11861, not assigned to this family but having homology to the A. fumigatus Gel5 protein. One M. oryzae β-1,3-glucanotransferase, MGG_06722, was up-suggested by chitin binding activity, was up-regulated at the 12 and 18 h germination time points (supplemental Table S4).
regulated in the wild type when compared with the ΔcpkA mutant and a second protein, MGG_11861, was up-regulated at 12 h following cAMP treatment. A third β-1,3-glucanosyltransferase, MGG_08370, was up-regulated at 8, 12, and 18 h during germination. Although no information exists regarding the catalytic nature of these proteins, their regulation patterns suggest specific roles during conidial germination and appressorium development.

A putative β-1,3-exoglucanase, MGG_00659, is strongly up-regulated in response to cAMP treatment at 8 and 12 h and is down-regulated in the ΔcpkA mutant at 18 h (Table II). β-1,3-exoglucanases catalyze the release of single glucose residues from the nonreducing end of β-1,3-glucan chains. The MGG_00659 protein is predicted to be extracellular and the CAZY database classifies it as a member of the glycosyl hydrolase 55 protein family (48). The protein is homologous to Lam1.3 (59% identity) of Trichoderma harzianum (50, 51) and Exg1 (46% identity) of Cochliobolus carbonum (52), both of whose β-1,3-exoglucanase activity has been experimentally characterized. The MGG_00659 transcript is induced during appressorium formation and a MGG_00659 deletion mutant retained pathogenicity and the ability to form appressoria (18).

A second putative β-1,3-exoglucanase, MGG_14087, with 50% identity to Lam1.3 was significantly up-regulated at 4 and 18 h during germination. Finally, a putative endo β-1,3-glucanase, MGG_05489, with homology to Eng11 of A. fumigatus (53) was also up-regulated at 18 h in the wild type when compared with the ΔcpkA mutant.

A host of additional proteins likely involved in cell wall modification were up-regulated by cAMP treatment or down-regulated in the ΔcpkA mutant. Included were a GH3 family putative β-glucosidase (MGG_10038), up-regulated at 18 h following cAMP treatment and previously demonstrated to be induced at the transcript level during appressorium formation (18). A second putative β-glucosidase, MGG_01885 belonging to the GH3 family, was down-regulated in the ΔcpkA mutant at 18 h. A GH92 family member encoding a putative α-1,2-mannosidase (MGG_07146) was up-regulated at 12 h in the cAMP treated sample. Two GH16 family proteins, MGG_00592 and MGG_01134, encoding putative glucosyltransferases were down-regulated in the ΔcpkA mutant at 18 h. MGG_00592, and to a lesser extent, MGG_01134, are homologous to the A. fumigatus Cfr1 protein (54) and the S. cerevisiae Crh1 protein (55), both of the Crh family of glycosylphosphatidylinositol (GPI)-anchored glucosyltransferases involved in the crosslinking of β-1,6-glucans to chitin. MGG_01134 also contains a CBM18 domain typically involved in the binding of chitin.

A critical role for melanin in the formation of functional appressoria has long been recognized (4, 56–58). Four genes involved in the synthesis of melanin have been identified including the hydroxynapthalene reductase BUF1 (MGG_02252), the polyketide synthase ALB1 (MGG_07219), the scytalone dehydratase RSY1 (MGG_05059), and 4HNR (MGG_07216) (18, 56, 59). Transcripts of all four genes were up-regulated during early appressorium formation on a hydrophobic surface and in response to cAMP treatment (18). We previously reported that Buf1, 4HNR and Rsy1 are among the most abundant proteins in M. oryzae conidia (29). All three proteins remained highly abundant during germination and appressorium formation with no significant regulation. However, Alb1, which catalyzes the first step in melanin biosynthesis and whose abundance is much lower in conidia was down-regulated during germination at 4 and 8 h, up-regulated by cAMP treatment at 4 and 8 h (supplemental Table S4) and strongly down-regulated in the ΔcpkA mutant at 18 h (supplemental Table S5).

