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EDITORIAL

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Unraveling the phosphorylation landscape: a leap forward in understanding the rice blast fungus pathogenicity

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Abstract

The rice blast fungus *Magnaporthe oryzae* stands as a formidable adversary to one of the world's most important crops, rice, which feeds over half of the global population. Its ability to rapidly evolve and adapt underscores the urgent need for a comprehensive understanding of its infection strategies. In a large-scale study published in Cell, Cruz-Mireles et al. (Cell 187:2557-73, 2024) utilized phosphoproteomics to globally map the phosphorylation landscape during the infection-related development by *M. oryzae*, identifying 2062 activated phosphoproteins carrying 8005 phosphosites. A subset of these phosphosites were conserved in the proteins of diverse fungal pathogens and appeared to be associated with biotrophic and hemibiotrophic infection. Thirty-two of these phosphoproteins are regulated by pathogenicity mitogen-activated kinase 1 (Pmk1), a central component of the MAPK signaling pathway, including VTi 1–2 suppressor, whose regulation by Pmk1 is essential for rice blast disease. Together, this global phosphorylation atlas offers a rich tapestry of potential therapeutic targets for developing green agrochemicals to control fungal diseases of plants.

Keywords Infection-related fungal development, Phosphorylation atlas, Mitogen-activated protein kinases, Pmk1, Vts1

The rice blast fungus *Magnaporthe oryzae*, which destroys enough rice to feed 60 million people annually, infects rice plants when fungal spores called conidia land on the plant surface and germinate to form melanized appressoria within which high turgor pressure equivalent to 8 MPa (the highest pressure reported in any biological system), enabling the pathogen to pierce the rice cuticle mechanically through infection pegs and subsequently colonize plant tissues (Ryder et al. 2023). The development of infection-related structures, such as appressoria, involves significant morphogenetic changes and signaling transduction events, including phosphorylation, regulated by the mitogen-activated protein kinases (MAPKs). The pathogenicity mitogen-activated kinase 1 (Pmk1) is a central component of MAPK signaling and is essential for appressorium development (Xu and Hamer 1996). The *M. oryzae* mutants lacking *Pmk1* can not form appressoria and, consequently, are non-pathogenic on rice plants. In addition, Pmk1 regulates nearly half of the fungus's transcriptome during appressorium development (Osés-Ruiz et al. 2021).

The components of the Pmk1 cascade have been identified based on their homologs in the yeast Fus3/Kss1 pathway, which is crucial for pheromone signaling

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and invasive growth (Elion et al. 1990, 1991). Upstream kinases Mst11 and Mst7 were characterized based on their similarity to yeast MAPKKK Ste11 and MAPKK Ste7 (Zhao et al. 2005). The adaptor protein Mst50, homologous to Ste50, regulates the Mst11-Mst7-Pmk1 MAP kinase module during appressorium formation (Park et al. 2006). Downstream interactors of Pmk1, including transcription factors Mst12, Hox7, Slf1, and Pic5, have also been identified, but their roles in regulating infection are not fully understood. Pmk1 homologs have been found in over 30 fungal pathogen species, all necessary for pathogenicity (Rispail et al. 2009; Jiang et al. 2018). These include pathogens responsible for major crop diseases like Septoria blotch of wheat (*Zymoseptoria tritici*), southern corn leaf blight (*Cochliobolus heterostrophus*), and Fusarium head blight (*Fusarium graminearum*), exhibit distinct infection lifestyles, such as biotrophy, hemibiotrophy, and necrotrophy (Savary et al. 2019). This suggests that the Pmk1 MAPK pathway is a conserved mechanism for fungal invasive growth across diverse pathogens. However, the global targets phosphorylated by Pmk1 have not been identified.

