Investigating signaling network adaptation by directive adaptive laboratory evolution in the fungus *Magnaporthe oryzae*

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Investigating signaling network adaptation by directive adaptive laboratory evolution in the fungus *Magnaporthe oryzae*

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Table of Content

Abbreviations

AC	Adenylate cyclase
ALE	Adaptive Laboratory Evolution
ATP	Adenosine triphosphate
Avr	Avirulence
CAC	Adenyl cyclase
cAMP	Cyclic adenosine monophosphate
ChIP	Chromatin immunoprecipitation
CWI	Cell wall integrity
DALE	Directed -adaptive laboratory Evolution
DDA	Data depentend acquisition
DHAP	Dihydroxyacetone phosphate
DIA	Data-independent acquisition
DMR	Differentially methylated regions
DNA	Deoxyribonucleic acid
Fps1	Glycerol facilitator
G3P	Glycerol-3-phosphate
GFD	Glycerol 3 phosphate dehydrogenase
GlcA	Glycerol kinase
GldB	Glycerol dehydrogenase
GPCR	G-protein coupled receptors
GPDH	Glycerol-3-phosphate dehydrogenase
GPP	Glycerol 1-phosphatase
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
Hik1p	Histidine kinase 1
HOG	High Osmolarity Glycerol
Hog1p	High osmolarity glycerol response
HPAEC-PAD	High-performance anion-exchange chromatography with pulsed amperometric detection
IG	Invasive grwoth
LC-MS/MS	Liquid Chromatography with tandem mass spectrometry
lof	Loss of function
MAPK	Mitogen-activated protein kinase
NADH	Nicotinamide adenine dinucleotide
Pbs2p	Polymyxin B sensitivity 2

PCR	Polymerase chain reaction
PDE	Phosphodiesterade
PKA	Protein kinase A
PTM	Posttranslational modifucations
ROS	Reactive oxygen species
Sln1p	Synthetic lethal of N-end rule
Ssk1p	Suppressor of sensor kianse 1
Ssk2p	Suppressor of sensor kianse
STL1	*Saccharomyces cerevisiae* Transporter Like 1
TCA	Tricarboxylic acid cycle
TOR	Target of Rapamycin
TORC	Target of Rapamycin complexes
XICs	Extracted ion chromatograms
Ypd1p	Tyrosine phosphatase dependent

List of illustrations

Nomcenclature

The following is an exemplary list of the spellings used in this work for genes, proteins and mutants:

MoHOG1: the HOG1 gene from *Magnaporthe oryzae*

Δ*Mohog1*: the *Magnaporthe oryzae* mutant in which the MoHOG1 gene is inactivated

MoHOG1p: the corresponding protein to the MoHOG1 gene

∆*Mohog1/*∆*Mostl1*: in the *Magnaporthe oryzae* mutant with the inactivated genes

∆*Mohog1/HOG1*: in the *Magnaporthe oryzae* mutant re-integration of the gene

If a different spelling was used in the respective literature, this was adopted.

Publications and manuscript presented in this thesis

Chapter 2:

Jacob, S. and Bersching, K. (2021) 'Controllable Bypass Suppression in Magnaporthe oryzae', in S. Jacob (ed.) Magnaporthe oryzae: Methods and Protocols. New York, NY: Springer US (Methods in Molecular Biology), pp. 225–231. Available at: https://doi.org/10.1007/978-1-0716-1613-0_18.

Chapter 3:

Bersching, K. et al. (2023) 'Data-Independent Acquisition (DIA) Is Superior for High Precision Phospho-Peptide Quantification in Magnaporthe oryzae', Journal of Fungi, 9(1), p. 63. Available at: https://doi.org/10.3390/jof9010063.

Chapter 4:

Jacob, S., Bühring, S. and Bersching, K. (2022) 'Recent Advances in Research on Molecular Mechanisms of Fungal Signaling', Encyclopedia, 2(2), pp. 840–863. Available at: https://doi.org/10.3390/encyclopedia2020055.

Chapter 5:

K.Bersching, C. Grünewald, J.Kannengießer and S. Jacob

Directed experimental adaptive evolution of osmoregulation in the fungal pathogen Magnaporthe oryzae is independent of glycerol metabolism associated genes.

Manuscript

Contributions to publications and manuscript presented in this thesis

Chapter 2:

K.B. and S.J. writing and review

Chapter 3:

Conceptualization, T.M., S.T. and S.J.; methodology, T.M. and K.B.; software, S.T.; validation, T.M. and K.B.; formal analysis, T.M. and S.T.; resources, S.T. and S.J.; writing—original draft preparation, K.B. and T.M; writing—review and editing, S.J. and S.T.; supervision, S.J. and S.T.; project administration, S.J. and S.T.; funding acquisition, S.J. and S.T. All authors have read and agreed to the published version of the manuscript.

Chapter 4:

K.B, S.B. and J.S. wrote the review

Chapter 5:

K.B. ,C.G. and S.J. designed research, K.B., C.G. and J.K. performed experiments, K.B.,C.G. and S.J. analyzed the data, K.B. and S.J. wrote the manuscript.

Zusammenfassung

Die Evolution wird traditionell als ein langsamer Prozess angesehen, der sich über Hunderte oder sogar Tausende von Jahren entwickelt. Jüngste Forschungen auf dem Gebiet der Evolutionsdynamik und der gezielten experimentellen adaptiven Evolution haben jedoch gezeigt, dass Mikroorganismen innerhalb eines viel kürzeren Zeitraums schnelle evolutionäre Veränderungen durchlaufen können. Trotz dieser Erkenntnisse sind die molekularen Mechanismen, die diese schnellen Anpassungen bewirken, nach wie vor kaum verstanden, was vor allem daran liegt, dass sie von seltenen und unvorhersehbaren Veränderungen abhängen. Die adaptive Evolution in Mikroorganismen beinhaltet in der Regel vorteilhafte genetische, transkriptomische oder proteomische Veränderungen, die durch natürliche Selektion erhalten bleiben. Das Verständnis dieser Mechanismen ist entscheidend für die Bewältigung globaler Herausforderungen wie des Klimawandels, des Auftretens neuer Krankheitserreger, der Ausbreitung invasiver Arten und der Entwicklung von Multiresistenzen.

Im Rahmen dieser Arbeit hat der Erreger der Reisbranderregers, *Magnaporthe oryzae*, die Komplexität von Signalnetzwerken aufgezeigt, die physiologische und biochemische Prozesse regulieren. Ein Beispiel dafür ist der „*High Osmolarity Glycerol*“ (HOG) Signalweg, der die zelluläre Anpassung an die Osmolarität der Umwelt regelt. „*Loss of function*“ (lof)-Mutanten des HOG-Stoffwechselwegs sind osmosensibel und produzieren nicht wie der Wildtyp-Stamm das wichtigste Solut der osmotischen Stressreaktion, Arabitol.. Interessanterweise entstehen, wenn diese lof-Mutanten einem konstanten osmotischen Druck ausgesetzt werden, stabile Suppressor-Stämme, die anstelle von Arabitol große Mengen Glycerin produzieren, was eine gezielte adaptive Laborevolution (DALE) zeigt.

Um die zugrunde liegenden Mechanismen besser zu verstehen, wurden verschiedene Suppressor-Mutanten durch DALE erzeugt. Ziel dieser Studie war es, die Gene zu identifizieren, die für die Anpassung an Langzeitstress und den Wechsel von der Arabitol- zur Glycerinproduktion verantwortlich sind. Kandidatengene wurden identifiziert und ihre Rolle bei der Produktion von Primärmetaboliten und ihrer Anpassungsfähigkeit durch gezielte Mutagenese untersucht. Im Rahmen dieser Arbeit wurde in Zusammenarbeit mit Experten Immunologie der Universitätsmedizin der Johannes Gutenberg-Universität in Mainz eine effektive Pipeline zur Analyse von Proteom- und Phosphoproteomdaten entwickelt. Unter Verwendung von *M. oryzae* als Modell wurde ein datenunabhängiger Erfassungsansatz (DIA) implementiert, der die Qualität und Vollständigkeit der Daten deutlich verbesserte. Diese Methode verkürzte die LC-MS/MS-Analysezeit von Peptiden und Phosphopeptiden und

erhöhte die Identifizierung von Phosphosites, wodurch eine verfeinerte Methodik und eine umfassende Grundlage für die Untersuchung von Signalprozessen in filamentösen Pilzen geschaffen wurde. Mit dieser Methode wurde eine neue Gruppe von Kandidatengenen identifiziert, die am Anpassungsprozess beteiligt sind. Diese Ergebnisse tragen zu einem tieferen Verständnis der komplexen evolutionären Mechanismen in *M. oryzae* bei und unterstreichen die Notwendigkeit weiterer Forschung, um die molekularen Grundlagen der schnellen evolutionären Anpassungen in Mikroorganismen zu entschlüsseln.

Summary

Evolution is traditionally perceived as a slow process, developing over a hundreds, or even thousands of years. However, recent research in evolutionary dynamics and directed experimental adaptive evolution has demonstrated that microorganisms can undergo rapid evolutionary changes within a much shorter timeframe. Despite these findings, the molecular mechanisms driving these rapid adaptations remain poorly understood, largely due to their dependence on rare and unpredictable changes. Adaptive evolution in microorganisms typically involves advantageous genetic, transcriptomic, or proteomic changes that persist through natural selection. Understanding these mechanisms is crucial for addressing global challenges such as climate change, the emergence of new pathogens, the spread of invasive species, and the development of multi-drug resistance.

Within this work the rice blast pathogen *Magnaporthe oryzae* has highlighted the complexity of signaling networks that regulate physiological and biochemical processes. The High Osmolarity Glycerol (HOG) pathway is an example, regulating cellular adaptation to environmental osmolarity. Loss of function (lof) mutants of the HOG pathway are osmosensitive and fail to produce the main critical osmotic stress response solute arabitol as it is in the wildtype strain. Interestingly, when these lof mutants are exposed to constant osmotic pressure, stable suppressor strains emerge that produce high amounts of glycerol instead of arabitol, demonstrating directed adaptive laboratory evolution (DALE).

To further understand the mechanisms behind, various suppressor mutants were generated using (DALE). This study aimed to identify the genes responsible for adaptation to long-term stress and the shift from arabitol to glycerol production. Candidate genes were identified and their roles in primary metabolite production and adaptive capacity were investigated through targeted mutagenesis. Within this work an effective pipeline for analyzing proteome and phosphoproteome data, in collaboration with experts at the Institute for Immunology at the University Medical Center of Johannes Gutenberg University Mainz was developed. Using *M. oryzae* as a model, a data-independent acquisition (DIA) approach was implemented, significantly improving the quality and completeness of the data. This method reduced the LC-MS/MS analysis time of peptides and phosphopeptides and increased the identification of phosphosites, establishing a refined methodology and a comprehensive basis for studying signaling processes in filamentous fungi. Within this method, a new set of candidate genes involved in the adaptation process was identified. These findings contribute to a deeper understanding of the complex evolutionary mechanisms in *M. oryzae* and underscore the need

for continued research to unravel the molecular basis of rapid evolutionary adaptations in microorganisms.

1. Introduction

1.1 *Magnaporthe oryzae*: From rice fields to laboratories

Food protection is of utmost importance in ensuring global food security and the well-being of human populations. Fungal phytopathogens, which are destructive plant pathogens, pose a significant threat to agricultural productivity and food supply chains (Case *et al.*, 2022). These pathogens can attack various crops, including staple crops such as rice, wheat (Monsur *et al.*, 2016), and maize (Schmidt *et al.*, 2020), as well as fruits and vegetables (Chandra *et al.*, 2022). The spread of the resulting diseases results in significant economic losses for farmers, affecting their livelihoods and agricultural economies. *Magnaporthe oryzae* is one of the most destructive fungal pathogens worldwide, known to cause the rice blast disease, and can be listet in line with (I) *Puccinia graminis*, (II) *Fusarium graminearum* and (III) *Phytophthora infestans*. By affecting one of the most important stable crops in the world it is a serious threat to global food security. While *P. graminis* causes wheat stem rust, (Sahu *et al.*, 2021), *F. graminearum* is responsible for *Fusarium* head blight or scrab on various cereal crops (Whetton *et al.*, 2018), including wheat, barley and corn. *P. infestans* is mainly known to infest potatoes (*Solanum tuberosum*) and tomatoes (*Solanum lycopersicum*) (Rojas-Estevez *et al.*, 2020).

Understanding the molecular mechanisms underlying the infection process is essential for developing strategies to control *M. oryzae* and other plant pathogenic fungi (Asif *et al.*, 2022). The infection cycle is characterized by differentiation steps, including spore germination, appressorium formation, penetration of the cuticula, colonization of the host plant and sporulation (Medyukhina *et al.*, 2015). The molecular basis of how *M. oryzae* interacts with the host is not yet completely understood. The infection cycle begins with the dispersal of conidia, the asexual spores of *M. oryzae*, onto the rice leaf surface. Conidia germinate upon sensing favorable environmental conditions such as moisture and nutrients. The germ tube that emerges from the spore adheres to the leaf surface and differentiates into a specialized infection structure called the appressorium. This structure is crucial for penetrating the host tissue (Maeda *et al.*, 2009). The ability of spores to survive in soil for extended periods allows them to persist in the absence of a host plant and to infect new plants when conditions become favorable (Liu and Zhang, 2022). Triglycerides are produced by produces glycerol, which is then accumulated in the appressorium, gradually building up the turgor. Once sufficient turgor pressure is generated, the appressorium forms a narrow penetration peg that breaches the rice leaf cuticle. This process is mechanical but also involves the secretion of a suite of

enzymes, such as chitinases and cellulases, which degrade the plant's cuticle and cell wall components, facilitating entry into the host tissue (Aro, Pakula and Penttilä, 2005). Appressoria are specialized structures formed at the tip of the germ tube and allow the fungus to penetrate the plant cuticle (Li, Zhou and Xu, 2012). The formation of appressoria is triggered by specific chemical signals from the plant surface, including cutin monomers and hydrophobic waxes (Liu *et al.*, 2011). This enables the fungus to extract nutrients from the host and to spread to adjacent cells and tissues (Keegstra, 2010; Luginbuehl and Oldroyd, 2017). The symptoms of rice blast can vary depending on the severity of the infection and the stage of plant development. Early infections are visible as small, circular lesions on the leaves, while more severe infections can cause the entire plant to wilt and die (Yao *et al.*, 2022). The fungus spreads cell-to-cell using a combination enzymatic degradations, colonizing the leaf tissue extensively (Xu, Staiger and Hamer, 1998). The disease can also affect the stem and panicle, leading to reduced grain quality and yield (Zhu *et al.*, 2016; Chen *et al.*, 2023). The fungus emerges on the surface of the infected tissue, producing new conidia on specialized structures called conidiophores. These conidia are disseminated by wind, rain, or mechanical contact to new sites, where they can initiate another infection cycle. The production of conidia is a critical phase for the propagation of the disease, allowing *M. oryzae* to spread rapidly across rice fields (Fernandez and Orth, 2018). Throughout its infection cycle, *M. oryzae* exhibits remarkable adaptability to environmental conditions. It can enter a latent phase under unfavorable conditions, surviving as dormant spores or within plant debris until conditions become favourable for growth and infection. This ability to adapt and persist in the environment contributes significantly to its effectiveness as a pathogen. Each stage of the infection cycle is characterized by specialized structures and strategies that enable the fungus to overcome plant defenses, extract nutrients, and spread to new hosts. Understanding this cycle provides insights into potential targets for disease control and the development of resistant rice varieties. The infection cycle of *M. oryzae* is highly dynamic and depends on various environmental and genetic factors (Fernandez and Orth, 2018). For example, the timing of spore production and the duration of infection depend on temperature, humidity, and the host's developmental stage. Host resistance genes can also affect the success of infection, with some genes conferring complete resistance, while others only delay disease onset (Couch *et al.*, 2005). The genetic diversity of *M. oryzae* populations also plays a crucial role in the infection cycle, with different pathotypes capable of overcoming host resistance and causing severe disease epidemics (Gladieux *et al.*, 2018). Controlling rice blast is a significant challenge for farmers and researchers (Fukuoka *et al.*, 2015), since rice is the primary source of food for over half of the world's population, and it is grown in over 100 countries (Nalley *et al.*, 2016). Most of the countries are located in south east Asia (Saleh *et al.*, 2014), but the regions are changing and due to global warming the habitat of fungi are changing worldwide (Zhong, Zhang and Fu,

2023). The production of rice is essential for food security and poverty alleviation in many parts of the world. It is estimated that *M. oryzae* and the causing rice blast annually contributes to loss enough rice to feed 60 million people (Fernandez and Orth, 2018). Traditional methods of disease management, such as crop rotation and the use of chemical fungicides, are limited in effectiveness, expensive and environmentally harmful (Manning and Soon, 2017). In recent years, there has been growing interest in developing alternative approaches in disease management, such as the use of resistant cultivars or biological control agents (Chung *et al.*, 2023).