Regulation of Transport and Extracellular Proteins—Conidia serve as agents of dispersal and as survival structures. Central to these roles is the ability to interact with their environment via the secretion of extracellular enzymes and uptake of external nutrients. GO analysis revealed an enrichment of transport functions in the set of proteins up-regulated by cAMP as well as those down-regulated during germination. The transporter activity GO category (GO:0005215) was enriched in the set of 166 proteins up-regulated by cAMP (supplemental Table S6) with 21 of the regulated proteins assigned to this category. An additional 12 of the 166 cAMP up-regulated proteins are assigned to at least one of the following GO categories; transport (GO:0006810), ion transport (GO:0006811), or protein transport (GO:0015031). These 33 transport related proteins up-regulated in response to cAMP are listed in supplemental Table S7. In addition, the regulation of nine of these 33 proteins was dependent on the presence of CpkA. Finally, the process of conidial germination resulted in the down-regulation of 77 proteins (supplemental Table S8) associated with at least one of the aforementioned GO categories and also an enrichment of the ion transport GO category (supplemental Table S6).

The golgi-localized P-type ATPase, Apt2 (MGG_02767), which is involved in exocytosis of an as yet undefined collection (60) of proteins responds strongly to cAMP treatment. Apt2 is required for normal pathogenicity and elicitation of the hypersensitive response during incompatible host-pathogen interactions (60). A putative plasma membrane localized H^+ ATPase, Pma2 (MGG_04994), was also induced by cAMP treatment at 18 h. The M. oryzae genome encodes a second H^+ ATPase, Pma1 (MGG_07200), which is one of the most abundant proteins of the conidial proteome (supplemental Table S4) and is not regulated during germination or appressorium formation. Pma1 is a homolog of yeast Pma1p and likely represents the housekeeping H^+-ATPase responsible for generation of proton gradients across the plasma membrane. On the other hand, Pma2 is much less abundant and up-regulated during appressorium formation. In addition, expression of PMA2 (referred to as Pma1 by Zhang et al. (61) and Pma2 by Gilbert et al. (60)) is higher when using ammonia rather than nitrate as a nitrogen source and its regulation is
partially dependent on the activity of the glucokinase Glk1 (61). However, its role in appressorium formation remains unclear.

The *M. oryzae* genome encodes four TRK family transporters, which are involved in the uptake of K⁺ and Na⁺ in other organisms (62). One member of the family, MGG_09119, was up-regulated during appressorium formation. Two putative K⁺-Na⁺ efflux P-type ATPases, MGG_02074 and MGG_010730, were up-regulated and a third, MGG_05078 was down-regulated by cAMP treatment. Interestingly all three were down-regulated by germination (supplemental Table S8). RNA silencing of these K⁺-Na⁺ efflux P-type ATPases resulted in strains with growth, conidiation, and pathogenic defects including deficiencies in establishment of appressoria (63).

Two putative Ca²⁺-transporting ATPases, MGG_02487 and MGG_07971, and a putative Ca²⁺/H⁺ antiporter, MGG_01381, were down-regulated by germination. MGG_02487 and MGG_07971 are homologs of the yeast vacuolar calcium pump, Pmc1p, which plays a role in cellular calcium homeostasis by transporting Ca²⁺ into the vacuole (64). RNA silencing-mediated knock down of MGG_02487 and MGG_07971 results in strains with severe growth defects and reduced conidiation (63) pointing toward an important role for calcium homeostasis during germination.

A number of other transport proteins are strongly down-regulated during germination. MGG_15745, a putative MFS family transporter, is a homolog of *S. cerevisiae* YMR221C, which was identified in the yeast mitochondrial proteome (65) and demonstrated to interact with *S. cerevisiae* Atg27 (66), a protein proposed to direct donor membranes to the pre-autophagosomal structure (67). MGG_00045, a putative alpha glucoside permease, shows 32% identity to the *S. cerevisiae* maltose transporters, Mph2 and Mph3 (68). In addition, two ABC type transporter proteins, MGG_01674 and MGG_03736, were down-regulated at all four germination time points and are homologous to the yeast proteins Ycf1 and Ybt1, respectively. Ycf1 is involved in transport of glutathione-metal conjugates into the vacuole (69) and Ybt1 was demonstrated to be a vacuolar ATP dependent bile acid transporter (70).