Cruz-Mireles et al. (2024) took advantage of recent advances in quantitative mass spectrometry to catalog phosphoproteins and phosphosites thereof during infection-related development by *M. oryzae*. A total of 8005 phosphosites were mapped onto 2062 proteins activated during the 6 h time course, during which *M. oryzae* conidia were incubated on glass coverslips for 6 h to form appressoria, mimicking the early infection phase of the pathogen on rice leaves. Using a label-free MS1-quantification approach (LFQ), the authors quantified 7048 phosphosite-carrying peptides, 5058 of which were differentially accumulated in germinating conidia (1–2 h) and appressorium formation (4–6 h) compared with conidia that, together with differential expression of up to 50% during appressorium formation (Osés-Ruiz et al. 2021), suggest a substantial re-wiring of phosphorylation-related signaling cascades. The phosphorylation dynamics of Pmk1 were also tracked, showing peak activity between 1 to 4 h post-germination and a second activation phase between 4 to 6 h. K-means clustering identified five clusters of differential phosphopeptides, corresponding to specific stages of infection development, with significant differences between the wild-type strain Guy11 and the Δ*pmk1* mutant. GO term enrichment analysis revealed that protein phosphorylation, signal transduction, autophagy, lipid binding, and intracellular transport are the key processes during infection-related development. These clusters showed distinct patterns: Cluster I included proteins present in conidia with reduced abundance during development; Cluster II contained proteins requiring Pmk1 for phosphorylation; Cluster III had early-appressorium development proteins, showing phosphorylation independent of Pmk1; Cluster IV and V included proteins crucial for appressorium formation and subsequent infection stages, with many showing Pmk1-dependent phosphorylation. Overall, the study demonstrated that the infection-related development of *M. oryzae* is accompanied by extensive changes in the phosphoproteome, with Pmk1 playing a central role in regulating these changes and ensuring the virulence of the fungus.

Cruz-Mireles et al. (2024) further explored the conservation of phosphosites across various fungal species with distinct pathogenic lifestyles. Phosphorylation sites, known to be functionally critical, are often conserved across species (Studer et al. 2016). To determine whether the identified phosphosites are conserved, the authors computed orthogroups using Orthofinder (Emms and Kelly 2019) with the proteins from 41 filamentous fungal species representing various lifestyles (e.g., biotrophy, hemibiotrophy, and necrotrophy) and generated species trees. The phosphosites identified in the *M. oryzae* phosphorylated proteins were mapped onto these orthogroups, and k-means clustering was employed to analyze conservation patterns, which resulted in the identification of nine conserved phosphorylated residue (CPR) groups containing 1198 CPRs. CPR-based clustering of species yielded a conservation tree with clades distinct from the clades of a phylogenetic tree contrived from orthogroups. This suggests phosphosite conservation extends beyond mere protein sequence conservation or phylogenetic proximity. Among the interesting clusters, cluster 4 included core signaling proteins conserved across most fungal species, featuring key kinases such as Pmk1, Osm1, and Mst7. Cluster 9 highlighted phosphosites conserved primarily among plant pathogens but not in saprophytes, linking these residues to plant host-dependent lifestyles. CPR cluster 6 showed conservation among hemibiotrophic plant pathogens, which initially invade living tissue before killing plant cells. This group includes major crop pathogens like *M. oryzae* and *Z. tritici*, with conserved phosphosites found in various transcription factors and metabolic enzymes. Cluster 3 was specific to the *Fusarium* genus, including *Fusarium graminearum*, and conserved phosphosites in pH-responsive and morphogenetic regulators. Cluster 5 demonstrated high conservation among Dothideomycete pathogens, responsible for diseases in cereals, suggesting a shared regulatory mechanism for invasive growth. Cluster 2 was unique to fungi producing pressurized, melanin-pigmented appressoria, such as *Colletotrichum* species, with conserved phosphosites in proteins linked to appressorium formation. Overall, the comparative analysis of these phosphosites across diverse fungal

pathogens provides valuable insights into the signaling mechanisms underlying fungal pathogenesis. The conserved phosphorylated residues among biotrophic and hemibiotrophic fungal pathogens offer a crucial resource for understanding and potentially disrupting the infection processes in these pathogens.