But why has *M. oryzae* become a model organism in the fundamental research?
M. oryzae is widely regarded as a model organism for several compelling reasons. First and foremost, *M. oryzae* is the causative agent of rice blast disease, one of the most devastating diseases affecting rice, a staple food for a significant portion of the world's population. Research on this pathogen is crucial for developing strategies to control rice blast and enhance food security. The genomic resources available for *M. oryzae* are extensive, with its genome fully sequenced and annotated. This genetic information provides a solid basis for molecular and genetic studies, enabling researchers to identify and manipulate genes involved in pathogenicity, stress response, and other critical functions. Additionally, *M. oryzae* serves as an excellent model for studying plant-pathogen interactions due to its well-characterized infection cycle, which includes spore germination, appressorium formation, and host tissue colonization. These stages can be examined in detail to understand the mechanisms of fungal infection and host defense. *M. oryzae* is also easy to cultivate in the laboratory making it accessible for experimental manipulation and high-throughput screening. The availability of advanced genetic tools and techniques, such as gene inactivations, RNA interference, and CRISPR/Cas9, allows to investigate gene function and regulatory pathways comprehensively. These techniques facilitate the study of genetic and epigenetic factors influencing fungal biology and pathogenicity. Moreover, the insights gained from studying *M. oryzae* can often be extrapolated to other fungal pathogens due to the conservation of many pathogenicity and stress response mechanisms across fungi. The combination of its economic importance, extensive genomic resources, ease of cultivation, advanced genetic tools, broader applicability to other pathogens, and a collaborative research community make *M. oryzae* an invaluable model organism for advancing our understanding of fungal biology, plant-pathogen interactions, and the development of effective disease management strategies.

1.2 Fungal Signaling: Cellular Communication in Fungi

Signaling pathways play a fundamental role in the complex of communication within cells, in particular for the response and adaptation to their environment (Combarnous and Nguyen, 2020). Intricate networks of molecular interactions transmit signals, integrate information from extracellular cues and translate them into specific cellular responses. In fungi, signaling pathways regulate critical processes, including growth, development, stress responses, and interactions with their environment (Zhou *et al.*, 2021). For instance, the cAMP-PKA pathway controls fungal morphogenesis and virulence, orchestrating cellular differentiation and infection strategy (Sun *et al.*, 2022). The mitogen-activated protein kinase (MAPK) pathway regulate responses to stress, nutrient availability, and host recognition (Sun *et al.*, 2022). Targeting key signaling components or disrupting critical signaling crosstalk, which are essential for fungal survival, aids in understanding and developing novel antifungal strategies. Furthermore, uncovering signaling pathways in fungi sheds light on evolutionary relationships, as they exhibit both conserved and unique signaling modules (Sun *et al.*, 2022). The study of signaling pathways in fungi unravels the complexities of cellular communication, illuminating their adaptation strategies, virulence mechanisms, and responses to environmental cues (Jacob *et al.*, 2014). Expanding this knowledge holds promise for combating fungal pathogens, improving agricultural practices, and deepening our understanding of the physiology world of fungi.

1.2.1. Navigating Stress and Survival: Unraveling the Significance of the High Osmolarity Glycerol Pathway in Fungal Adaptation

Fungal signaling pathways are intricate networks of molecular interactions within cells that transmit and process information, enabling cells to respond to various stimuli and coordinate their activities (Jacob, Bühring and Bersching, 2022). One prominent example of a signaling pathway is the High Osmolarity Glycerol (HOG) pathway, which plays a crucial role in cellular response to osmotic stress in fungi (Jacob *et al.*, 2015). The HOG pathway is a signaling pathway found in eukaryotic organisms, including yeast and fungi (Zhao *et al.*, 2022). It is also involved in the regulation of biotic interactions and responses to abiotic stresses in plant pathogenic fungi (Zhang *et al.*, 2021) (Figure 1).

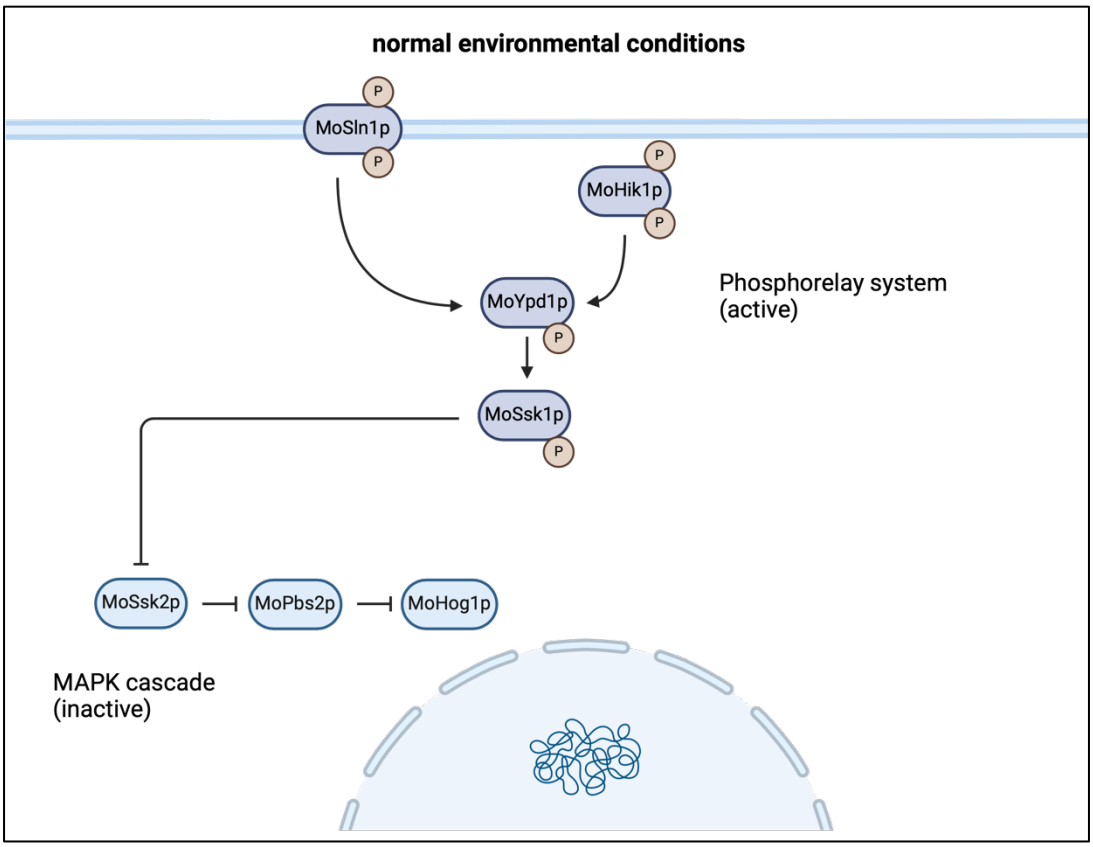

Figure 1: A schematic overview of the High Osmolarity Glycerol (HOG) signaling pathway in Magnaporthe oryzae. *The two-component hybrid histidine kinases, MoSln1p and MoHik1p, act as sensors for high osmolarity conditions. The phosphotransfer protein MoYpd1p facilitates the transfer of phosphate to MoSsk1p. Under typical environmental conditions, the phosphorelay system comprising MoSln1p, MoHik1p, MoYpd1p, and MoSsk1p remains phosphorylated, while the MAPK cascade involving MoSsk2p, MoPbs2p, and MoHog1p remains inactive. Created with BioRender.com.*

The HOG pathway exemplifies the complexity and significance of signaling pathways in cellular physiology and adaptation, because it integrates multiple environmental signals to regulate osmoregulation, different stress responses, and survival mechanisms in fungi, highlighting the intricate network of molecular interactions essential for maintaining cellular homeostasis under varying conditions. Signaling pathways, including the HOG pathway, serve as cellular communication systems, transmitting signals from receptors on the cell surface or within the cell to specific target molecules (de Nadal and Posas, 2022). The pathways involved in cellular signaling often encompass a series of enzymatic reactions that trigger the activation or inhibition of crucial molecules such as protein kinases, transcription factors, or effector proteins (Parundekar and Viswanathan, 2021). This pathway is a mitogen-activated protein kinase (MAPK) cascade that activates the expression of certain genes, leading to the accumulation of glycerol under osmotic stress in *Saccharomyces cerevisiae* (Zhao *et al.*, 2016). The activation the HOG pathway involves coordinated activity with other pathways, such as the TOR (Target of Rapamycin) pathway (Li *et al.*, 2024) or the cAMP (Cyclic adenosine monophosphate) pathway (Yin *et al.*, 2016). While the TOR pathway is involved in the regulation of cellular growth, proliferation, metabolism, and response to environmental cues

(So *et al.*, 2019) the cAMP pathway plays a central role by mediating a variety of physiological processes, including growth, development, and response to environmental stimuli (Sun *et al.*, 2022).

The HOG pathway is an essential mechanism in fungi like *M. oryzae* for adapting to osmotic stress. It consists of a phosphorelay system with a downstream MAPK (mitogen-activated protein kinase) cascade. Under normal, unstressed conditions, the phosphorelay system is constitutively phosphorylated, keeping the downstream MAPK cascade inactive or repressed (Stojanovski *et al.*, 2017; Kennedy *et al.*, 2019) (Figure 1). When the extracellular environment becomes hypertonic, osmosensors located within the plasma membrane, such as the Sln1 ("synthetic lethal of N-end rule") histidine kinase in yeast, or the cytosolic MoHik1p ("Histidine kinase 1") in *M. oryzae*, detect these changes and initiate the cellular response (Tanaka *et al.*, 2014) (Figure 2).

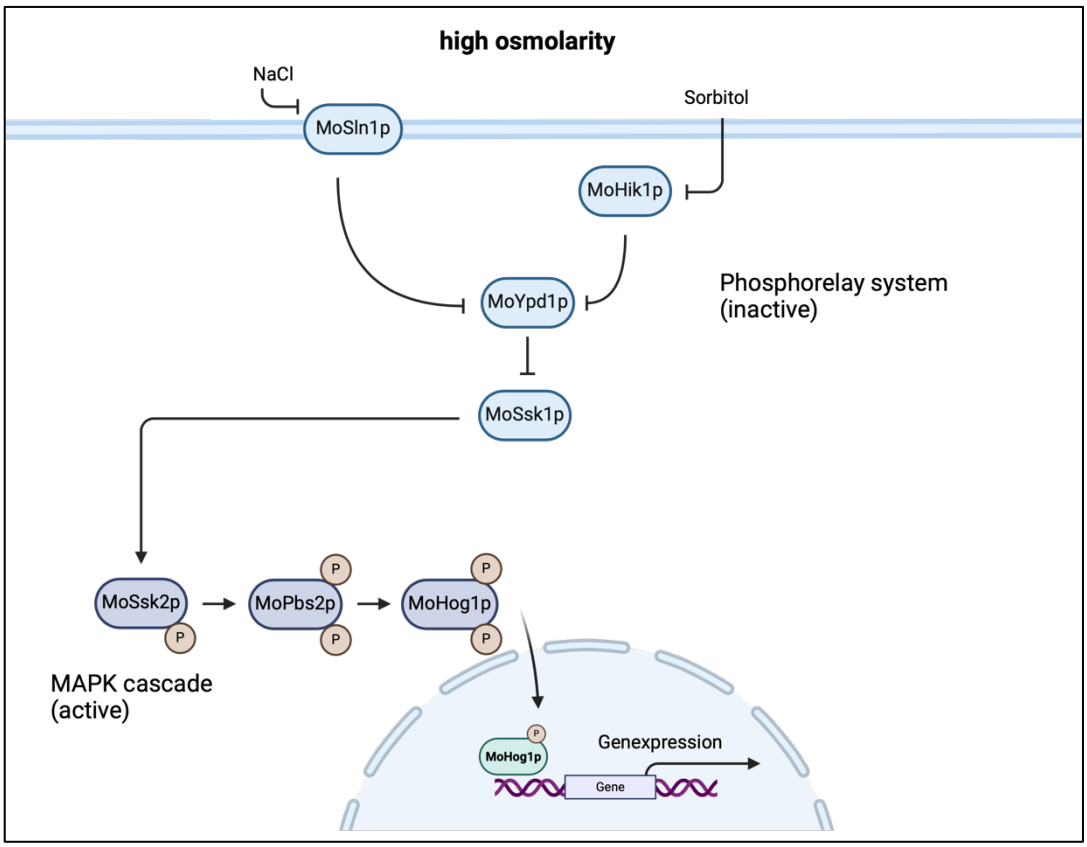

Figure 2: A schematic summary of the High Osmolarity Glycerol (HOG) signaling pathway in Magnaporthe oryzae*. Exposure to high osmolarity conditions, such as NaCl or Sorbitol, triggers the dephosphorylation of the signaling components, resulting in the dephosphorylation of MoSsk1p. Consequently, this leads to the activation of the MAPK cascade involving MoSsk2p, MoPbs2p, and MoHog1p. remains phosphorylated, while the MAPK cascade involving MoSsk2p, MoPbs2p, and MoHog1p remains inactive. Created with BioRender.com.*

Upon detection of osmotic stress, the osmosensors activate a series of phosphorylation events within the HOG pathway (Jacob, Bühring and Bersching, 2022). In *S. cerevisiae* and *M. oryzae*, the phosphorelay system remains phosphorylated through autophosphorylation of

the histidine kinase Sln1p. Phosphotransfer occurs via Ypd1p (phosphotransfer protein “tyrosine phosphatase dependent”) to the response regulator Ssk1p (“suppressor of sensor kinase”). This phosphorylation of Ssk1p prevents the activation of the downstream MAPK cascade, meaning that Ssk1p's phosphorylation inhibits the activation of Ssk2p (MAPKKK), Pbs2p (“polymyxin B sensitivity”; MAPKK), and Hog1p (“high osmolarity glycerol response”; MAPK). Upon osmotic stress (e.g., high salt or sugar concentrations), osmosensors such as MoSln1p or MoHik1p (only in *M. oryzae*) detect the hyperosmotic conditions and activate the HOG pathway. The osmosensors in *M. oryzae*, upon detecting stress, autophosphorylate the sensor kinase domains (e.g., MoSln1p or MoHik1p). The phosphorylated sensor kinase transfers the phosphate to the response regulator MoYpd1p, which then transfers it to Mossk1p. Phosphorylated MosSk1p activates the MAP kinase kinase kinase (MAPKKK) MoSsk2p. MoSsk2p phosphorylates and activates the MAP kinase kinase (MAPKK) MoPbs2p. MoPbs2p, in turn, phosphorylates the MAP kinase (MAPK) MoHog1p at specific threonine and tyrosine residues, crucial for its full activation. Activated MoHog1p translocates to the nucleus, where it regulates the expression of genes involved in osmotic stress response (Figure 2). These genes encode proteins that help the cell adapt to high osmolarity conditions, such as those involved in the production of primary metabolites (Jacob, Bühring and Bersching, 2022). Disruption in the phosphorelay system, such as the termination of MoSln1p autophosphorylation due to extracellular hyperosmotic stress, increases the proportion of unsphosphorylated Mossk1p. This leads to an interaction between MoSsk1p and MSsk2p, resulting in the autophosphorylation of MoSPk2p. Subsequently, the MAPKK MoPbs2p and the MAPK MHog1p are activated through dual phosphorylation by their respective upstream kinases (Hohmann, 2002).

In comparison to *S. cerevisiae*, *M. oryzae* contains at least nine other known histidine kinases besides MoSln1p, including the group III HK MoHIK1, which is sensitive to fludioxonil and sugar. In contrast, the genome of *S. cerevisiae* only contains the Group VI HK Sln1p. The involvement of MoHik1p in the phosphorelay system and as the target of fludioxonil in *M. oryzae* has been demonstrated (Jacob, Bühring and Bersching, 2022). The HOG pathway is also involved in regulating diverse environmental stress responses, including oxidative stress, nutrient limitations, high temperatures, and other chemical and mechanical stresses. It is crucial for the survival of pathogenic fungi in hostile environments (Silva *et al.*, 2020; Jacob, Bühring and Bersching, 2022).

The translocation of MoHog1p from the cytoplasm to the nucleus upon phosphorylation is a crucial step in its function and promotes stress-responsive gene expression either directly or by phosphorylating other transcription factors (Hong and Huh, 2021; Husain *et al.*, 2022). These include genes encoding osmolyte transporters, enzymes involved in the biosynthesis of compatible solutes (such as glycerol), and other factors that promote cell survival under high

osmolarity conditions. The upregulation of these genes helps restore cellular homeostasis and protect the cell from osmotic stress-induced damage (Alonso-Monge *et al.*, 2020). While initially discovered in yeast, homologs of the HOG pathway components are found in other eukaryotes, including filamentous fungi and higher eukaryotes. This suggests its evolutionary conservation and importance in cellular adaptation to osmotic stress across diverse organisms (Liu *et al.*, 2021).

1.2.2 The Importance of Metabolism in Fungi

Primary and secondary metabolites in fungi include a wide range of molecules, including amino acids, nucleotides, lipids, polyketides and carbohydrates. Regulation of the underlying metabolic pathways in fungi has been observed in response to light, impacting carotenoid metabolism, polysaccharide and carbohydrate metabolism, fatty acid metabolism, and the production of secondary metabolites (Tisch and Schmoll, 2010). Primary metabolites are molecules that are essential for the growth and survival of the organism, while secondary metabolites are molecules that are produced by the organism but are not essential for growth or survival (Pott, Osorio and Vallarino, 2019). Additionally, specific metabolic pathways, such as mannitol metabolism and phenylalanine metabolism, have been studied in the context of pathogenic fungal-host interactions, highlighting the intricate metabolic responses of fungi under changing conditions (Upadhyay *et al.*, 2015).

Fungal metabolism plays an important role in ecological and anthropogenic contexts, primarily through its involvement in decomposition and nutrient cycling. As primary decomposers, fungi are instrumental in breaking down complex organic materials, facilitating the release and recycling of nutrients essential for ecosystem sustenance (Voříšková and Baldrian, 2013). This metabolic function extends to symbiotic relationships, particularly in the form of mycorrhizal associations, where fungi enhance nutrient absorption for plants in exchange for carbohydrates, thus promoting ecological stability and plant health (Treseder *et al.*, 2016). Additionally, the diverse metabolic pathways of fungi have been exploited in biotechnological and industrial applications, including the production of antibiotics, alcohols, organic acids, and enzymes (Zhang *et al.*, 2020). In the food industry, fungi are crucial for the fermentation processes in bread, beer, and cheese production (Dupont *et al.*, 2017). However, the metabolic activities of fungi also have detrimental effects, such as the production of mycotoxins, which pose health risks to humans and animals (Tran-Dinh, 2013). Understanding these metabolic processes is essential for harnessing their benefits and mitigating their threats, illustrating the dualistic nature of fungal metabolism in environmental and human contexts.