Pathogenic development by *M. oryzae* on the leaf surface is characterized by the production and secretion of extracellular enzymes that aide in the penetration phase of the infection process. Analysis of the subcellular localization of wild-type proteins up-regulated by cAMP treatment (Figs. 3A and 3C) or when compared with the ΔcpkA mutant in the presence of cAMP (Figs. 3C and 3D) identified a higher percentage of extracellular proteins relative to the whole set of proteins identified in each experiment. The 166 proteins up-regulated by cAMP treatment contain 39 proteins predicted to be extracellular by WolfPSORT (71). Likewise, another 32 predicted extracellular proteins were more abundant in the wild type when compared with the ΔcpkA mutant. Collectively, 60 unique putative extracellular proteins were identified as up-regulated either in response to cAMP treatment or by loss of regulation in the ΔcpkA mutant (Table S9).

Several extracellular proteins likely involved in degradation of cell walls were induced during cAMP-mediated appressorium formation. A cutinase, Cut2 (MGG_09100), whose transcripts are up-regulated during appressorium formation (72)
and is required for normal development of appressoria and complete virulence (73), was strongly up-regulated by cAMP treatment at 18 h. Interestingly, treatment of a Δcut2 mutant with cAMP or other inducers of the cAMP signaling pathway restored appressorium formation to wild-type levels indicating that Cut2 lies upstream of the cAMP signaling pathway (73). A putative fungal lignin peroxidase, MGG_07790, was up-regulated 7.4-fold by cAMP treatment at 8 h and up-regulated 7.2-fold at 18 h when compared with the ΔcpkA mutant (Table II). Transcripts for MGG_07790 were up-regulated during appressorium formation in response to cAMP and on an inductive hydrophobic surface (18). Two putative feruloyl esterases, MGG_09404 and MGG_05366 and, were induced during appressorium formation at 8 and 18 h respectively (Tables II and supplemental Table S4). Both proteins contain a tannase and feruloyl esterase family domain (pfam07519) and show homology to the Aspergillus niger FaeB protein, which is involved in the degradation of plant cell walls via release of ferulic acid or other aromatic compounds from cell wall polysaccharides with a preference for plant pectins (74). Finally, a multicopper oxidase, MGG_13764, with homology to fungal laccases and billirubin oxidases was 45.7-fold more abundant in the wild type than the ΔcpkA mutant. This protein was recently demonstrated to contain billirubin oxidase activity (75). However, like billirubin oxidases from other filamentous fungi, its biological substrate and function remains unclear. Related laccases from filamentous fungi catalyze oxidation of phenolic compounds and play roles in both development and degradation of plant lignins (76, 77).