Cruz-Mireles et al. (2024) further examined the phosphorylation patterns in proteins related to infection development in *M. oryzae* by mapping 201 differentially phosphorylated residues to signaling pathways critical for appressorium morphogenesis and plant infection. In the Pmk1 MAPK signaling pathway, 11 out of 17 proteins exhibited changes in phosphopeptide abundance. These included upstream components like MAPKK Mst7 and adaptor protein Mst50 (Li et al. 2017), as well as downstream targets, such as transcription factors Sfl1, Znf1, Hox7, and Mst1 (Cao et al. 2016). Additionally, 12 out of 25 proteins in the Sln1 histidine kinase signaling pathway, crucial for appressorium turgor sensing (Ryder et al. 2019), also showed changes in phosphorylation. The study found that proteins involved in autophagy, essential for appressorium function and dependent on Pmk1 (Kershaw and Talbot 2009), displayed changes in eight of 23 autophagy-related proteins. In the cAMP protein-kinase-A signaling pathway, which works with Pmk1 to regulate appressorium development and turgor generation, three proteins showed phosphorylation changes. The recently identified Vast1 pathway, important for appressorium maturation (Zhu et al. 2021), had four out of five associated phosphoproteins with altered abundance, suggesting its critical role in plant infection. Across all signaling pathways analyzed, distinct differences in phosphopeptide abundance profiles between the wild-type strain Guy11 and the Δ*pmk1* mutant indicated Pmk1's significant regulatory influence on various physiological and morphogenetic processes vital for forming a functional appressorium. This underscores the comprehensive regulatory role of Pmk1 in infection-related development.

To determine the direct potential substrates of the Pmk1 MAPK, essential for plant infection by *M. oryzae*, Cruz-Mireles et al. (2024) conducted targeted quantitative phosphoproteomic analysis using parallel reaction monitoring (PRM) on samples from both wild-type Guy11 and the Δ*pmk1* mutant. This approach aimed to validate differentially phosphorylated peptides identified through LFQ and to enhance the accuracy of these identifications. The authors hypothesized that Pmk1 direct targets would show phosphorylation only in the presence of Pmk1. They selected 286 phosphopeptides from 101 proteins based on differential phosphorylation in both genotypes, known Pmk1 targets, and hierarchical transcriptomic analysis. Out of these, 182 phosphopeptides exhibited differential abundance, representing 86 proteins. Among these, 63 phosphopeptides from 32 proteins were differentially phosphorylated in Guy11 but not in the Δ*pmk1* mutant, identifying them as "putative targets of Pmk1." The study confirmed previously reported Pmk1 targets, such as phosphosites in the transcription factor Hox7 (Osés-Ruiz et al. 2021), and components of the Pmk1 pathway like Mst11, Mst7, and Mst50. The PRM analysis identified a set of 32 putative Pmk1 substrates involved in various cellular processes crucial for appressorium morphogenesis and virulence. Functional annotation revealed roles for these targets in kinase activity, transcription regulation, autophagy, and cAMP signaling. Yeast-two-hybrid screening utilizing Pmk1 as bait showed direct interactions between Pmk1 and nine of these 32 putative targets, including transcription factors Far1 and Som1 (bin Yusof et al. 2014; Yue et al. 2017), and several previously uncharacterized proteins, such as a potential regulatory protein VTi 1–2 suppressor (Vts1). This study demonstrated that quantitative comparative phosphoproteomics effectively identifies putative direct targets of the Pmk1 MAPK, advancing the understanding of the signaling mechanisms in fungal pathogenesis.

PRM analysis identified three phosphorylation sites in Vts1, with S175 and S420 being differentially phosphorylated in the presence of Pmk1. Further, using a Pmk1 analog-sensitive mutant, the researchers demonstrated that inhibiting Pmk1 affects phosphorylation at these specific sites. In vitro kinase assays confirmed Pmk1 specifically phosphorylates Vts1 at S175 and S420. To understand Vts1's role in pathogenicity, a targeted deletion mutant of Vts1 was created in *M. oryzae* Guy11, which resulted in aberrant appressoria development and severe impairment in causing rice blast disease. Introducing phosphomimetic and phosphodead alleles of *Vts1* into the Δ*Vts1* mutant showed that Pmk1-dependent phosphorylation of S175 is crucial for virulence, while S420 played a lesser role. These findings demonstrate that quantitative phosphoproteomics can uncover novel regulators like Vts1 and provide insights into the signaling pathways essential for fungal virulence and plant infection.

In conclusion, the study published in Cell represents a tour de force in the field of fungal pathogenesis, unveiling the intricate phosphorylation landscape governing infection-related development by the rice blast fungus. The insights gained from this study will help develop targeted interventions to thwart fungal pathogenesis and safeguard agricultural productivity.

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YP conceptualized the manuscript and VB wrote the manuscript.

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