1.2.3 Primary Metabolism

Primary metabolism in fungi refers to the essential biochemical processes involved in the maintenance of cellular functions, such as energy production, biosynthesis of basic building blocks (e.g., amino acids, nucleotides), and central metabolic pathways (e.g., glycolysis, tricarboxylic acid cycle (TCA)) (Chroumpi, Mäkelä and de Vries, 2020). These processes are fundamental for the survival and growth of fungi under normal physiological conditions. In addition to their basic roles in cellular metabolism, primary metabolites in fungi also play important roles in a range of ecological and industrial processes (Zhang *et al.*, 2020). Fungi are used in the production of food and beverages such as bread, beer, and cheese (Dupont *et al.*, 2017). In these applications, the fungi use primary metabolites such as carbohydrates and amino acids to produce a range of secondary metabolites such as ethanol and flavor compounds. The formation of these metabolites typically occurs under nutrient scarcity, i.e., during the stationary phase of growth, and is often closely associated with differentiation processes.

1.2.4 Glycerol Metabolism's Role in Fungal Adaptation

Glycerol not only serves as a compatible solute in order to compensate extracellular osmotic changes but rather is a versatile compound with significant importance in various biological processes. It serves as a carbon and energy source, a compatible solute for osmotic regulation, and as a precursor for the biosynthesis of essential molecules, such as lipids and phospholipids (Lee *et al.*, 2001; De Vries *et al.*, 2003). In filamentous fungi, glycerol plays a crucial role in osmotolerance, serving as a major osmolyte and contributing to adaptation to environmental cues. Research has revealed the essentiality of glycerol in the early stages of spore germination and its involvement in the transition between different morphological forms, which is critical for fungal pathogenicity and environmental adaptation (Dušková *et al.*, 2015).
Why is glycerol significant for cellular metabolism in the filamentous fungi *M. oryzae*?:
(I) Glycerolipid synthesis: Glycerol serves as a backbone for the synthesis of glycerolipids, which are crucial components of cellular membranes. Glycerol-3-phosphate can be esterified with fatty acids to form triglycerides, which are storage lipids involved in energy storage and insulation. Additionally, glycerolipids such as phospholipids are vital constituents of cell membranes, playing a role in maintaining membrane structure and integrity (Yao *et al.*, 2014).
(II) Energy production: Glucogenesis is particularly relevant during periods of nutrient deprivation or when alternative energy sources are required (Zhang *et al.*, 2018). Glycerol can be converted to glucose through the process of glyceroneogenesis, providing an alternative

source of glucose for energy production and maintaining glucose homeostasis. This is especially important during periods when glucose availability is limited (Shi *et al.*, 2018).
(III) Osmoregulation and cellular homeostasis: Glycerol plays a role in osmoregulation, helping to maintain cellular water balance. Eukaryotic organisms, especially those living in environments with fluctuating osmotic conditions, may accumulate glycerol as an osmolyte Glycerol acts as a compatible solute, helping to balance water movement and prevent cellular dehydration or swelling (Bohnert *et al.*, 2019).

Overall, glycerol is crucial for eukaryotic organisms due to its involvement in energy metabolism, membrane synthesis, osmoregulation and glucose homeostasis. Its versatile properties make it an essential molecule for cellular function and adaptation to various physiological conditions (Klein *et al.*, 2017). With the focus on the HOG pathway in *M. oryzae* is glycerol being produced as a compatible solute in order to compensate the osmotic imbalance between extracellular osmotic changes and cellular homeostasis (Jacob *et al.*, 2015). Eukaryotes have evolved different pathways to process glycerol, depending on the specific cellular needs and metabolic conditions:
(I) Glycerol uptake: Eukaryotic cells can acquire glycerol from the environment through various transporters located on the plasma membrane, like Glycerol/H^+ Symporter MoStl1p (Luyten *et al.*, 1995). Glycerol can also be generated internally through the breakdown of triglycerides or the glycerolipid component of membranes (Thines, Weber and Talbot, 2000).
(II) Conversion to glycerol-3-phosphate (G3P): Once inside the cell, glycerol is typically converted to glycerol-3-phosphate (G3P) by the action of glycerol kinase. G3P serves as an important intermediate in multiple metabolic pathways (Bailoni and Poolman, 2022).
(III) Glycerol-3-phosphate dehydrogenase (GPDH) pathway: In this pathway, G3P is oxidized to dihydroxyacetone phosphate (DHAP) by the enzyme glycerol-3-phosphate dehydrogenase. DHAP can enter the glycolytic pathway to produce ATP or be used in other biosynthetic processes (Klein *et al.*, 2017).
(IV) Glycerol-3-phosphate shuttle: G3P can also be utilized in the mitochondria through the glycerol-3-phosphate shuttle. In this shuttle, G3P is converted to DHAP by cytoplasmic GPDH. DHAP can then cross the mitochondrial membrane and be reconverted to G3P by mitochondrial GPDH. This G3P can enter the glycolytic pathway or contribute to the production of ATP through oxidative phosphorylation (Shi *et al.*, 2018).
(V) Glyceroneogenesis: Glyceroneogenesis is a pathway that allows the synthesis of glycerol from non-carbohydrate precursors such as pyruvate, lactate, or amino acids. It involves the conversion of these substrates to G3P through a series of enzymatic reactions (Xue, Chen and Jiang, 2017).

(VI) Glycerol utilization: Glycerol can also be utilized as a carbon source for energy production. It can be converted to DHAP through the action of glycerol dehydrogenase, and subsequently enter the glycolytic pathway to generate ATP (Chen and Liu, 2016).
(VII) Glycerolipid synthesis: Glycerol can be utilized for the synthesis of glycerolipids, such as triglycerides and phospholipids, which are essential components of cellular membranes and energy storage molecules. Glycerol-3-phosphate acyltransferase enzymes catalyze the esterification of G3P with fatty acids to produce glycerolipids (Gao *et al.*, 2013).
Overall, glycerol metabolism in eukaryotic organisms involves the uptake, conversion, and utilization of glycerol for energy production and the synthesis of important biomolecules (Foster *et al.*, 2017). Glycerol, recognized for the function in osmotic regulation and cellular resilience, has a multifaceted role as a precursor and active participant in various primary metabolic pathways. Glycerol is involved in central carbon metabolites and helps to the synthesis of fundamental molecules essential for the fungi's growth and survival like the primary metabolism.

1.3 Rapid adaptation – The current knowledge in *Magnaporthe oryzae*

Evolution is generally perceived as an incredibly slow process occurring over thousands of years. However, recent research in evolutionary dynamics has shed light on rapid evolutionary adaptations in microorganisms, revealing that these processes can occur much faster than previously thought (Selmecki *et al.*, 2015). Despite this, the molecular mechanisms driving these rapid adaptations are not well understood, as they often involve rare, unpredictable spontaneous mutations. Understanding these mechanisms is crucial not only for academic purposes but also for addressing global challenges such as ecosystem changes, climate change, the emergence of pathogens, the spread of invasive species, and the development of (multi-)resistance to vaccines and drugs (Gladieux *et al.*, 2018). Advantageous genetic changes that evolve and persist through natural selection are referred to as "adaptive walks." A notable example is the development of multidrug resistance in microbial organisms. For pathogenic microbes, the ability to rapidly evolve is essential for survival (Naranjo-Ortiz and Gabaldón, 2020). Rapid adaptation can occur within a few generations, making it possible to observe genetic evolution, population dynamics, and competitive interactions in real time. Most research has focused on bacterial systems (Good *et al.*, 2017), but similar principles apply to other microorganisms.

Adaptive Laboratory Evolution (ALE) is a powerful methodology within evolutionary biology that allows researchers to observe and manipulate the evolutionary trajectories of

microorganisms under controlled laboratory conditions. By subjecting microbial populations to specific environmental stresses or selective pressures over multiple generations, ALE facilitates the rapid accumulation of beneficial mutations and the emergence of adaptive traits. This approach provides real-time insights into evolutionary dynamics, revealing the genetic and physiological mechanisms driving adaptation. ALE has practical applications in biotechnology, medicine, and environmental science (Nam, Conrad and Lewis, 2011)

In the rice blast pathogen *M. oryzae* traditional evolutionary mechanisms have been documented through high nucleotide variation, high substitution rates, and frequent polymorphisms (Huang *et al.*, 2014). Pathogenic organisms like *M. oryzae* have to continuously adapt to changing environmental stimuli and dynamic host-pathogen interactions (Naranjo-Ortiz and Gabaldón, 2020). Besides long-term adaptations mediated by genomic or epigenetic changes, the facultative pathogenic lifestyle of these fungi requires rapid adaptive responses for swift regulation of cellular processes. This rapid adaptation is facilitated by posttranslational modifications (PTMs), which allow dynamic alterations in protein structure and protein-protein interactions, being both highly flexible and partially reversible (Wang et al., 2022).

Evolutionary adaptation in microorganisms is often driven by genomic instability following whole-genome duplication and the activity of transposable elements, both of which restructure the genome and contribute to species diversification (Fouché *et al.*, 2020). These processes are believed to play a role in the emergence of disease resistance genes in plants, while the evolution of avirulence genes (Avr genes) in plant-pathogen interactions remains less studied (Figueroa, Dodds and Henningsen, 2020). The HOG pathway in *M. oryzae* exemplifies the complexity and importance of signal transduction pathways in cellular adaptation. This pathway controls the adaptation to increased osmolarity in the environment. The HOG pathway in *M. oryzae* has proven to be a valuable model for fundamental research on physiological functions and agricultural fungicides' modes of action (Bersching and Jacob, 2021). Loss of function (lof) mutants of the HOG pathway have been used to characterize individual functional elements (Jacob, Schüffler and Thines, 2016). These lof mutants are not only osmosensitive but also fail to produce important osmoprotectants such as arabitol. In 2019, Bohnert *et al.* cultivated these osmosensitive lof mutants under continuous osmotic stress and observed the emergence of stable individuals from each mutant. Interestingly, these rapidly evolved suppressor strains were able to cope with osmotic stress by producing glycerol instead of arabitol (Bohnert *et al.*, 2019; Jacob and Bersching, 2021). These suppressor strains, classified as either reversible or irreversible based on their ability to respond to stress, exhibited a notable intracellular adaptation. One main difference is that the irreversible suppressor mutants are able to survive under osmotic stress (notably like the wildtype), the reversible mutants are unable to cope with osmotic stress (notably like the original lof mutants).

The adaptation experiment has been replicated over 100 times with consistent results. Remarkably, this phenomenon has only been observed in inactivation mutants related to the HOG pathway *(*Δ*Mohik1,* Δ*Moypd1,* Δ*Mossk1,* Δ*Mossk2,* Δ*Mopbs2,* and Δ*Mohog1*), and not in other osmosensitive mutants. The only exception is that the adaptation process has not been successful with the two-component hybrid histidine kinase MoSln1p.

1.4 Scope of the dissertation

This dissertation investigates the molecular and genetic mechanisms driving adaptive evolution in the rice blast pathogen *M. oryzae* using directed adaptive laboratory evolution (DALE). The study focuses on understanding the adaptation processes in suppressor strains that emerge from the HOG pathway inactivation mutants *(*Δ*Mohik1,* Δ*Moypd1,* Δ*Mossk1,* Δ*Mossk2,* Δ*Mopbs2,* and Δ*Mohog1*) under osmotic stress. These suppressor strains exhibit a metabolic shift, producing glycerol as a primary metabolite in response to osmotic stress, contrasting with the wildtype's production of arabitol and are so called adapted or suppressor strains (reversible and irreversible).

A key element of this research is the development and validation of a protocol for the generation of suppressor mutants by DALE, which facilitates the study of adaptive evolution in a controlled environment. Through detailed characterization of these suppressor mutants, the study identifies and analyzes the genetic and metabolic pathways implicated in this metabolic shift, particularly focusing on genes involved in glycerol metabolism as determined by transcriptome analyses.

Further investigations include creating and analyzing double mutant strains. The suppressor mutants that lack specific glycerol metabolism-related genes to distinguish the contributions of these genes from alternative drivers of adaptation. Additionally, the reintegration of HOG pathway genes into suppressor strains is explored to evaluate the stability of the suppressor phenotype and its primary metabolite production in response to osmotic stress.

The study integrates transcriptomic, proteomic, and phosphoproteomic analyses to provide a comprehensive framework for understanding rapid adaptation mechanisms in *M. oryzae*. The findings contribute to the broader understanding of microbial adaptation, with potential applications in managing pathogenic evolution and addressing microbial responses to environmental stressors.

2. Controllable Bypass Suppression in *Magnaporthe oryzae*

Jacob, S. and Bersching, K. (2021) 'Controllable Bypass Suppression in Magnaporthe oryzae', in S. Jacob (ed.) *Magnaporthe oryzae: Methods and Protocols*. New York, NY: Springer US (Methods in Molecular Biology), pp. 225–231. Available at: https://doi.org/10.1007/978-1-0716-1613-0_18.

Full text article:
https://link.springer.com/protocol/10.1007/978-1-0716-1613-0_18

To investigate the empirical work and validate the adaptation mechanism in *Magnaporthe oryzae*, a stable assay was implemented early in this study. A key aspect of this research is the development and validation of a protocol for generating suppressor mutants through Directed Adaptive Laboratory Evolution (DALE), enabling the study of adaptive evolution in a controlled environment. Evolutionary adaptation is conventionally seen as a slow process over extended periods, yet this study shows that *M. oryzae* can rapidly adapt its osmoregulatory function under salt stress. Given the reproducibility of this rapid adaptation, establishing a validated, standardized assay is essential, as a well-designed, reliable method allows researchers to consistently generate and analyze suppressor strains, ensuring robust and reproducible results.

Chapter 18

Controllable Bypass Suppression in *Magnaporthe oryzae*

Stefan Jacob and Katharina Bersching

Abstract

Evolutionary adaptation of living organisms is commonly thought to be the result of processes that have taken place over long periods of time. By contrast, we found that the filamentous rice blast fungus *Magnaporthe oryzae* rapidly suppresses the osmosensitive "loss of function" (lof) phenotype in knockout mutants of the high-osmolarity glycerol (HOG) pathway. That suppression occurs highly reproducibly after 4 weeks of continuous growth upon salt stress. Stable mutants reestablished in osmoregulation arise independently out of individual osmosensitive lof mutants of the HOG pathway. The major compatible solute produced upon salt stress by these suppressor strains was found to be glycerol, whereas it is arabitol in the wildtype strain. We aim to address the molecular or biochemical mechanisms behind this rapid suppression and characterize the associated factors and signaling pathways which enable or prevent suppression. Therefore, we present a protocol to generate these suppressor mutants in *M. oryzae* easily to study the molecular basis of evolutionary processes or even epigenetic modulation. This protocol may be applicable to many other fungi and will open a door for researchers worldwide since the HOG pathway is worked on intensively in many different model organisms.

Key words High-osmolarity glycerol (HOG) pathway, Adaptation, Bypass suppressor, Osmolyte production, Glycerol, Arabitol, *Magnaporthe oryzae*, Suppressor mutation, Rewiring, Polyol metabolism

1 Introduction

Genetic suppression is a term describing mutations which restore the original phenotype seen prior to the original background mutation. Historically, suppressors have proven to be extremely valuable since the early 1970s for determining the relationship between two gene products in vivo, even in the absence of cloning or sequence information [1–5]. Suppressor mutations are useful for identifying new genetic sites which affect a biological process of interest. They also provide evidence between functionally interacting molecules and intersecting biological pathways [2]. On one hand, mutations that restore a wildtype phenotype despite the continued presence of the original mutation are termed suppressors. On the other hand, modifiers that result in a more severe phenotype are termed

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https://doi.org/10.1007/978-1-0716-1613-0_18,

enhancers. The term suppressor was first introduced into *Magnaporthe* research by the identification of one of the most prominent bypass suppressors, the Mac1 phenotype (sum), involved in cAMP signaling [6]. The diversity of possible suppressor mechanisms can present a challenge in defining the relationship between a suppressor and the original gene. The simplest suppression mechanism is *intragenic suppression*, where a phenotype caused by a primary mutation is complemented by a second mutation in the same gene. *Informational suppressors* alter the passage of information from DNA to protein, in apparent violation of the genetic code. Furthermore, the class of *amount suppressors* consists of mutations that increase the amount of the original protein. Another mechanism for increasing the overall activity of a defective protein is to increase its specific activity. The respective mutations are called *activity suppressors*. A mutation that alters one step of the multistep pathway can often be suppressed by mutations in genes that affect other steps within that same pathway. This class of suppressors is often extremely informative, because in addition to identifying other components of the pathway of interest, the suppressors can also reorder the pathway [7]. A mutation that inactivates one pathway can often be suppressed by altering a second pathway. This *bypass suppressor* might affect the regulation of a pathway that has a related or overlapping function, or the suppressor could alter the specificity of a functionally unrelated pathway. The use of innovative suppressor hunts is important for deciphering biological pathways now and in the future. However, given the importance and the various possibilities of different types of suppression, knowledge of the molecular mechanisms behind and the molecular basis of what exactly facilitates, respectively, constrains suppressions are not well-documented to date. Therefore, a better understanding of the molecular mechanisms behind suppressors is required.