Regulation of Ribosomal Proteins During Conidial Germination—Previous studies demonstrated that translation is essential for conidial germination in fungi (78, 79). In this study, 114 ribosomal proteins were identified of which 12 are up-regulated and 16 are down-regulated during germination. Based on homology to the well characterized cytoplasmic and mitochondrial ribosomes of S. cerevisiae (80–82), the 12 up-regulated proteins are components of the cytoplasmic ribosome and 14 of the 16 down-regulated proteins belong to the mitochondrial ribosome consistent with an enrichment of the mitochondrial GO category in the proteins down-regulated during germination (supplemental Table S6). In addition, the process of germination resulted in an increase in abundance of six proteins predicted to be associated with the nuclear preribosome complex. MGG_02505, shares homology to the yeast cytoplasmic proteins Reh1p and Rei1p, which are involved in stabilization of the cytoplasmic 60S ribosomal subunit and also nuclear import of the 60S ribosomal subunit export factor, Arx (83). MGG_01561 is a homolog of yeast Nog2p, a putative GTPase required for processing of the 27S rRNA precursor of the mature 25S rRNA and export of the pre 60S ribosome from the nucleus (84). MGG_00482 is a homolog of the yeast Nip7 protein, which is required for pre-rRNA processing and biogenesis of the 60S ribosomal subunit (85). MGG_06242 contains a Kr1-like family C-terminal domain also found in the yeast Kr11 protein, which is required for biogenesis of the 40S ribosomal subunit (86). MGG_07258 is similar to the yeast Mpp10 protein, which is required for processing of pre-18S rRNAs in yeast (87). Finally, MGG_03080 contains two WD40 like domains and shows weak homology to the yeast SQT1 protein, which is required for normal assembly of the 60S ribosomal subunit (88). The up-regulation of numerous cytoplasmic ribosomal proteins and putative ribosomal biogenesis factors indicates that conidial germination stimulates production of ribosomes.

Down-regulation of Mitochondrial Proteins During Germination—As mentioned previously, the set of 383 proteins down-regulated during conidial germination were enriched in the mitochondrial cellular component GO category (GO: 0005739). Forty-five of the down-regulated proteins were assigned to this category (supplemental Table S10). An examination of the oxidative phosphorylation pathways in filamentous fungi identified 76 M. oryzae genes likely to encode components of this pathway (89). A total of 57 of these proteins were identified in the proteomics data, 17 were down-regulated in at least one germination time point and none were up-regulated (Table S10). Included in the down-regulated proteins were 11 putative NADH dehydrogenase subunits of electron transport complex I, two putative alternative NADH dehydrogenases and nine probable components of the mitochondrial import complexes of the inner (TIM23 complex) and outer (TOM complex) mitochondrial membranes involved in the mitochondrial import of nuclearencoencoded preproteins synthesized in the cytoplasm (90). Also down-regulated was a putative mitochondrial presequence protease, MGG_02440, whose yeast homolog, Cym1, is involved in the degradation of mitochondrial presequence peptides after their removal from imported preproteins (91). The elevated abundance of mitochondrial proteins in conidia before germination suggests a need for significant energy production to initiate germ tube development.

Regulation of Calpain-like Proteases—Previous reports have established a link between protein processing and turnover and appressorium development (18, 92). Two putative thiol proteases, MGG_08526 and MGG_14872, are both strongly up-regulated at 18 h in the presence of cAMP, with MGG_08526 being the most strongly up-regulated protein in the data set (Tables II and supplemental Table S4). MGG_08526 is also regulated in a CpkA-dependent fashion (Table II). Transcripts for both proteins were up-regulated during appressorium formation (18). MGG_14872 contains a cysteine proteinase domain with similarity to domain II of the calpain family of calcium-dependent cysteine proteinases but lacks other protein domains typically associated with calpains. A total of four M. oryzae proteins contain this cysteine proteinase domain and all are predicted to be localized to the nucleus. The three remaining cysteine proteinase domain containing proteins (MGG_06335, MGG_07573 and MGG_15810) were not detected in this study. MGG_08526 is also predicted
to be nuclear in localization and contains a partial cysteine proteinase domain lacking the N-terminal portion of this domain harboring a conserved cysteine residue required for catalytic activity suggesting it may not have proteolytic activity. Analysis of a MGG_08526 gene deletion mutant revealed no gross defects in appressorium formation or pathogenicity (18). Calpain-like cysteine proteinases have been identified in other fungi and include the *Aspergillus nidulans* PalB and *S. cerevisiae* YMR154c, both of which are involved in the adaptation to alkaline conditions and the latter also being required for efficient sporulation (93–95). In mammalian systems, nuclear calpains are known to mediate cleavage of calcium-dependent protein kinases and thereby influence Ca$$^{2+}$$ signaling pathways (96). Based on the roles of similar signaling proteinases in other organisms and their regulation during appressorium formation it suggests that these putative proteinases have a role in signal transduction during infectious development in *M. oryzae*.

**Regulation of Secondary Metabolism**—During the process of appressorium formation a number of enzymes central to the biosynthesis of secondary metabolites were induced including enzymes representing key entry points to secondary metabolism. A putative phenylalanine ammonia lyase (PAL), MGG_10036, was up-regulated by cAMP treatment in a CpkA-dependent manner (Table II). MGG_10036 transcripts were previously reported to be up-regulated during appressorium formation (18). PAL catalyzes the production of cinnamic acid and ammonia from phenylalanine and serves as the entry point to the phenylpropanoid pathway. Although MGG_10036 is up-regulated at the transcript and protein level during appressorium formation, deletion of this gene had no effect on appressorium development or pathogenicity (18).

The mevalonate pathway converts acetyl-CoA to isopentenyl diphosphate from which sterol and nonsterol isoprenoid biosynthesis is initiated. HMG-CoA synthase (MGG_01026) and HMG-CoA reductase (MGG_08975) are involved in synthesis of mevalonate and represent the rate-limiting steps of the pathway. Both proteins are up-regulated at 18 h during appressorium formation (Table II). In addition, up-regulation of transcripts of both genes was observed in appressoria (18). Interestingly, the induction of both proteins by cAMP treatment is CpkA dependent as both proteins were more abundant in wild-type appressoria than the ΔcpkA mutant (Table II). The three proteins downstream of HMG-CoA reductase responsible for the conversion of mevalonate to isopentenyl diphosphate, including, MGG_16219 (a putative mevalonate kinase), MGG_05812 (a putative phosphomevalonate kinase) and MGG_09750 (a putative diphosphomevalonate decarboxylase) were all identified but not significantly regulated. The up-regulation of enzymes required for production of secondary metabolite precursors during appressorium formation indicates an enhanced flux through these pathways during the developmental process.

**Validation of the Global Proteomics Data Via Absolute Quantification**—To further validate the quantification and differential expression of proteins within the global proteomics data sets an absolute quantification of a select group of regulated proteins was generated by selected reaction monitoring (SRM) (97). The selected proteins included two proteins up-regulated by cAMP treatment (MGG_08526 and MGG_04994), a protein down-regulated by cAMP treatment (MGG_09355) and a protein up-regulated during germination (MGG_05580). Synthetic peptides containing a heavy isotope labeled arginine or lysine were spiked into triplicate protein samples produced from conidia and conidia germinated in the presence or absence of 50 mM cAMP at 4, 8, 12, 18, and 24 h for both the wild type and ΔcpkA mutant. Quantification of the target peptides was performed on a TSQ Vantage triple stage quadrupole mass spectrometer. The quantification of the four selected proteins is presented in Fig. 4. In each instance the regulation of the target protein as determined by SRM is in agreement with the global proteomics data. A putative CAIB/BAIF family CoA transferase, MGG_05580, was up-regulated during germination (supplemental Table S4 and Figs. 4A and 4B). However, the abundance of this protein is reduced by cAMP treatment. Interestingly, the abundance of this protein is greater in the ΔcpkA mutant than the wild type and the reduction in protein levels by cAMP treatment is also observed in the ΔcpkA mutant suggesting a suppression of MGG_05580 protein levels by cAMP signaling. A protein of unknown function, MGG_09355, containing a NACHT domain typically found in components of signaling pathways with scaffold functions was strongly repressed by cAMP treatment (supplemental Table S4 and Figs. 4D and 4E). This protein is abundant in conidia and remains abundant during germination in the absence of cAMP. Similar to MGG_05580, the MGG_09355 protein is more abundant in the ΔcpkA mutant but still responds negatively to cAMP treatment. Conversely, Pma2, a putative plasma membrane-localized H$$^{+}$$-ATPase and the putative protease, MGG_08526, are up-regulated by cAMP treatment in the global proteomics data and the SRM data (supplemental Table S4 and Figs. 4G, 4H, 4J, and 4K). Pma2 increases in abundance during germination but responds strongly to cAMP treatment in a CpkA-dependent manner. MGG_08526 is the protein most strongly up-regulated by cAMP treatment and was undetected in the SRM analysis of germinated samples not treated with cAMP. The agreement of protein regulation observed in the global proteomics data with that observed in the SRM data indicates that the spectral counting based label free quantification used in this study provides an accurate means of identifying regulated proteins. Finally, the SRM data includes a 24 h time point not included in the global proteomics studies. It is apparent from this data that changes in protein abundance in response to cAMP treatment were stronger or at least equivalent for the analyzed proteins at 24 h as compared with 18 h.
suggesting that further analysis of mature appressoria at later time points is warranted.