We describe a method in this protocol which explains how to generate suppressor mutants arising from "loss of function" (lof) mutants of the high-osmolarity glycerol (HOG) signaling pathway in *M. oryzae*. Inactivation of the components of the HOG pathway results in mutant strains, which are viable but impaired in osmoregulation [8]. After 4 weeks of cultivation upon high osmolarity, stable individuals with reestablished osmoregulation capacity arise independently from each of the lof mutants [9]. This phenomenon is highly reproducible and occurs only in osmosensitive lof mutants related to the HOG pathway and not in other osmosensitive *Magnaporthe* mutants. The major compatible solute produced by these suppressor strains to cope with high osmolarity is glycerol, whereas it is arabitol in the wildtype strain. Scientists will be able to follow up the question of how eukaryotic signaling pathways evolve to adapt toward changing environmental situations with the following protocol. This is one of the challenging questions in biology.

Adaptive evolution is a central biological process that underlies diverse phenomena, from the acquisition of antibiotic resistance to the evolution of niche specialization.

2 Materials

2.1 Generation of Suppressor Mutants (Suppressor Assay)

1. Fungal lof mutant strains of the HOG pathway (e.g., *Magnaporthe oryzae ΔMoypd1*, *ΔMossk1*, *ΔMossk2*, *ΔMopbs2*, or *ΔMohog1*) (*see* **Note 1**).
2. Petri dishes.
3. Complete medium (CM). To make 1 L, mix 10 g glucose, 1 g yeast extract, 2 g peptone, 1 g casamino acids, 50 mL nitrate salt solution (containing per liter H_2O: 120 g $NaNO_3$, 10.4 g KCl, 30.4 g KH_2PO_4, 10.4 g $MgSO_4{\cdot}7H_2O$), and 1 mL of a trace element solution (containing per liter H_2O: 22 g $ZnSO_4{\cdot}7H_2O$, 11 g H_3BO_3, 5 g $MnCl_2{\cdot}4H_2O$, 5 g $FeSO_4{\cdot}7H_2O$, 1.7 g $CoCl_2{\cdot}6H_2O$, 1.6 g $CuSO_4{\cdot}5H_2O$, 1.5 g $Na_2MoO_4{\cdot}2H_2O$, 50 g Na_2EDTA, pH 6.5 adjusted by 1 M KOH).
4. Osmotic stress medium. Mix the CM with stress-inducing agents, for example, KCl, NaCl, or sorbitol (*see* **Note 3**).
5. Osmotic stress stock solutions. Exemplarily, solve 2.5 M KCl in H_2O.
6. Autoclave.
7. Growth chamber or incubator with light.

All chemicals used were p.a. quality unless otherwise stated. All preparations should be carried out with deionized water (H_2O).

2.2 Verification of the Suppressor Strains

1. DNeasy® Plant Mini Kit (Qiagen).
2. Individual restriction enzymes (depends on which gene knockout is to be validated).
3. Reagents and equipment for standard Southern analysis.
4. Further HPAEC-PAD analysis is recommended for a reliable and comprehensive testing of the suppressor strains generated (*see* Chapter 4). Consequently, reagents and equipment for HPAEC-PAD analysis are needed.

3 Methods

3.1 Generation of Suppressor Mutants (Suppressor Assay)

1. Use the lof mutant strains with an inactivated HOG pathway (e.g., *ΔMoypd1*, *ΔMossk1*, *ΔMossk2*, *ΔMopbs2*, and *ΔMohog1*).
2. Begin growing each fungus from the filter stocks (*see* **Note 2**) and incubate them about 8–10 days at 26 °C on solid CM, light/dark 16/8 h (in petri dishes).

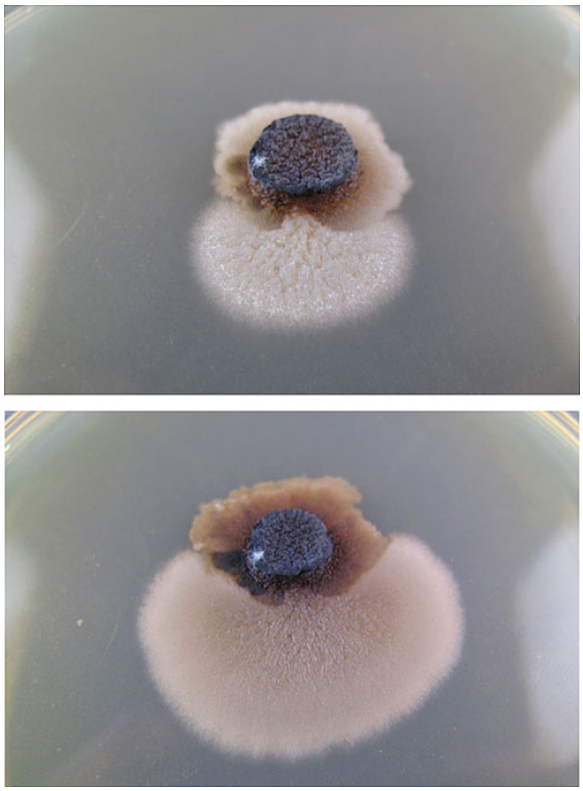

Fig. 1 Example of *Magnaporthe oryzae* "loss of function" (lof) strain *ΔMohog1* and *ΔMohog1(suppressor)* after 5 weeks of cultivation upon 1 M KCl salt stress. The lof strain *ΔMohog1* (dark brown) was grown on CM including 1 M KCl at 26 °C with light/dark 16/8 h. The *ΔMohog1* mutant was found to be highly sensitive toward 1 M KCl, whereas the outgrowing *ΔMohog1(suppressor)* strain (bright) was able to grow much faster

3. Transfer a small plug from each plate to new CM plates and incubate for 8–10 days before starting the suppressor assay.
4. Transfer a small plug from a plate that is growing in the incubator onto osmotic stress medium (e.g., solid CM including 1 M KCl) (*see* **Note 3**).
5. Cultivate the fungal cultures at 26 °C with a light/dark rhythm of 16/8 h.
6. After about 4 weeks, small mycelium filaments begin to grow out of the original cultures (Fig. 1; *see* **Note 4**). These mycelium filaments grow much faster as compared with its "parent" lof strain.
7. Transfer a small plug from the rapidly growing "adapted" suppressor mutant from the 1 M KCl salt stress medium to normal CM (unstressed conditions). Cultivate the cultures for

3–4 weeks under stress-free conditions at 26 °C with light/dark 16/8 h, transferring the cultures every 8–10 days onto freshly prepared CM.

8. Then, transfer the suppressor strains back to the repeated salt stress (e.g., CM + 1 M KCl) and incubate them further at 26 °C with light/dark 16/8 h.
9. After a few days, the suppressor strains will be found to grow as fast as those taken directly from stress conditions. The phenotype appears to be stable and memorized (*see* **Note 5**).
10. Finally, before using the suppressor strains in other assays, take a single spore of the suppressor culture and propagate it to obtain a "pure strain" of one individually suppressed strain.

3.2 Further Verification of the Suppressor Strains

Please check two highly relevant parameters initially in the suppressor strains to avoid any possibility of contaminations or confusions about mixed cultures. Firstly, determine whether the genes originally inactivated in the "parent" lof mutants are still inactivated, for example, by means of standard Southern analysis. Additionally, point out whether the suppressor strains produce glycerol instead of arabitol as an osmotic stress response (for detailed procedure, *see* Chapter 4).

An example of the results of carbohydrate analytics for verification of suppressor strains of *M. oryzae* is shown in Fig. 2.

1. Impose hyperosmotic shock by adding osmotic stress stock solutions to the fungal strains, so that the final concentration in the culture broth is 0.5 M KCl.
2. Determine the intracellular levels of the major osmolytes (e.g., arabitol and glycerol) after 7 h (*see* **Note 6**).

4 Notes

1. It is important to know that it has not been possible for us to isolate suppressor strains from HOG pathway-independent osmosensitive *Magnaporthe* mutant strains, i.e., *ΔMostu1* (transcription factor in cAMP/PKA signaling pathway), *ΔMogpd1* (glycerol-3-phosphate dehydrogenase), or *ΔMoskn7* (response regulator protein), to date.

   Furthermore, long-term cultivation upon salt stress or other osmotic stress agents do not lead to a "better" osmoregulation or osmotolerance in *Magnaporthe* wildtype strains.
2. Fungal stocks are obtained from filter paper disks containing fungal mycelia. Stocks must be dehydrated in a desiccator for at least 2 weeks and stored in dry sterile tubes at −20 °C. The reason is that *M. oryzae* produces spontaneous mutations after a lot of subcultures. Therefore, the process of subculturing of

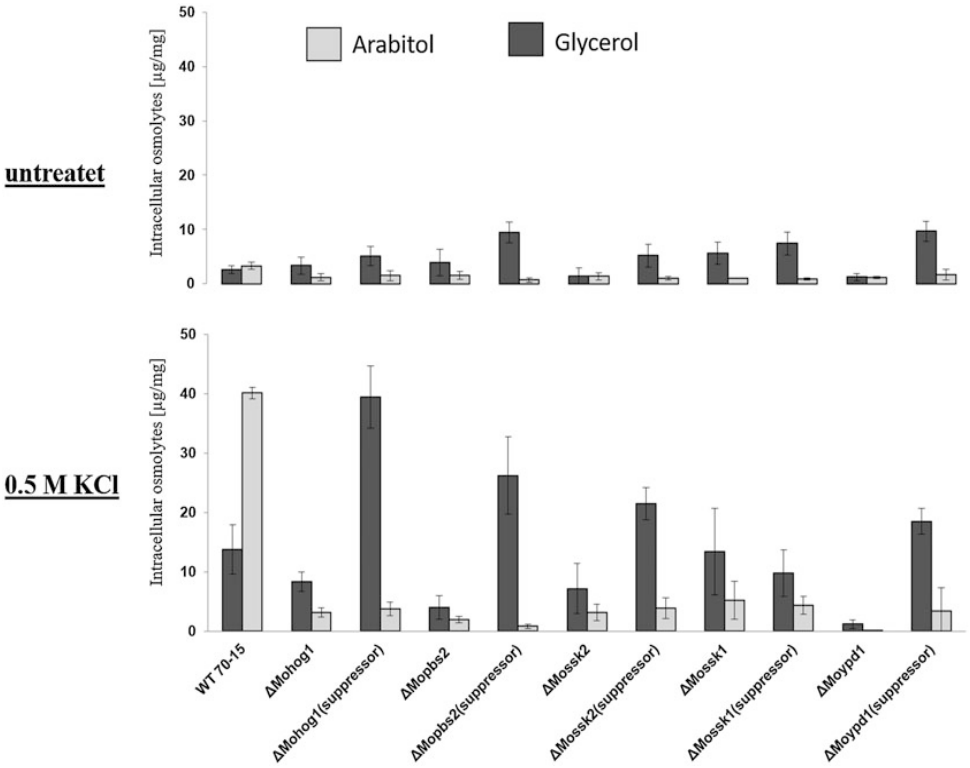

Fig. 2 Carbohydrate production in the *Magnaporthe oryzae* wildtype strain (WT 70-15), the lof mutants, and the corresponding "suppressor" strains after 0.5 M KCl treatment. Compatible solute accumulation of glycerol (in dark gray) and arabitol (in light gray) was determined in mycelium after salt stress for 7 h. Carbohydrates were extracted and quantified by HPAEC-PAD analysis. Error bars represent the standard deviation of three biological replicates of each strain

this fungus should be avoided. Put the filter paper onto a CM agar plate and incubate at 26 °C with light/dark 16/8 h when using the fungal culture from stock.

3. The most effective concentration to obtain suppressor mutants should be determined previously in growth assays for each fungal species. Find the concentration in which fungal growth of the lof mutants with an inactivated HOG pathway is strongly restricted, whereas the wildtype strain should be affected but able to grow much better compared to the mutants.
4. The period of the suppressor event could vary between 4 and 12 weeks, whereby most of the events take place after 4–6 weeks. Do not transfer the cultures to freshly prepared stress medium several times; you have to leave them on the same plate for the whole time.
5. There will be some suppressor candidate strains which cannot cope with the osmotic stress. These strains should be discarded because the suppression is unstable.

6. Arabitol is the major intracellular compatible solute produced by the wildtype strain after osmotic shock. By contrast, the lof mutants *ΔMohog1*, *ΔMopbs2*, *ΔMossk2*, *ΔMossk1*, and *ΔMoypd1* could not produce either arabitol or glycerol in significant amounts. Interestingly, it was found that all suppressor strains *ΔMohog1(suppressor)*, *ΔMopbs2(suppressor)*, *ΔMossk2(suppressor)*, *ΔMossk1(suppressor)*, and *ΔMoypd1(suppressor)* responded to hyperosmotic stress by accumulating high amounts of glycerol rather than arabitol.

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3. Data-Independent Acquisition (DIA) Is Superior for High Precision Phospho-Peptide Quantification in *Magnaporthe oryzae*

Bersching, K. *et al.* (2023) 'Data-Independent Acquisition (DIA) Is Superior for High Precision Phospho-Peptide Quantification in Magnaporthe oryzae', *Journal of Fungi*, 9(1), p. 63. Available at: https://doi.org/10.3390/jof9010063.

Supplementary Materials: The following supporting information can be downloaded at: https:// www.mdpi.com/article/10.3390/jof9010063/s1, Table S1: Variable window sizes foe DIA acquisition with Orbitrap Exploris 480.

Author Contributions: Conceptualization, T.M., S.T. and S.J.; methodology, T.M. and K.B.; software, S.T.; validation, T.M. and K.B.; formal analysis, T.M. and S.T.; resources, S.T. and S.J.; writing—original draft preparation, K.B. and T.M; writing—review and editing, S.J. and S.T.; supervision, S.J. and S.T.; project administration, S.J. and S.T.; funding acquisition, S.J. and S.T. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Not applicable.

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Data Availability Statement: All raw data have been uploaded via JPOST [27] to be available on proteo-meXchange [28] and can be accessed with the identifier PXD034481.

Conflicts of Interest: The authors declare no conflict of interest.

Full-text article:
https://www.mdpi.com/2309-608X/9/1/63

In collaboration with Prof. Dr. Tenzer from Institute for Immunology at the University Medical Center of the Johannes Gutenberg University in Mainz, we established a robust method for phospho-peptide quantification. Protein phosphorylation, a reversible process central to major cellular signaling networks, is increasingly analyzed through quantitative phospho-

peptidomics, evolving from a specialized technique to a powerful platform for comprehensive profiling. We applied data-independent acquisition (DIA) using *Magnaporthe oryzae* as a model, achieving a dramatic increase in data completeness while maintaining phosphosite and sequence confidence. Our method shortens LC-MS/MS analysis time from 3 hours to 1 hour and identifies up to 10 times more phosphosites than previously reported studies on *M. oryzae*, offering an enhanced methodology and valuable resource for studying signaling in filamentous fungi.

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Data-Independent Acquisition (DIA) Is Superior for High Precision Phospho-Peptide Quantification in *Magnaporthe oryzae*

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Abstract: The dynamic interplay of signaling networks in most major cellular processes is characterized by the orchestration of reversible protein phosphorylation. Consequently, analytic methods such as quantitative phospho-peptidomics have been pushed forward from a highly specialized edge-technique to a powerful and versatile platform for comprehensively analyzing the phosphorylation profile of living organisms. Despite enormous progress in instrumentation and bioinformatics, a high number of missing values caused by the experimental procedure remains a major problem, due to either a random phospho-peptide enrichment selectivity or borderline signal intensities, which both cause the exclusion for fragmentation using the commonly applied data dependent acquisition (DDA) mode. Consequently, an incomplete dataset reduces confidence in the subsequent statistical bioinformatic processing. Here, we successfully applied data independent acquisition (DIA) by using the filamentous fungus *Magnaporthe oryzae* as a model organism, and could prove that while maintaining data quality (such as phosphosite and peptide sequence confidence), the data completeness increases dramatically. Since the method presented here reduces the LC-MS/MS analysis from 3 h to 1 h and increases the number of phosphosites identified up to 10-fold in contrast to published studies in *Magnaporthe oryzae*, we provide a refined methodology and a sophisticated resource for investigation of signaling processes in filamentous fungi.

Keywords: proteomics; LC-MS/MS; phospho-peptide enrichment; bioinformatics; cellular signaling; *Magnaporthe oryzae*; phosphorylation; DDA; DIA; phospho-peptidomics

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1. Introduction

The phosphorylation of proteins is among the most prominent and significant post-translational modifications [1]. Protein kinases make this reversible modification possible by the addition of a phosphate group (PO_4) to the polar residual of amino acids. As a consequence, this addition modifies the protein from an apolar (hydrophobic) to a more polar (hydrophilic) state. The subsequent conformational changes enable interactions with other molecules [2]. The biochemical nature of phosphorylated amino acids facilitate interaction with other proteins, which enables, e.g., the assembly of proteins or protein complexes. The fundamental challenge in the research of signal transduction pathways is the highly dynamic nature of reversible phosphorylation of the involved signaling proteins [3,4]. Apart from the static information of whether a peptide, peptide fragment or a certain amino acid residue is phosphorylated or not ("on" or "off"), it is of the utmost interest to understand the dynamic alteration of quantitative changes in phosphorylation levels over time associated with a given stimulus or cellular process [5].