Comparison of the cAMP Responsive Transcriptome and Proteome—A DNA microarray-based analysis of gene expression revealed the up- and down-regulation of 644 genes and 370 genes, respectively, in a comparison of cAMP treated and untreated M. oryzae conidia germinated for 9 h on a hydrophilic surface (18). A comparison of differentially expressed genes derived from this transcriptome data and proteins regulated at 8 h during appressorium development described here is presented in supplemental Fig. S2. No correlation between changes in mRNA and protein abundance is observed when genes for which both transcript and protein information is available are considered (supplemental Fig. S2). A similar lack of correlation was observed when proteomes from the other time points were compared with the

Fig. 4. Absolute quantification of four proteins by SRM. SRM-based absolute quantification (A, D, G, J), average spectral count data (B, E, H, K), and relative transcript abundance (C, F, I, L) of four regulated proteins including MGG_05580 (A, B, C), MGG_09355 (D, E, F), MGG_04994 (G, H, I) and MGG_08526 (J, K, L). Protein abundance in SRM data is reported as fmol of target protein per 10 μg of total protein with averages from three biological replicates and standard errors shown. Average normalized spectral counts from experiment 1 are plotted with standard errors shown. Transcript abundance from qRT-PCR is presented as average measurements of expression relative to actin from three biological replicates with standard errors shown. Wild-type (blue and red lines) and ΔcpkA mutant (green and purple lines) conidia were germinated on a hydrophilic surface in the presence (red and purple lines) or absence (blue and green lines) of 50 mM cAMP and three biological replicates were analyzed for each condition.
9 h transcriptome (data not shown). However, 53 genes whose transcripts were significantly regulated (29 up- and 24 down-regulated) also showed a significant change in protein abundance at 8, 12, or 18 h in the same direction (supplementary Fig. S3 and supplemental Table S11). Specifically, at the 8 h time point, proteins for 20 of the 53 genes were significantly regulated and the remaining 33 proteins, although not significantly regulated, showed a positive correlation to the transcriptome data. To further explore the relationship between transcript and proteins levels we performed qRT-PCR analysis for a set of 12 genes whose proteins were significantly regulated during one or more stages of development. Samples for qRT-PCR analysis were generated in a manner consistent to those for proteomics and transcriptomics studies. A comparison of fold changes for cAMP treated and untreated samples from four time points (4, 8, 12, and 18 h) of qRT-PCR data to the 9 h DNA microarray-based transcriptomics data is presented in supplemental Fig. S4. As expected, the 8 h qRT-PCR data shows a strong correlation ($R^2 = 0.84$) to the 9 h transcriptome with weaker correlations observed for other qRT-PCR time points indicating that changes in gene expression are consistent and reproducible under our experimental conditions even when samples are generated years apart.