In the last decade, liquid chromatography-tandem mass spectrometry (LC-MS/MS) and variations thereof were the method of choice to quantify thousands of proteins across multiple biological samples with high throughput, robustness and sensitivity [6]. An unsolved problem in quantitative phospho-peptidomics by mass spectrometry is still the low abundance of phosphorylated proteins as compared to the complete proteome and to the complement of a given protein, from which naturally only a small portion is phosphorylated in a particular way [7–9]. The drive to solve this problem and constantly improve the precision of protein measurements pushes MS-techniques and the related methods forward, following the overarching goal of proteomics to comprehensively identify and quantify all proteins and protein modifications in a biological system [10]. For example, the enrichment of phospho-peptides is absolutely necessary, including immunoprecipitation (IP), metal oxide/immobilized ion affinity chromatography (MOAC/IMAC), fractionation strategies such as high-pH reversed-phase chromatography (HpH RP), strong cation exchange (SCX), or electrostatic repulsion hydrophilic interaction liquid chromatography (ERLIC) [11].

One of the major bottlenecks to obtaining a comprehensive and precise analysis nowadays is not the accuracy of the instruments and measurements used but rather important processes such as data acquisition and data processing [12]. Most of the MS-based proteomic workflows use the "data-dependent acquisition" (DDA) strategy [13–16], often in combination with "dynamic exclusion" (DE), which rules out a selection of fragmented peptides within a specific time window [17]. In DDA, precursor ions are stochastically selected on the basis of their signal intensity and subsequently fragmented, separated and finally detected by a mass analyzer such as a "time-of-flight" (TOF) or an Orbitrap [18]. In more detail, the top N most intensive m/z ions are identified from the MS1 scan (precursor spectrum, in proteomics typically precursors are peptides) by the operating software of the mass spectrometer and sequentially selected with a very narrow window (e.g., $\pm$0.5 Dalton) by the quadrupole for fragmentation, so their MS2 spectra (fragment spectra) can be collected. The resulting fragment m/z values vary by the corresponding masses for amino acids according to their sequence. This way, the processing software (or the analyzing scientist) can compare the obtained amino acid sequence with the measured m/z of the intact peptide. Depending on the amount of amino acid sequence evidence and the congruence between theoretical and measured precursor m/z, a score for the probability of a correct identification is calculated [19]. The selected number N of most intense ions is typically between 10 and 25 and can be chosen depending on the instrument speed and on the analytical need. When short LC gradients and highly complex MS1 spectra are present, a high N is needed for deep peptide coverage. On the other hand, a high N costs measurement time and MS1 quantification accuracy. In general, the DDA strategy decides, depending on the MS1 information, which precursors are selected for fragmentation. It provides clean and high-quality spectra that can also be used for de novo sequencing with certain prerequisites. In addition to that, the data processing is not computationally intensive and implements easy and straight forward algorithms that are accessible to a broad community.

In contrast, to alleviate the limitations associated with DDA and DE, strategies on unbiased "data-independent acquisition" (DIA) are available in which every peptide within a specific time window is fragmented [20]. That means that, in data independent acquisition strategy, no preselection is performed. The fragmentation is independent of any MS1 information. Instead of choosing a very narrow window for selecting the precursors for fragmentation, a wide window of precursor m/z are allowed to pass through the quadrupole [21]. This way, multiple precursors co-fragment and create chimeric MS2 spectra, where the assignment of the precursor and their corresponding fragments is not easily possible. More complex bioinformatic algorithms have to be applied to elucidate the amino acid evidence for each precursor [22]. This also includes the use of spectral libraries, which are either labor intensive or computationally intensive to create. Recent developments in the proteomics community show improvements in algorithms and software to be able to process DIA generated raw data in a comprehensive and user-friendly way.

The accessibility of high performing computer systems has paved the way for increasing use of DIA [23]. The major advantage of DIA is a robust and accurate quantification as well as the decrease of missing values, due to the fact that no selection of precursors is performed. Instead, borderline signal intensities are also fragmented and have the chance to be identified and quantified.

Prior to this study, it was generally assumed that DIA can quantify the same number of proteins as typically identified by DDA methods, but with better accuracy and reproducibility across many samples [24]. In DDA, one major problem was the high number of missing values caused by the experimental procedure due to either a random phospho-peptide enrichment selectivity or borderline signal intensities, which both cause the exclusion for fragmentation. From this follows an incomplete dataset reducing confidence in the subsequent statistical bioinformatic processing.

Here, we successfully developed a method including DIA for data acquisition by using the filamentous fungus *Magnaporthe oryzae* as model organism. Application of this method resulted in an absolutely reliable dataset of *M. oryzae* under osmotic stress with high data quality (such as phosphosite and peptide sequence confidence), while at the same time data completeness increases dramatically. We are convinced that this is an excellent basis for further research on the dynamic processes of phosphorylation in signaling networks in a high quality as never seen before.

2. Materials and Methods

2.1. Sample Preparation

2.1.1. Cultivation of Magnaporthe Oryzae

The fungal strain used in this study was *Magnaporthe oryzae* (*M. oryzae* 70-15 strain (MoWT), Fungal Genetics Stock Center). The strain was maintained at 26 °C on complete medium (CM) according to [25]. For protein isolation, the *M. oryzae* cultures were grown in 250 mL liquid CM in 500-mL glass flasks for 96 h at 26 °C and 120 rpm. Samples were then taken and the mycelium was immediately separated from the culture fluid and ground into powder with the TissueLyserII (Qiagen) according to the user manual. In order to generate a resource for research on osmotic stress in *M. oryzae*, the samples were stressed by the addition of KCL to a final concentration of 0.5 M, and samples were taken at 0 min (as control sample), 10 min, 60 min, 240 min and 24 h. All samples were generated in biological quadruplicates, making in total 20 samples. In addition to that, three mutated variants with loss of function of MoHOG1, a central osmostress MAPK signaling protein, were included in this research. Details about mutant types and preparation are provided in [26].

2.1.2. Cell Lysis and Protein Digest

If not stated otherwise, all reagents were used in LC-MS/MS grade from common vendors. The sample preparation for all *Magnaporthe oryzae* samples has been performed as described in [11]. In short, a sample aliquot of lyophilized and grinded mycelium was suspended in boiling SDS/DTT lysis buffer with following treatment of ultrasound. Proteins were precipitated by chloroform/methanol precipitation and resolubilized in urea containing buffer. DNA/RNA removal by benzonase and tryptic digest was performed overnight, followed by desalting and lyophilization. An aliquot of lyophilized peptides was used for proteome analysis, and 1000 µg was subjected to phospho-peptide enrichment by TiO_2 spin tips.

2.1.3. Phospho-Peptide Enrichment

Phospho-peptide enrichment of *M. oryzae* samples was performed as described in [11].

2.2. Peptide Identification

2.2.1. LC-MS/MS of M. oryzae Samples for Resource

A total of 3 µL of the reconstituted phospho-peptides were separated on an Ultimate 3000 nanoUPLC (Thermo Scientific, Waltham, MA, USA) with 300 nL/min by a reversed

phase C18 column (HSS-T3 C18 1.8 µm, 75 µm × 250 mm, Waters Corporation, Milford, MA, USA) at 55 °C using a 45 min linear gradient from 95% Eluent A (0.1% TFA, 3% DMSO in water) to 35% Eluent B (0.1% TFA, 3% DMSO in ACN) with additional 15 min of equilibration (60 min LC runtime total) followed by ionization in positive mode using a Nanospray Flex electrospray ionization source (Thermo Scientific). Mass-to-charge analysis of the eluting peptides was performed using an Orbitrap Exploris 480 (Thermo Scientific) in data independent acquisition (DIA) mode. MS1 scans were acquired with a resolution of 120,000 at 200 m/z in a range of 345–1250 m/z. The RF lens was set to 40% and AGC target to 300% (i.e., corresponding to 3×10^6 charges). DIA MS2 scans were acquired with a resolution of 30,000 at 200 m/z with a variable window scheme (as shown in supplementary Table S1). The normalized collision energy was set to 27%, RF lens to 40% and AGC target to 1000% (i.e., corresponding to 10×10^6 charges).

2.2.2. LC-MS/MS of M. oryzae DIA Samples for Comparison

A total of 2 µL of the reconstituted phosphor-peptides were separated on a nanoElute LC system (Bruker Corporation, Billerica, MA, USA) at 400 nL/min using a reversed phase C18 column (Aurora UHPLC emitter column, 25 cm × 75 µm 1.6 µm, IonOpticks) which was heated to 50 °C. Peptides were loaded onto the column in direct injection mode at 600 bar. Mobile phase A was 0.1% FA (v/v) in water and mobile phase B 0.1% FA (v/v) in can. Peptides were separated, running a linear gradient from 2% to 37% mobile phase B over 45 min. Afterwards, the column was rinsed for 5 min at 95% B followed by equilibration. Eluting peptides were analyzed in positive mode ESI-MS using parallel accumulation serial fragmentation (PASEF) enhanced data-independent acquisition mode (DIA) on a timsTOF Pro 2 mass spectrometer (Bruker Corporation). The dual TIMS (trapped ion mobility spectrometer) was operated at a fixed duty cycle close to 100% using equal accumulation and ramp times of 100 ms each, spanning a mobility range from $1/K_0 = 0.6\,Vs\,cm^{-2}$ to $1.6\,Vs\,cm^{-2}$. We defined 36 × 25 Th isolation windows from m/z 300 to 1165, resulting in fifteen diaPASEF scans per acquisition cycle. The collision energy was ramped linearly as a function of the mobility from 59 eV at $1/K_0 = 1.3\,Vs\,cm^{-2}$ to 20 eV at $1/K_0 = 0.85\,Vs\,cm^{-2}$.

2.2.3. LC-MS/MS of M. oryzae DDA Samples for Comparison

A total of 2 µL of the reconstituted phospho-peptides were separated on an Ultimate 3000 nanoUPLC (Thermo Scientific) with 300 nL/min by a reversed phase C18 column (HSS-T3 C18 1.8 µm, 75 µm × 250 mm, Waters Corporation) at 55 °C using a 45 min linear gradient from 95% Eluent A (0.1% TFA/3% DMSO/Water) to 35% Eluent B (0.1% TFA/3% DMSO/ACN), followed by ionization using a Nanospray Flex electrospray ionization source (Thermo Scientific). All samples were measured in triplicates. Mass-to-charge analysis of the eluting peptides was performed using an Orbitrap Exploris 480 (Thermo Scientific) in data dependent acquisition (DDA) mode. Full scan MS1 spectra were collected over a range of 350–1600 m/z with a mass resolution of 60,000 @ 200 m/z using an automatic gain control (AGC) target of 100%, maximum injection time was set to "Auto" and RF lens to 40%. Within a fixed cycle time of 1.5 s the most intense peaks above the signal threshold of 2×10^4, harboring a charge of 2–6, were selected within an isolation window of 1.4 Da as precursors for fragmentation using higher energy collisional dissociation (HCD) with normalized collision energy of 30. The resulting fragment ion m/z ratios were measured as MS2 spectra over an automatically selected m/z range with a mass resolution of 15,000 @ 200 m/z, AGC target was set to "Standard" and maximum injection time to "Auto".

2.2.4. Data Processing Parameters DIA

Peptides were identified and label-free quantification of proteins was performed using DIA-NN (v1.8). Full proteome samples from *M. oryzae* were processed using library free mode with standard parameters, except for tryptic cleavage sites considering no cleavage before proline. The FASTA protein database contained 12.790 protein entries of the *M. oryzae* reference proteome and 172 common contaminant proteins (both from Uniprot). For

phospho-peptide analysis of *M. oryzae*, a phospho-peptide spectral library was predicted in silico using the built-in library free prediction algorithm provided by DIA-NN. For *M. oryzae*, the aforementioned FASTA database was used as basis.

The spectra library was predicted with the precursor charge range set between 1 and 4, and the range for fragment ions and precursor mass to charge ratio was limited to 250–1250 m/z. The peptide length was set to 7–30. Tryptic cleavage considering no cleavage after the lysine or arginine is followed by proline, and maximum one missed cleavage was allowed. N-terminal methionine excision was enabled and cysteine carbamidomethylation was set as fixed modification. The maximum number of variable modifications was set to 3, allowing exclusively UniMod:21 modifications, i.e., mass delta of 79.9663 corresponding to phosphorylation at serine, threonine and tyrosine. The generated spectral libraries were used for follow-up identification and quantification in DIA-NN using the standard settings.

2.2.5. Data Processing Parameters DDA

The DDA raw files were processed by PEAKS X Pro (BSI, Mississauga, ON, Canada) using the FASTA file described in Section 2.2.4. Precursor tolerance and fragment ion tolerance were set to 15 ppm and 0.03 Da, respectively, two missed cleavages were allowed, camabidomethylation at cysteins was set as fixed modification while oxidation on methionine, and phosphorylation on serine, threonine and tyrosine were set as variable modifications with a maximum of 4 variable modifications per peptide.

2.2.6. Availability of Raw Data

All raw data for *M. oryzae* proteome and phosphoproteome resource have been uploaded via JPOST [27] to be available on proteomeXchange [28] and can be accessed with the identifier PXD034481. All files for the DIA/DDA comparison have been uploaded separately to the archive PXD038605.

3. Results and Discussion

Comparison of DDA vs. DIA Approach for Phospho-Peptide Identification

A promising approach to gain more confidence in phospho-peptide data is the data independent acquisition (DIA) approach. Per definition, DIA generates MS2 spectra of higher complexity compared to DDA. The identification of the phosphosites especially requires sophisticated bioinformatic methods that had not been available in the past. Recent implementations in proprietary software such as Spectronaut [29], and developments of open source software such as DIA-NN [23], in combination with affordable high-performance computational resources made the analysis of phospho-peptides in DIA possible with sufficient confidence within a reasonable time frame. There are only a few publications describing the use of DIA for phospho-peptides [29–31] and thus the differences in the data quality have not yet been reviewed comprehensively, especially in the context of predicted spectral libraries. Furthermore, recent developments in coupling tandem ion mobility spectrometry to high resolution TOF instruments, leading to the commercialization of the timsTOF by Bruker Daltonics, promise a deeper understanding of proteomics datasets by adding an additional identification feature and more confident identification by less complex MS spectra. To investigate the use of DIA for phosphoproteomics in general, and especially the use of the Bruker timsTOF Pro 2, we took the opportunity of available measurement time and produced a dataset of three biological replicates of wildtype *M. oryzae* in DDA with an Orbitrap Exploris 480 and in DIA with a Bruker nanoElute coupled to a timsTOF Pro 2. The datasets were processed with PEAKS and DIA-NN, respectively, and the results summarized in Figure 1.

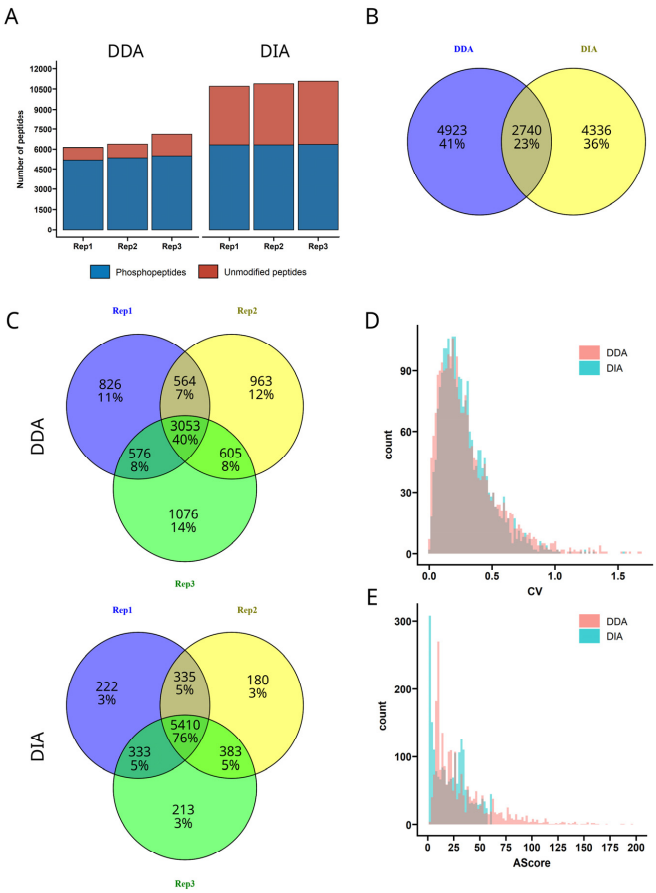

Figure 1. Performance comparison of three *M. oryzae* biological replicates measured in DDA and DIA regarding (**A**) peptide counts (**B**) overlap of identified phosphor-peptides (**C**) overlap within DDA and DIA replicates (**D**) precursor quantity reproducibility and (**E**) phosphosite identification confidence.

The number of identified phospho-peptides is comparable between DDA and DIA, while the number of unmodified peptides in the DIA samples is significantly higher. Consequently, the apparent enrichment efficiency decreases from around 80% in DDA to 50% in DIA (Figure 1A). This observation is explained by the DIA scheme, as no criterion for fragmentation is applied, and also unmodified peptides with low signal intensity are selected for fragmentation. Interestingly, all unique phospho-peptide identifications of the three replicates combined are roughly 10% higher in DDA (7.663 peptides) compared to DIA (7076 peptides), and the overlap of peptide IDs is small (23%), as shown in Figure 1B. The overlap of peptide sequences without considering the phosphosite was slightly increased with 44%, so roughly 20% differ in the assigned phosphosite. First, the type of mass spectrometer used certainly influences the selection of peptides to be ionized and thus selected for fragmentation. Additionally, it has not yet been shown to what extent the software DIA-NN actually provides false positive identifications. To exclude a higher false positive rate as the reason for the low number of overlapping identifications, both datasets (DDA and DIA) were searched in either PEAKS or DIA-NN against a connected database of *Mus musculus* and *Magnaporthe oryzae* proteome. As the sample was generated from *M. oryzae*, the number of identified *Mus musculus* proteins are expected to be not

more than the previously set up false-discovery rate of 1%. For the DDA dataset, from 36.006 total identifications 112 peptides were identified from *Mus musculus* (i.e., 0.3%) and in the DIA dataset, from 40.817 total identifications only 65 were identified from *Mus musculus* (i.e., 0.2%). In conclusion, the false identification rate can be excluded as a reason for the low overlap between the data acquisition strategies. Comparing the intra-sample group overlap of the identifications within the replicate measurements (Figure 1C) reveals another possible reason for the difference in peptide numbers. DIA consistently provides more reproducible identifications, while the overlap for DDA measurements is much less. When accepting only peptides with at least two out of three identifications, the number of quantifiable peptides is 35% higher in DIA (6.461 peptides) compared to DDA (4.798 peptides), while the number of complete peptide data (three out of three) also increased in DIA measurement. Thus, not only the number of quantifiable peptides but also data completeness is increased.