Measurements of transcript abundance by qRT-PCR for 12 regulated proteins revealed three trends regarding changes in gene expression and protein abundance. Four of the genes examined showed a positive relationship between changes in transcript and protein abundance. Three of these genes (MGG_08526, MGG_4994, and MGG_00659) were up-regulated during appressorium formation and a fourth (MGG_05580) was up-regulated during germination. As shown in Fig. 4 and supplemental Fig. S5, changes in transcript abundance for these four genes tend to occur before observed changes in protein abundance although the pattern of regulation remain highly similar. A second set of three genes were characterized by transcripts that are most abundant in conidia and fall sharply following germination whereas protein levels generally increase in one treatment and fall in the other. At the protein level, MGG_04714 and MGG_00150 are up-regulated by cAMP treatment even though transcript levels fall dramatically after germination (supplemental Fig. S5). Transcripts of MGG_09355 are also reduced upon germination, falling more quickly in the cAMP treated sample reflecting the down-regulation of the protein during appressorium development whereas protein levels trend upward at later stages of germination (Fig. 4). Finally, the remaining five genes (MGG_00302, MGG_01679, MGG_06660, MGG_09272, and MGG_02570) show complex patterns of regulation from which no obvious relationships emerge.

**DISCUSSION**

Technological advances in the field of mass spectrometry-based proteomics now allow proteome analysis at depths unobtainable just a few years ago. The introduction of the FASP methodology and StageTip-based peptide level fractionation resulted in a doubling of M. oryzae conidial proteome coverage relative to previous work using GeLC-MS/MS (28, 29) while requiring only small amounts of starting material (50 μg). In the present study, we have identified 3200 unique proteins present in conidia, germinating conidia, or cAMP-induced appressoria. Interestingly, only 193 of the identified proteins were not detected in conidia and therefore are specific to germination or appressorium formation. Recent investigations have revealed that although conidia are considered dormant structures necessary for survival and dispersal in the environment, their proteomes are highly complex and proteins of the central metabolic pathways as well as those required for cellular maintenance are well represented (29, 98, 99). This observation, in addition to bias in LC-MS/MS based proteomics toward identification of proteins of high abundance, suggests that the number of identifiable proteins unique to germination or appressorium development may be small as observed here. Finally, the protein identifications presented here and by Gokce et al. (29) total 3540 proteins, which represents 27.6% of the predicted M. oryzae proteome.

Conidia were germinated in the presence and absence of cAMP to allow investigation of the germination and appressorium formation processes independently. Using spectral counting-based label free quantification we were able to observe temporal changes in protein abundance for hundreds of proteins highlighting the dynamic nature of the M. oryzae proteome. As expected, by four hours a large number of proteins were regulated in response to germination and relatively few responded to cAMP treatment. However, by the onset of appressorium formation at 8 h a larger number of proteins showed cAMP dependent regulation. In addition, a direct comparison of wild type and ΔckpA mutant conidia germinated in the presence of cAMP identified proteins whose regulation during appressorium formation was dependent on CpkA activity, which is required for pathogenicity and normal appressorium formation (10, 11). A total of 59 proteins were regulated (33 up-regulated and 26 down-regulated) by cAMP treatment in a CpkA dependent manner and are therefore responsive to cAMP signaling.

The emergence and elongation of conidial germ tubes followed by the formation of an appressorium is a dynamic process that necessitates de novo cell wall biogenesis and substantial remodeling of existing structures. The importance of this process is reflected in the abundance and regulation of a large number of proteins involved in cell wall metabolism including proteins central to chitin, β-1,3-glucan, and melanin production in addition to proteins containing domains associated with carbohydrate modification whose specific catalytic activities remain unclear. Six of the seven M. oryzae chitin synthase proteins were identified with two being up-regulated during germination. Two proteins central to chitin degradation, a putative glucosamine-6-phosphate isomerase and a
putative N-acetylglucosamine-6-phosphate deacetylase were also up-regulated during germination and a chitin deacetylase was up-regulated during appressorium formation. These observations indicate a recycling of chitin during germ tube elongation for use where new cell wall material is being produced and a conversion of cell wall chitin to chitosan in the maturing appressorium. Chitosan has been documented on the surface of infection structures in planta for a number of plant pathogenic fungi and it was proposed that conversion of chitin to chitosan prevents degradation of the fungal cell wall by plant-derived chitinases (46, 100). Finally, the four proteins involved in the synthesis of melanin are among the most abundant proteins in the conidial proteome and remain highly abundant throughout the development of an appressoria. Interestingly, the least abundant of the four proteins and the first enzyme of melanin biosynthesis pathway, the polyketide synthase, ALB1, was the only protein of the group shown to be differentially regulated at the protein level. This suggests that resting conidia are primed for melanin biosynthesis and regulation of the first enzyme in the pathway enables sufficient melanin production for appressorium development.