In order to understand the biology, it is not only the number of quantifiable peptides is important, but also the reproducibility and quality. Therefore, the coefficients of variation (CVs) for every quantifiable peptide (at least two out of three replicates) have been calculated from the replicate measurements and plotted as a histogram (Figure 1D). The difference between both datasets is not significant, with median CVs around 25%, which is reasonable due to technical variability in LC-MS/MS measurement. A beneficial effect of DIA on data quality has been shown on the proteome level [32], which results from the higher number of peptides that are available for quantification. A second important aspect in phospho-peptide identification is the correct localization of the phosphosite. Both approaches, DDA and DIA, offer a confidence measure for the correct site. Nevertheless, even when no evidence for the correct phosphosite is present in the spectrum, the peptide still harbors a phospho-group at some amino acid, otherwise the peptide precursor mass would not be correct. Thus, we can be confident that, due to common quality control measures (e.g., false discovery rate calculation at peptide level), there is a phospho-group present somewhere in the peptide, but the correct phosphosite identification can remain ambiguous. Therefore, DIA-NN calculates a site localization probability and PEAKS provides the Ascore, which is calculated by multiplying the negative decadic logarithm of the *p*-value for incorrect identification by 10. Consequently, the higher the AScore the more confident the identification, with a maximum possible value of 1000. Typically, a confidence of at least 75% (for calculation of AScore: 25% probability of false localization) is desired [33] as a prerequisite for class I phosphosite. Therefore, a common cut of value for the AScore is a value of 6, corresponding to 25% false localization probability. In Figure 1E, the distribution of AScores obtained from both acquisition strategies is shown. The DDA AScores peak is around a value of 10, whereas DIA data seem to provide two different peaks, the first peak with an AScore below 6 and the second peak with an AScore around 30, which equals a site confidence of 99.9%. Thus, the median site confidence is roughly the same, due to the inhomogeneous distribution of the DIA-NN confidences. The reason for this difference is presumably the higher complexity of MS2 in DIA data. There, confidence is only achieved in the presence of strong fragment evidence, whereas the algorithm of PEAKS for processing DDA MS2 spectra seems to have a more refined algorithm to assign calculate variances in probability with high sensitivity. A possible reason for the clear separation of either very low confidence or very high confidence of the phosphosite localization in DIA analysis with ion mobility included might be a combination of ion mobility separation before fragmentation, yielding in cleaner spectra, and the de-noising capability of MS2 spectra in DIA-NN, that contributes to increased identification of evidence fragments for the correct phosphosite. Therefore, the assumption that DDA data provide more confidence in the site localization by higher quality spectra is only partly true. Nevertheless, discovery phase in phospho-proteomics, the correct phosphorylation site is of less importance anyway. More importantly, both algorithms provide equally high confidence that these peptides are phosphorylated (regardless of the phosphosite). Conclusions about active/inactive pathways or protein phosphorylation with approximate protein sites can be drawn anyway.

Based on these findings, we measured a sample set of *M. oryzae* samples including KCL salt stress to build a resource for further research. Across all samples, 29.494 unique phospho-peptides could be identified, corresponding to a total number of 45.291 phospho-sites. The most recent phosphoproteomics study in *M. oryzae* from 2015 by W.L. Franck et al. in the group of R.A. Dean [34] reported 4894 phosphosites, which we were able to increase, outperforming by a factor of roughly 10-fold with our methodology. In addition to that, W.L. Franck et al. used a chromatography method which took as long as 3 h, which we could outperform by a factor of 3-fold by developing an LC method with only a 45 min gradient and 60 min runtime in total.

In conclusion, the application of DIA is a promising strategy for the comprehensive description of a phospho-proteomics dataset. We have shown that data completeness increases dramatically while the data quality remains at least equal. The downsides of the DIA application are resource intensive and time consuming bioinformatic processing and the lack of intuitive spectra visualization. A possible solution to this is provided by the proprietary software Spectronaut, which is able to visualize XICs of precursors and fragments in a user-friendly way [29]. Nevertheless, DIA-NN has been shown to provide superior identification performance, utilizing neuronal networks while being open source at the same time. A direct phospho-peptide ID benchmark of both types of software has not been described in the literature yet and would serve as interesting starting point for further bioinformatics research.

Furthermore, we not only provide a refined methodology for phospho-peptide analysis in filamentous fungi but also a large dataset that can serve as valuable resource for further signaling research in *M. oryzae*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jof9010063/s1, Table S1: Variable window sizes foe DIA acquisition with Orbitrap Exploris 480.

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4. Recent Advances in Research in Molecular Mechanisms of Fungal Signaling

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Understanding molecular mechanisms, particularly biochemical signaling, is essential for this work as it underpins how organisms respond to environmental changes. Signaling pathways enable cells to process external signals through a limited set of proteins in complex networks. This published review provides a comprehensive overview of key mechanisms in fungal signaling, supporting the research into the adaptive responses of *Magnaporthe oryzae* and enriching the study of signal transduction in eukaryotic microorganisms.

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Review

Recent Advances in Research on Molecular Mechanisms of Fungal Signaling

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Abstract: Biochemical signaling is one of the key mechanisms to coordinate a living organism in all aspects of its life. It is still enigmatic how exactly cells and organisms deal with environmental signals and irritations precisely because of the limited number of signaling proteins and a multitude of transitions inside and outside the cell. Many components of signaling pathways are functionally pleiotropic, which means they have several functions. A single stimulus often results in multiple responses, a distinct response can be triggered by numerous stimuli and signals initiated by different stimuli are often transduced via commonly used network components. This review sheds light on the most important molecular mechanisms of cellular signaling in fungi and consequently provides a comprehensive overview about the current state of research on the road to understand the impact of signal transduction in eukaryotic microorganisms.

Keywords: cAMP signaling; quorum sensing; alternative splicing; lipid signaling; MAPK cascade; multistep phosphorelay; pheromone signaling; glucose signaling; light signaling; fungal signaling; fungi

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1. Introduction or History

Adaptation and resilience to environmental changes is a prerequisite for cells and organisms to live, survive and evolve. The expansion of signaling pathways in three kingdoms—Archaea, Bacteria and Eukarya—came about through the horizontal transfer of bacterial genes and the coevolution of the components of the respective systems [1–3]. Consequently, in terms of their functional properties and molecular architecture, signaling systems in unicellular eukaryotes represent an intermediate stage in the evolution of signaling systems between pro- and higher eukaryotes [2]. All living cells have in common that the functional organization of fundamental processes of the cell—growth, metabolism, differentiation and apoptosis—includes four basic components: (i) a signal receptor, which specifically recognizes a signal molecule; (ii) a signal transport, which is associated to the receptor; (iii) a signal amplifier, which is an ion channel or an enzyme producing second messengers; and (iv) an effector (signal receiver), which initiates single or multiple intracellular signal cascades, resulting in the response to the external changes [1].

Here, we aim to map the great diversity of molecular signal transduction processes in fungi to show how signaling proteins encrypt information, coordinate different transmission routes and deploy response to various environmental stimuli. Therefore, we present an overview of the most important mechanisms of molecular cellular signal transduction by showing selected and prominent examples.

2. Mitogen-Activated Protein Kinase Signaling

Mitogen-activated protein kinase (MAPK) signaling pathways represent one of the most important cellular architectures for the perception and transition of extracellular information ubiquitous in all eukaryotic organisms [4,5]. They have a myriad of cell functions in

all species of fungi, for example, mating, cell cycle control, differentiation, stress-response and -resistance, resilience, adaptation, cell wall assembly and integrity, autophagy and apoptosis, virulence, cell–cell communication and plant-fungus interaction [4,6].

In contrast to metabolic enzymes, which are known to be efficient for catalytic chemistry reactions within and outside of the cell, MAPK evolved to be dynamic molecular switches for signal transduction that can be controlled by membrane recruitment, dimerization and phosphorylation [7]. The signal propagation in the MAPK cascade follows a multistage process in which the amplification of signals by sequential events of phosphorylation make this system sensitive to the lowest stimulation patterns. The MAPKs are serine/threonine kinases, activated by a MAPK kinase (MAPKK), which is a 'dual-specific' kinase that phosphorylates its substrates at both Ser/Thr and Tyr motifs, i.e., targeting a Thr/x/Tyr motif at the MAPK (x represents glycine, proline or glutamate) [8,9] (Figure 1).

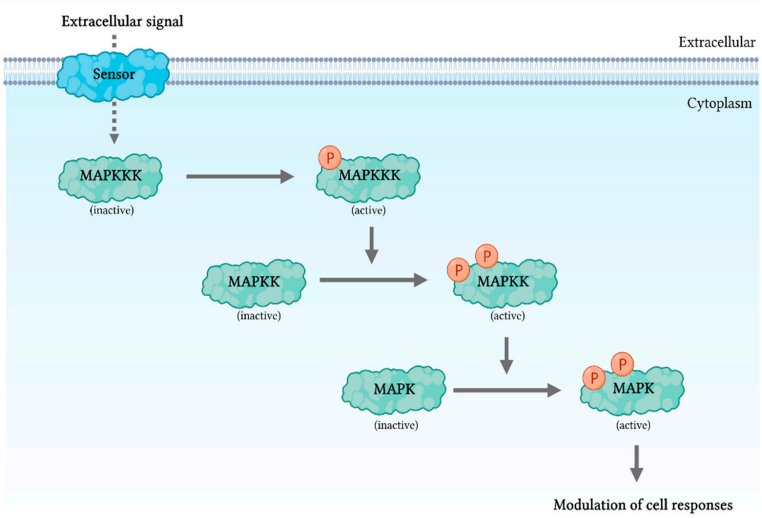

Figure 1. Schematic representation of MAPK signal transduction cascade. A membrane receptor perceives an extracellular signal. The sensor then activates the MAP kinase module (MAPKKK activates MAPKK, which in turn activates the MAPK) by phosphorylation. That may occur via different routes and intermediate steps. The modulation of cell responses by the active MAPK may activate different protein kinases or cytosolic components. Furthermore, the activated MAPK may translocate to the nucleus and regulates transcription factors. Arrows indicate activation.

The MAPKK is, in turn, activated by a MAPKK kinase that transmits signals from stimulus-activated upstream effectors, i.e., response regulators, GTP-binding proteins, other kinases, respectively, so-called pattern-recognition receptors [10,11]. The MAPK suffers conformational changes upon phosphorylation that subsequently increases the activity about 1000–50,000-fold [5,12]. Consequently, MAPKs are not really active unless phosphorylated by their respective upstream kinases. The molecular conformational change, i.e., involves the movement of the phosphorylated loop toward the active site and the rotation of the whole C-terminal lobe. The activation involves the opening of the A-loop, the relative alignment of the N-lobe and C-lobe and the rotation of the αC-helix [13]. In other words, phosphorylation in the activation loop area triggers the reorganization of the flexible C-terminal lobe, which rotates to the N-terminal lobe, thereby forming the ATP-binding active site for catalysis [12].

The most prominent example of MAPK signaling is the eukaryotic p38 MAPK pathway, which is well conserved in all eukaryotes. This signaling cascade is assumed to be a key player in a wide array of cellular processes associated with ageing, inflammatory diseases

and tumor development in mammals or differentiation, virulence and environmental stress signaling in fungi [14–17]. In the latter, the respective signaling cascade is called the high osmolarity glycerol (HOG) pathway with the p38 MAPK Hog1p. Signal transduction at the central MAPK Hog1p is achieved by phosphorylation of the dual Thr/x/Tyr phosphorylation motif. Apart from osmoregulation, Hog1p activation in fungi is addressed by many stimuli, including ultraviolet light, heat, fungicides and reactive oxygen species [14,18–21]. There are very few studies with statements about the molecular function of single amino acids (aas) in the Thr/x/Tyr motif of a Hog1/p38 MAPK. The studies concerning phosphorylation of p38 MAPKs are based mostly on immunoblot analysis using antibodies targeting the doubly phosphorylated Thr/x/Tyr motif of MAPK (null)mutant strains [22,23]. These methods are not suitable to distinguish or individually quantify Thr and Tyr phosphorylation and particularly their relationship to each other. Some rare studies with statements on single Thr or Tyr functionality are also based exclusively on immunoblot techniques [24–27]. In one of them, the role of Thr174 and Tyr176 phosphorylation in the yeast MAPK Hog1p is only addressed by the examination of the vegetative growth of hyperactive mutants [28]. The authors conclude that Tyr176 is required mainly for enhancing the catalytic activity following osmostress, whereas Thr174 is essential for the biological and catalytic activity, although not necessarily as a phosphor-acceptor. This is in line with results in cell culture assays and in vitro experiments with human p38, which postulate that phosphorylation at Thr180 might be more important for TGFβ-activated protein kinase (TAK)-1 mediated signaling than at Tyr182 [26]. These observations point to a complex but only partially understood imagination of regulatory molecular mechanisms that present putative functions of MAPK, but this does not reflect anything about the mechanisms of signal coding or signal encryption.

In fact, these studies show a limited 'on/off' mapping without the possibility of distinguishing between the intensity and dynamic of phosphorylation at the single aas. Thus, gaining specific insights about the molecular programming of the Thr/x/Tyr motif by temporal site-specific quantification of phosphorylation and its contribution to (dis)regulation of cellular processes is absolutely mandatory.

3. cAMP Signaling

It is widely accepted that the secondary messenger cyclic adenosine monophosphate (cAMP) plays an extraordinary role in cellular signaling, and the spatial regulation of the cAMP level is critical for faithful signal transduction. However, our knowledge of how receptors, cAMP signaling enzymes, effectors or other key proteins regulate specific cell responses is limited [29]. The cAMP regulates a variety of physiological processes in eukaryotic cells and is produced in response to extracellular stimuli, such as hormones [30,31].

The most studied target of cAMP in eukaryotic cells, and in particular in the model fungus *Saccharomyces cerevisiae*, is the cAMP-dependent protein kinase A (PKA). The PKA mediates almost all physiological effects of cAMP in fungi, and this is also valid for other multicellular eukaryotes [32,33]. In a classical cAMP signaling pathway, a specific extracellular signal is perceived by a transmembrane receptor and transmitted into the cells through heterotrimeric G-proteins structured of α, β and γ subunits [34] (Figure 2).

The G-proteins are activated through the binding of an inducing ligand under GDP-to-GTP exchange of the guanine nucleotide, which is bound to the Gα subunit. After that, the Gα subunit is released from the Gβγ dimer [35]. Subsequently, the Gα or the Gβγ subunit transfers the signal by stimulation of effectors, such as the adenylyl cyclase, which, in turn, starts to synthesize the second messenger cAMP [36,37]. The activities of the synthesizing adenylyl cyclase and the degrading phosphodiesterase affects the intracellular cAMP level. The cAMP biosynthesis occurs as a consequence of various extracellular stimuli, such as light, temperature, nutrients and hormones, thereby regulating a high number of physiological processes. The PKA is a tetramer consisting of two catalytic and regulatory subunits, which rest in the inactive state under non-inducing conditions when the cAMP level is low. Both subunits are highly conserved among eukaryotes and

fungi [34]. Upon inducing conditions, when cAMP levels increase, the interaction of cAMP with the two regulatory subunits results in a conformational rearrangement that triggers disaggregation of the tetramer and consequently the release of the catalytic subunits. These catalytic subunits start the phosphorylation of downstream substrates in the cytosol or translocate into the nucleus (Figure 2). Within the nucleus, the PKA catalytic subunits can either activate or inhibit transcription factors or transcriptional repressors thereby regulating cellular functions [38,39].

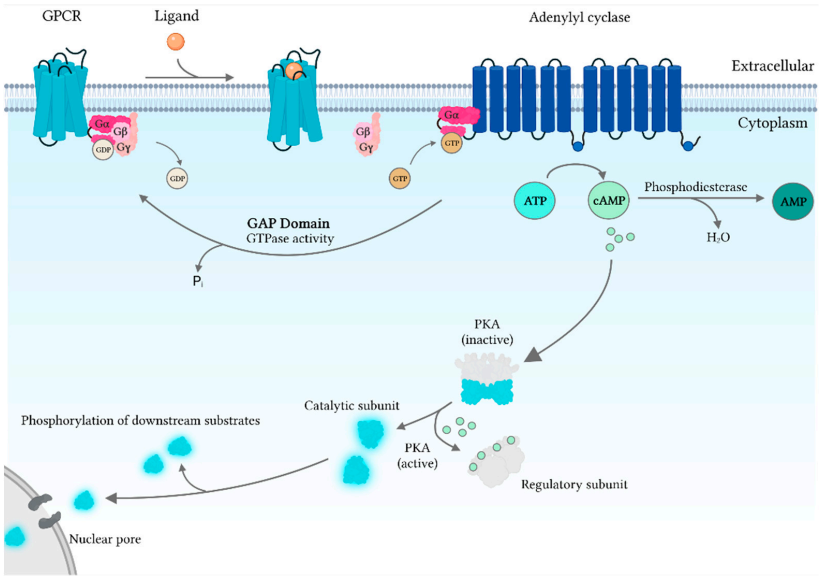

Figure 2. Schematic representation of cAMP and PKA signaling pathway in *S. cerevisiae*. Activation of the G-protein-coupled receptor (GPCR) by the ligand leads to the activation of adenylyl cyclase, which in turn triggers GTPase-activating proteins (GAPs) with GTP hydrolysis activity to stimulate formation of the inactive, GDP-bound protein and the release of free phosphate (Pi). Additionally, adenylyl cyclase synthesizes cAMP from ATP. When cAMP binds to the regulatory subunits of the inactive PKA, the catalytic subunits will be released and phosphorylate downstream substrates in the cytosol or translocate into the nucleus.

The cAMP pathway in filamentous saprophytes is very important for vegetative growth and sporulation and mating. That also includes the formation of appressoria, invasive hyphae, sclerotia differentiation and sporulation [34]. Thus, cAMP regulates virulence and morphogenesis in a wide range in various fungi, including the plant pathogens. The cAMP signaling pathway in *S. cerevisiae* mediates nutrient sensing and controls pseudohyphal differentiation upon nitrogen-limiting conditions [40–43]. In *Saccharomyces pombe*, the cAMP is responsible for the effects of glucose on spore germination and gluconeogenesis and regulates mating [44]. It furthermore regulates hyphal growth and morphogenesis, conidiation and spore germination in model filamentous fungi, such as *Aspergillus* sp. and *Neurospora* sp. [45–48].

4. Quorum Sensing

Microorganisms living close together in high numbers need to communicate with each other. Quorum sensing (QS) is a mechanism of microbial communication reliant on cell density that regulates highly important developmental processes. In order to achieve this communication, microorganisms release and detect small molecules known as QS molecules (QSMs) able to control their biological activities and behaviors [49]. The concentration

of QSMs increases pro rata to the population and a regulatory response is started after a critical threshold is achieved. Consequently, that leads to the coordinated expression or repression of QS-dependent genes [50]. Quorum sensing as a mechanism of signaling and communication was first observed in bacteria in studies on genetic competence in *Streptococcus pneumoniae* and bioluminescence in marine *Vibrio* species about 50 years ago [51]. Subsequently, QS was found in many bacteria regulating important processes, including biofilm formation, secretion of virulence factors, sporulation and biosynthesis of antibiotics [52–54].

Apart from bacteria, studies have revealed that many population-density behaviors in fungi, such as biofilm formation or virulence and pathogenesis, are regulated by QS [52]. The discovery of filamentation control in the pathogenic fungus *Candida albicans* by the QSM farnesol promoted the phenomenon of QS in fungi as well [55]. Farnesol was the first QSM isolated from a eukaryotic microorganism [56,57], but it was rapidly followed by the identification of the QSMs phenylethanol, tyrosol and tryptophol [50,58,59]. Since then, the role of QSMs in fungi has been widely studied in both yeasts and filamentous fungi, for example, *S. cerevisiae*, *C. albicans*, *Candida dubliniensis*, *Aspergillus niger*, *Aspergillus nidulans* and *Fusarium graminearum* [50,56,60].

The signaling molecules are not generally strain-specific and a huge diversity of those molecules has been reported in fungi. In more detail, among the most important examples of QSM in fungi are lipids (oxylipins), peptides (pheromones), alcohols (tyrosol, farnesol, tryptophol and 1-phenylethanol), acetaldehydes and some volatile compounds [61]. These compounds are actively involved in fungal QS, regulating diverse key functions, such as pathogenesis, morphogenesis and filamentation. It was documented for the first time in 2006 that the cell culture supernatant of the stationary phase from a culture of *S. cerevisiae* strain induced filamentation [62]. In this study, two aromatic alcohols were identified, phenylethanol (PheOH) and tryptophol (TrpOH), as the active principle of QS inducing filamentation. The production of these two molecules was shown to be dependent on the cell density. A high cell density results in an increase of the expression level of the ARO9 and ARO10 genes and subsequently stimulated the production of the aromatic alcohols [62,63]. The aromatic aminotransferase Aro9p and decarboxylase Aro10p are required for their synthesis outgoing from the metabolism of the aa phenylalanine and tryptophan. This aromatic alcohol production can also be stimulated by tryptophol itself. The latter activates the transcription factor Aro80p in a positive feedback loop resulting in the expression of the transaminase and decarboxylase genes ARO9 and ARO10 [64]. Consequently, yeast cells at a high population density produce more aromatic alcohols per cell than yeast cells at a low population density [62,64]. PheOH and TrpOH appear to trigger the upregulation of the FLO11 gene synergistically via the cAMP-dependent PKA subunit Tpk2p and the transcription factor Flo8p [52,62]. The glycosylphosphatidylinositol-anchored cell surface flocculin protein Flo11p is essential for filamentous growth [65–67]. *S. cerevisiae* strains with inactivation of either FLO8 or TPK2 do not form filaments upon the presence of PheOH and TrpOH [62]. Apart from cell density, it is known that the key morphogenesis-inducing stimulus in *S. cerevisiae*, nitrogen starvation, strongly induces the production of PheOH and TrpOH. In the end, the signaling sensors and signal transport mechanisms of QS in fungi have not yet been sufficiently elucidated. There is also evidence for strain differences in QS, which requires more research [68]. The importance of understanding the molecular mechanisms by which microorganisms interact is key to assessing how they might affect biofilms, cause diseases, influence the quality and safety of fermented food and behave in biotechnological applications.

5. Alternative Splicing

Alternative splicing (AS) is a pervasive mechanism in eukaryotic organisms that generates multiple different transcript and protein isoforms from one single gene sequence [69–72]. During gene expression, the spliceosome, a multi-protein complex of five snRNP (small nuclear ribonucleoprotein: U1, U2, U4, U5 and U6), orchestrates the removal of noncoding

sequences (introns) of the primary mRNA and assembles different combinations of coding sequences (exons) into mature mRNA. Each snRNPs contains one snRNA (small nuclear RNA) and several proteins [73]. The molecular splicing process is a two-step transesterification reaction that removes introns as lariat intermediates (looped structures) and ligates the remaining exons [74].

Introns are defined by a 5′-splice site (5′SS), an adenosine branch point (BP), the polypyrimidine tract (pY tract) and the 3′SS (Figure 3, top).

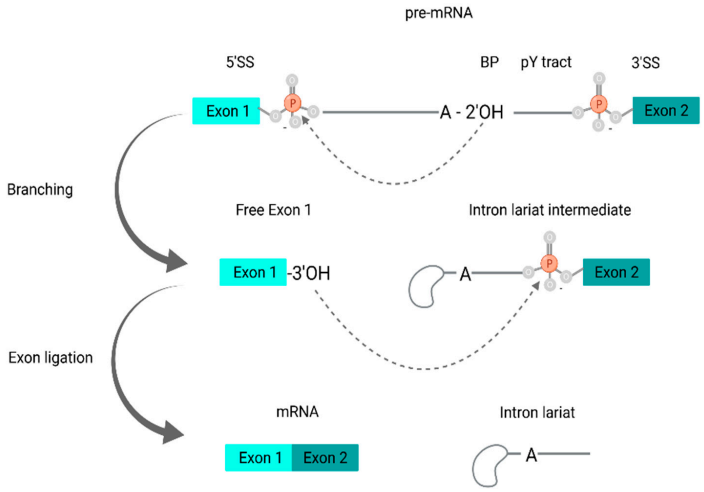

Figure 3. Two-step transesterification mechanism of pre-mRNA splicing. In the branching step, the 2′-OH of the branch point (BP) adenine of exon 2 attacks the phosphate of the guanine at the 5′ end of the 5′ splice site (5′SS) of exon 1. During exon ligation, the 3′-OH of the free exon attacks the phosphate of the 5′ end of the intron lariat intermediate.

The first step of the splicing process is called branching and entails the nucleophile attack by the 2′-OH group of the BP adenosine on the phosphate at the 5′SS. As a result, the 5′-2′ phosphodiester linkage between the 5′end of the intron and the BP adenosine forms an intron-lariat-3′exon intermediate and a free 5′exon with a 3′OH group (Figure 3, middle). In the following exon ligation, the 3′-OH group of the 5′exon attacks a phosphate at the 3′SS, resulting in the ligation of the 5′ and 3′exons and the excision of the lariat intron (Figure 3, bottom) [75–77]. Thereby, the spliceosome assembles the different exons in a stepwise manner (overview in Figure 4).

Initially, the intron is recognized by its 5′SS, BS and 3′SS of U1 snRNP and splicing factors, forming the pre-spliceosome (E complex) [78]. In subsequent steps, the E complex recruits U2 snRNP to generate the pre-spliceosome (A complex), which assembles with the tri-snRNP (U4, U5 and U6) into the pre-catalytic spliceosome (B complex). The dissociation of U1 and U4 snRNP results in the activation of the spliceosome (B^{act} complex), which is then converted into the catalytically activated complex (B*) (Figure 4, bottom). The first step of the transesterification reaction occurs in B*, resulting in the catalytic step I spliceosome (C complex) and then remodeling into the step II-activated spliceosome (C* complex). Next, the second step of the transesterification reaction is catalyzed in the complex C*, followed by its conversion into the post-catalytic complex. Ligated exons (mRNA) were found in the post-catalytic complex for the first time and the excised lariat intron could be identified. The newly formed mRNA is then released, resulting in the intron lariat spliceosome. After the latter dissociates, all snRNPs can be recycled for additional rounds of splicing [75,79–82]).

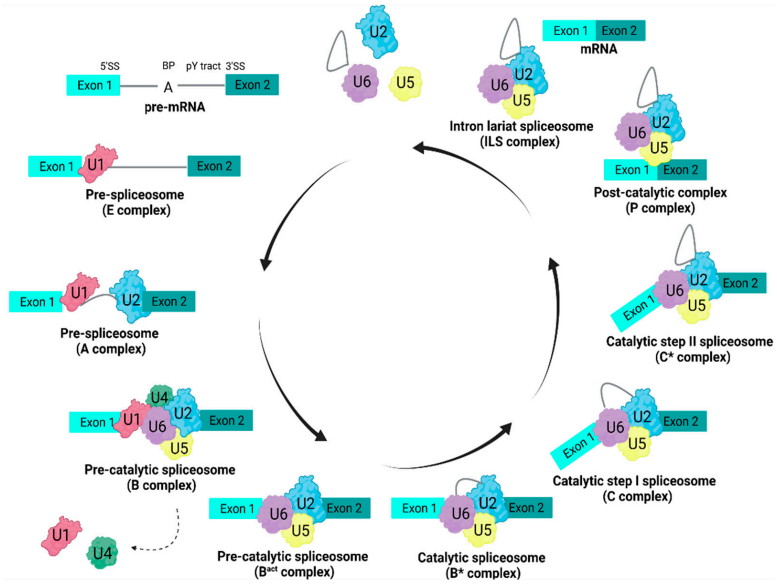

Figure 4. Assembly and the catalytic cycle of the spliceosome. Initially, the 5′SS, BP and 3′SS are first recognized by the U1 small nuclear ribonucleoprotein (snRNP), forming a pre-spliceosome (E complex). The U2 snRNP attaches at exon 2 to subsequently form the A complex, which interacts with the U4, U5, U6 tri-snRNP to assemble into the pre-catalytic spliceosome (B complex). There are at least six additional distinct spliceosome complexes: B^{act}, B*, C, C*, P and the intron lariat spliceosome (ILS complex). Each complex has a unique architecture.

About 95 % of genes containing intron in humans are alternatively spliced, resulting in approximately 100,000 splicing decisions [83,84]. Varying AS events can result in altered protein isoforms with potentially dramatic consequences for the organism. Thus, AS resulting in changed protein interactions or the inhibition of enzymes can induce cancer development or the impairment of drug efficacy [85,86]. The most prominent AS patterns are classified into five categories: exon skipping, intron retention, alternative 5′splice site, alternative 3′splice site and mutually exclusive exons (Figure 5).

Even though AS is widely accepted for increasing transcriptome and proteome diversity in higher eukaryotes, a comprehensive understanding of the molecular mechanisms in fungi and its putative downstream functional effects in signaling is mainly unexplored [87,88]. The fungal kingdom is a species-rich group of organisms with genome sizes ranging from 10 to 90 Mb [89–91]. According to the most recent studies, Ascomycota, Basidiomycota and Deuteromycota have a higher incidence of AS than previously thought [78,90,92–96]. Various physiological processes are affected by AS, such as growth, a pathogenic lifestyle, dimorphic changes and stress adaptation [90,92,94,96–100]. Thereby, numerous precursor messenger RNAs are differently alternatively spliced depending on different environmental conditions, such as changes in extracellular phosphate concentration, temperature and ambient pH [101].

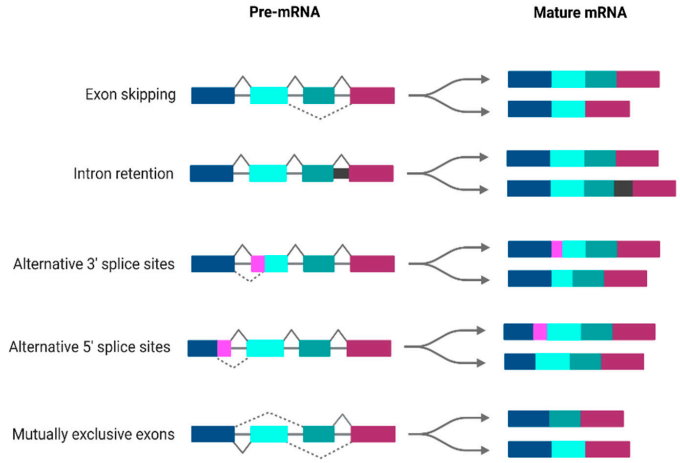

Figure 5. An overview of some of the most prominent types of alternative splicing. Exon skipping: This process removes certain exons and their adjacent introns from mRNA prior to translation. Intron retention: non-coding regions of a gene are retained in the final mRNA transcript. Alternative 5′ or 3′ splice sites: The exons join at alternative 5′ or 3′ splice sites. Mutually exclusive exons: two (or more) splicing events are not independent, but are executed or disabled in a coordinated manner.

Interestingly, most genes encoding proteins of nonpathogenic fungi, such as *S. cerevisiae* and *C. albicans*, have a simple gene structure with only one intron, whereas genes of pathogenic fungi, such as *Cryptococcus neoformans*, contain multiple introns [95,102–104]. However, the specific function of fungal introns in pathogenicity or signaling remains unclear. Intron retention is the most common pattern of AS [103,105]. The percentage of genes containing intron ranges from 2.5 % (*Candida glabrata*) to 99 % (*C. neoformans*) [104]. Annotations of fungi in public databases typically include only one or two transcript isoforms per gene [105,106]. Over 20 % of the genes in *Magnaporthe oryzae*, the causal agent of rice blast disease, undergo AS [92]. However, a recent study report has shown that the PTEN gene (*MoPTEN*), a homolog of the human dual-phosphatase tumor suppressor, has two protein isoforms that differ in their lipid and phosphatase activity. One isoform is essential for conidia and appressorium formation, while the other is required for the invasive hyphal growth in rice grains [100]. Consequently, different isoforms of this protein are of use in different stages of the pathogenic life cycle. Host cell invasion by *M. oryzae* starts with conidial development outside plant cells, followed by conidial germination, tube elongation, maturation and differentiation into the dome-shaped appressorium (isoform 1). A successful development of the invasive hypha after penetration will determine the severity of colonization and, thus, the fate of neighboring cells (isoform 2) [107].

In conclusion, the number of reports including AS in fungi are increasing rapidly, consistent with the evidence of the role of AS in essential regulatory mechanisms, as described in higher eukaryotes. However, accurate isoform prediction, identification and biological characterization remains a key issue for a better understanding of the signal diversity in fungi.

6. Multistep Phosphorelay Systems

It is important to take a deeper look into the perception, transduction and processing of signals within the living cells in order to understand the molecular mechanisms underlying the adaptation of microorganisms toward changes in the environment [108,109]. Whereas signaling processes in prokaryotic organisms are achieved in a two-component system, eukaryotic organisms have developed a more complex multistep phosphorelay system

(MSP) [110]. The detection of environmental changes and the signal transduction in both of these signaling systems occurs by phosphorylation through a phosphoryl group transfer within a signaling cascade [110–113]. External stimuli are perceived by a histidine protein kinase (HK) within the prokaryotic two-component system and are then transmitted to a response regulator protein [114,115] (Figure 6A).

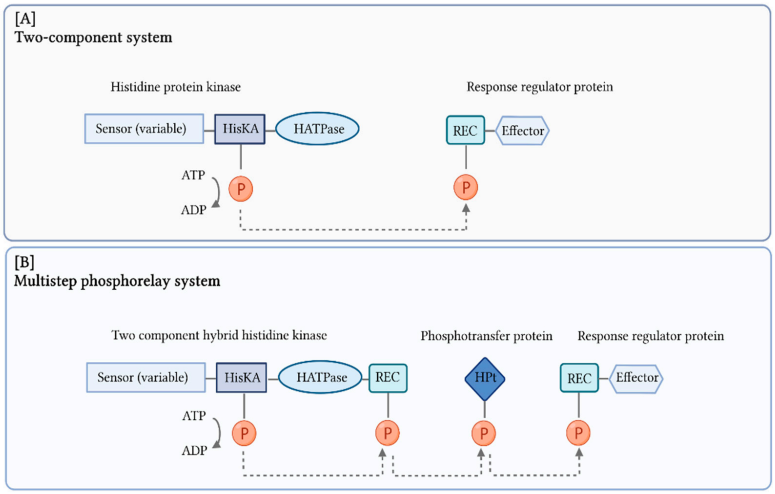

Figure 6. Schematic representation of the prokaryotic two-component system (TCS) and the eukaryotic multistep phosphorelay system (MSP). (**A**) For the two-component system, the phosphate transport is shown from the histidine kinase phosphoacceptor domain (HisKA) of the histidine protein kinase to the signal receiver domain (REC) of the response regulator protein. (**B**) The MSP system includes additional regulatory steps: the phosphate is transferred from the HisKA of the two-component hybrid histidine kinase to a REC domain within the same protein and is subsequently transferred via a phosphotransfer protein to the response regulator protein. The phosphate transfer is indicated by arrows.