Conidia mobilize storage reserves to provide energy and biosynthetic precursors for germination. Rapid use of these reserves is required for timely germination when environmental conditions are favorable. The extensive down-regulation of mitochondrial proteins during germination indicates that mitochondria or at least mitochondrial proteins are generally more abundant in conidia and suggests a need for rapid energy generation during the initial stages of germination. Furthermore, a large number of transport-related proteins were down-regulated during the germination process, supporting a reliance on storage reserves for germination in nutrient poor environments.

During appressorium formation an up-regulation of predicted extracellular proteins was observed. Previous studies examining gene expression during the formation of appressoria revealed the regulation of a large number of genes encoding putative extracellular proteins (18, 20). Many of the up-regulated extracellular proteins belong to families of carbohydrate modifying enzymes. These proteins likely fall into two classes, those that are involved in assembly and modification of the fungal cell wall as mentioned previously and those that are involved in degradation of the plant cell wall during the penetration phase of infection. A number of proteins likely involved in the breakdown of the plant cell wall were strongly up-regulated at the later stages of appressorium formation including the cutinase, Cut2, previously shown to be required for host penetration (73), and a number of putative peroxidases, laccases, and feruloyl esterases potentially involved in the degradation of plant lignin and pectin. Likewise, three enzymes representing key regulatory points of secondary metabolism including a putative phenylalanine ammonia lyase, HMG-CoA synthase and HMG-CoA reductase were up-regulated during appressorium formation, suggesting that in addition to the production of extracellular proteins during appressorium formation, M. oryzae may also be producing secreted secondary metabolites that are important for pathogenicity.

Finally, changes in protein abundance observed in proteomic studies often fail to correlate well with changes in gene expression observed in transcriptomic studies performed on similar biological samples (101) and a similar trend was observed here during appressorium formation. This is lack of correlation is typically attributed, in part, to the independent analysis of different experimental samples at separate times and often in different laboratories. On the other hand, increasing evidence suggests that translational regulation may be the most important factor controlling cellular protein levels and therefore significant discordance between protein and mRNA expression is expected (101). Restricting the analysis to those proteins that are significantly regulated resulted in a marked improvement in correlation to transcript abundance during appressorium development indicating a more positive relationship between protein and transcript changes for regulated proteins. Interestingly, a detailed analysis of gene expression during development by qRT-PCR indicated that changes in transcript abundance are directly reflected in protein levels, with a time delay, for some genes. Transcript abundance for another set of genes is highest in conidia and decreases substantially following germination although this change is not reflected in protein levels pointing toward the existence of pools of mRNA residing in conidia that are actively transcribed during germination to produce stable proteins. Furthermore, in several instances no obvious link between mRNA and protein levels was observed and the patterns of regulation were complex, which further supports the lack of correlation between the global transcriptomic and proteomic studies. Although changes in gene expression do not accurately reflect changes in protein abundance following cAMP treatment, a set of 53 proteins whose differential expression is in agreement at both the transcript and protein level was identified. Many of these proteins have known roles in appressorium development and pathogenicity as discussed above and the remainder are likely candidates for further investigation.

The data presented here provides the most extensive protein level analysis of appressorium formation performed to date and provides key insights into the development and physiology of the early stages of infection-related development. The data set also serves as a resource for the M. oryzae research community and provides a foundation for future research on post translation modification of the M. oryzae proteome.

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