By contrast, the more complex eukaryotic MSP senses and transmits external changes by the use of different but also more components: a two-component hybrid histidine-kinase (HHK), a phosphotransfer protein and a response regulator protein [111,112,116] (Figure 1). The main difference between the prokaryotic two-component system and the eukaryotic MSP is the HHK containing an additional receiver domain compared to the simpler architecture of the HK from prokaryotes. Additionally, a phosphotransfer protein is working between the HHK and the response regulator, refining but also complicating the signaling process [117,118]. Kinases phosphorylate proteins by using ATP as a phosphate donor and are named and categorized based on the aa residue they phosphorylate [118,119]. These specific aa residues are serine, threonine, tyrosine or histidine [120].

The transfer of the phosphoryl group within the MSP is from His-Asp-His-Asp [118,119,121]. The HHKs are the primary sensor proteins of the signaling cascade, with variable sensory domains at the N-terminus (e.g., HAMP or HAMP-like linker domains (poly-HAMP)), an HK domain with an autophosphorylation site [122–124] and a Histidine similar ATPase catalytic domain (HATPase domain). The C-terminal response regulator domain (REC) within the HHK contains the Asp phosphoacceptor residue [124]. The next step is the transfer of the phosphate group from the Asp to the Histidine residue of the histidine containing phosphotransfer domain (HPt) of the phosphotransfer protein and, in the last step of the phosphorelay system, to the Asp of the REC within a response regulator protein (Figure 6) [114]. The HPts are attached to HKs at the C-terminal end in prokaryotes. By contrast, eukaryotic HPts are separated as an individual protein that can communicate

between HK and RR, and shuttle into the nucleus and back to the cytosol. Hence, it serves as a mediator protein between the two units and is responsible for interacting with proteins or signaling pathways in addition to the MSP [121]. The first identification of an MSP in a signaling pathway was documented in the yeast *S. cerevisiae*. This MSP, with the HHK Sln1p and the phosphotransfer protein Ypd1p, is known to be part of the HOG pathway [123]. In contrast to the single HHK coding gene *SLN1* in *S. cerevisiae*, the genomes of filamentous pathogenic fungi possess multiple HHK-coding genes [111], where the HHKs are widespread, for example, in *A. nidulans*, *Botrytis cinerea*, *C. albicans*, *Cochliobolus heterostrophus*, *Fusarium verticillioides*, *Neurospora crassa* and *M. oryzae* [112].

Although the MSP within the HOG pathway is one of the signaling pathways most studied in fungi, the exact molecular mechanisms of phosphotransfer are not yet fully understood and documented. One example is the activation of the HOG pathway within the filamentous fungus *M. oryzae* by osmotic stress, which triggers cytosolic MoHog1p via phosphorylation at T171 and Y173 in the dual phosphorylation motif [125]. Subsequently, MoHog1p migrates into the nucleus, starting the cellular stress response [126]. Whereas some of the details concerning the phosphorylation pattern in the HOG pathway have already been identified in *M. oryzae*, signal perception and transformation of extracellular signals into phosphorylation at the sensor HHK MoHik1p remains unclear [123]. The exact aa positions of the phosphorylation events at the HHK differ slightly between organisms but are comparable on the protein level by blast. An example of different aa position can be illustrated with Nik1p in *C. albicans* and MoHik1p in *M. oryzae*. The phosphotransfer, for example, in the HKK Nik1p in *C. albicans* occurs from aa H510 within the HisKA domain to aa D924 within the REC domain [127,128]. The His-Asp phosphorylation pattern within a HKK in *M. oryzae* is located at aa position H736 in the HisKA domain [108,122,124], and the phosphoryl group is transferred to the phosphoacceptor at position D1153 in the REC domain. The aa position His69 is predicted to play an important role in the phosphoryl transfer activity of the HPt domain within the phosphotransfer protein Ypd1p in *C. albicans* [120,129]. The phosphoryl group is then transferred to the aspartate residue D556 in the REC domain of the response regulator protein in *C. albicans* [130]. Apart from osmoregulation, MSPs regulate key cellular regulatory processes and responses within the fungi when exposed, for example, to osmotic stress [108], oxidative stress [131,132] or light [133], and plays an important role in the regulation of all aspects of fungal physiology [109,111,120].

It is important to focus on the research of MSP in filamentous fungi not only to unravel fundamental basics in order to understand the molecular mechanisms of signaling in fungi, but it is also of high interest due to HOG pathway-specific fungicides. The HHKs MoHik1p in *M. oryzae*, Drk1p in *D. hansenii* or Nik1p in *C. albicans* are group III HHKs and, therefore, specific to filamentous fungi. This means that no homologues have been found in plants or mammals to date. These HHKs are known to be involved in the mode of action of the commercial fungicide fludioxonil [18,122,134]. Consequently, research on MSP leads to new opportunities to develop novel antifungal compounds without causing significant toxicity to other organisms in the environment [110,120,135].

7. Lipid Signaling

The study of lipid signaling networks has increased significantly in recent years. Lipid signaling, although best studied in mammalian cells, is now also appreciated in microbial cells, particularly in yeasts and molds [136]. Lipids are well characterized in mammalian cells as signaling molecules in pathophysiological processes, such as cancer, autoimmune diseases, inflammation, cardiovascular diseases and neurological disorders. Changes in the network of lipid signaling most probably results in these diseases because of the alteration in cellular homeostasis [137–145].

Lipids perform signaling and regulatory roles in plants, apart from structural roles, in various cellular processes, particularly the sphingolipids as regulatory signaling molecules. These lipids have signaling functions in programmed cell death, cell-to-cell interactions and cell wall formation, endoplasmic reticulum integrity, stomatal closure, membrane

stability, temperature-induced signal transduction, salt and drought tolerance, pollen development, cell division and growth, cell type differentiation and organogenesis, mineral ion homeostasis, cellular organization and plant-microbe interactions [146–155].

Similarly, fungal lipid signaling renders the fungi hypervirulent. That means, lipids help them to get more resistant to cell death by host or environmental stresses, e.g., to the host immune responses. The lipid signaling molecules in pathogenic fungi are mainly sphingolipids, farnesol and oxylipins [136]. They mediate specific cellular processes, such as growth, differentiation and apoptosis. Shedding light on the molecular lipid signaling events in fungi can lead to a significant understanding of the pathophysiological events regulated by lipids and open up the possibility of exploiting new means for the development of novel therapeutic strategies [156,157].

Sphingolipids are being studied in detail in the yeast-like fungus *C. neoformans*. The studies highlight that sphingolipids play a significant role in the regulation of virulence. Sphingolipids were found to regulate many cellular processes in *C. neoformans*, including the production of melanin by the formation of diacylglycerol, which affects protein kinase C1 (Pkc1p) [158,159] (Figure 7).

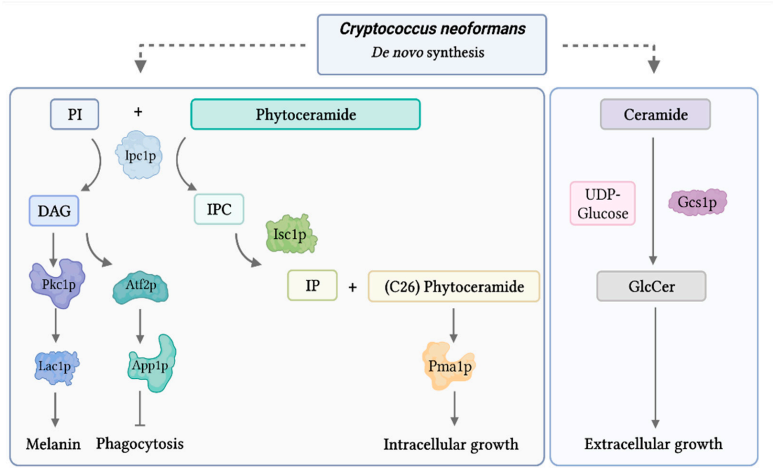

Figure 7. Regulation of lipid pathway in *C. neoformans*. PI = phosphatidylinositol; Ipc1p = inositol phosphoryl ceramide synthase; IPC = inositol phosphoryl ceramide; DAG = diacylglycerol; Pkc1p = protein kinase C1; Lac1p = laccase; Atf2p = activating transcription factor 2; App1p = antiphagocytic protein 1; Isc1p = inositol phosphosphingolipid phospholipase C; IP = inositol phosphate; Pma1p, plasma membrane ATPase 1; Gcs1p = glucosylceramide synthase; UDP = uridine diphosphate; GlcCer = glucosylceramide.

Sphingolipids modulate signaling events by the activation of transcription factor 2 (Atf2p). This process renders phagocytosis through the activation of the antiphagocytic protein 1 (App1p) [160,161]. Furthermore, they are involved in the regulation of fungal growth in the intracellular and extracellular environments by the biochemical performance of inositol phosphosphingolipid phospholipase C1 (Isc1p) and the GlcCer synthase (Gcs1p) [158,159] (Figure 7). These lipid-regulated processes strongly affect the virulence of *C. neoformans* in the lung environment with important impact on the course of the disease. Interestingly, intra- and extracellular growth of *C. neoformans* was found to be regulated by environment-specific sphingolipids, indicating that the fungus has an efficient arsenal of different lipid-molecules that might be used depending on which host cell compartment it is currently in [162]. To explain in more detail, when *C. neoformans* is located within the macrophages of its host, the expression pattern of only some specific sphingolipid-metabolizing enzyme(s) coding genes increase, such as Ipc1p. To further support this

hypothesis, it was found that when the fungal cells are shifted from an alkaline or neutral pH to an acidic pH, Ipc1p and Isc1p are required for adaptation towards the changing environment [163]. These observations make complete sense because *C. neoformans* enters the body through the respiratory tract and inhalation, finding an environment with neutral pH in the alveolar spaces and an acidic pH later on within the phagolysosome of alveolar macrophages. Thus, a better understanding of lipid signaling and how *C. neoformans* adapts to different environments will give us to a better understanding of how the pathogen interacts with the host.

8. Pheromone and Glucose Signaling

Sophisticated molecular mechanisms have evolved in microorganisms sensing the environment to respond to pheromone and nutrient signals. These environmental signals are perceived by G-protein-coupled receptors (GPCRs), which comprise the largest family of transmembrane receptors are likely to be key mediators of host–microbial interactions in eukaryotes [164,165]. Apart from a conspicuous sequence and functional diversity, all the GPCR family members have a fundamental basic architecture that includes seven transmembrane domains and a common molecular mechanism of signal transduction [166]. The GPCRs are crucial conduits for pheromone and nutrient sensing in many fungi [165,167–170].

The involvement of GPCRs in fungal pheromone sensing has been well studied [171,172]. The fact that binding of pheromones to a GPCR, which is located on the cell surface, initiates fungal mating is also documented in detail [173,174]. Two different pheromones in ascomycetes, such as *S. cerevisiae*, are sensed and secreted by opposite mating types by two different GPCRs, Ste2p and Ste3p [175] (Figure 8).

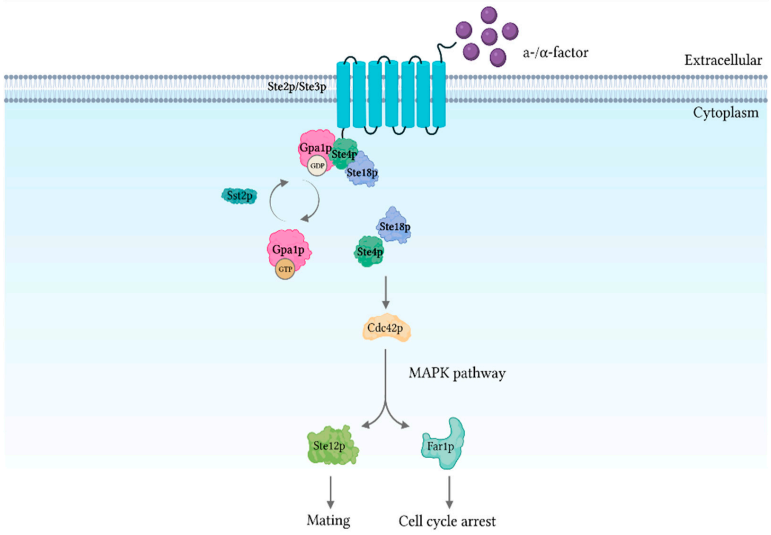

Figure 8. Schematic representation of the pheromone signaling pathway in *S. cerevisiae*. Pheromone signaling relies on the two sensors Ste2p and Ste3p that bind a- and α-factor. The signal is transported to the GPCR consisting of the Gα protein Gpa1p and the βγ subunit Ste4p and Ste18p. Ste4p and Ste18p are released and subsequently stimulate Cdc42p, which results in activation of the mating MAP kinase cascade or in cell cycle arrest.

These pheromones are named the a and α sex peptide pheromones, which trigger Ste2p and Ste3p to activate the Gα protein Gpa1p upon GDP-GTP exchange. Gpa1p dissociates from the βγ dimer of Ste4p and Ste18p and that leads to the activation of the MAPK cascade, resulting in either cell fusion with the opposite mating type or cell cycle arrest [176–178].

The architecture of the GPCRs is well conserved in the ascomycete phylum, including *C. neoformans*, *M. oryzae*, *N. crassa*, *Schizosaccharomyces pombe* or *A. nidulans* [179–181].

Glucose is one of the main carbon energy sources for many organisms and has dramatic effects on the regulation of carbon metabolism and many other properties of cells. Consequently, all organisms have evolved elaborated mechanisms to sense this molecule. Elucidation of the molecular basis of the initial glucose-sensing mechanisms has proven to be very difficult for a long time. This is largely due to the dual function as a signaling and nutrient molecule and the overlapping of the two functions [182]. Fungi have developed multiple strategies to perceive and transport glucose. One example is the GPCR sugar receptor Gpr1p in *S. cerevisiae* that senses glucose and sucrose and, subsequently, triggers Gpa2p. That results in activation of the adenylyl cyclase, the amount of cAMP increases and that activates the PKA [170] (Figure 9).

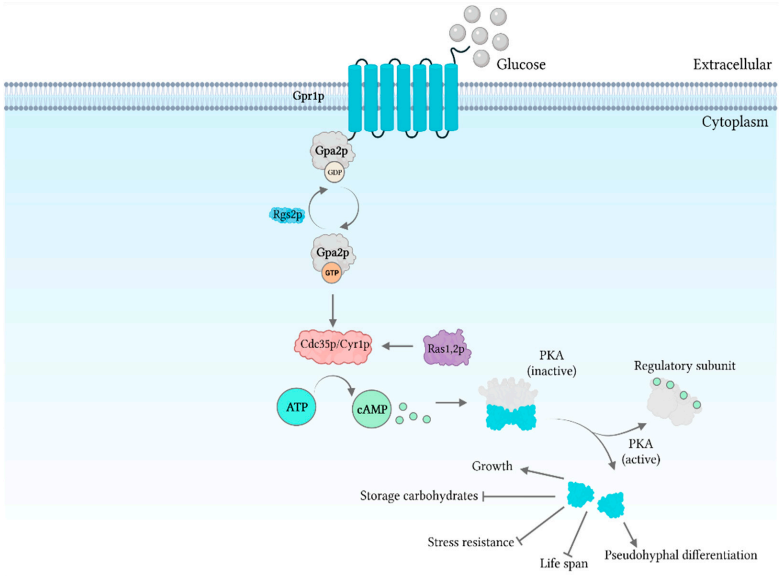

Figure 9. Schematic representation of the glucose signaling pathway in *S. cerevisiae*. The putative glucose receptor Gpr1p activates the Gα protein Gpa2p. Rgs2p stimulates the GTPase activity of Gpa2p and inhibits glucose-induced cAMP signaling. Gpa2p in turn activates adenylate cyclase (Cdc35p/Cyr1p). The function of adenylate cyclase also depends on the Ras1p and Ras2p. Activation of the PKA by cAMP results in stimulation of growth and differentiation, loss of stress resistance, mobilization of carbohydrates and in reduced life-span.

Similarly, Gpr1p homologues in *S. pombe* (Git3p receptor) and *C. albicans* (CaGpr1p) sense glucose and activate cAMP signaling, regulating morphogenesis and yeast-to-hyphal transition [183,184]. It was documented concerning the Gpr1p homologue Gpr4p in *N. crassa* that a carbon source-dependent interaction with the Gα subunit Gna1p influences cAMP production and, consequently, asexual development [185]. Many glucose-induced effects require the metabolization of the glucose molecule. Therefore, it is possible to distinguish between the nutrient function of glucose and its regulatory or signaling function: most glucose-induced signal transduction pathways apparently require no metabolization for their activation [182]. In line with this, apart from the GPCRs, *S. cerevisiae* also possesses a family of hexose transporters (Hxts) that are involved in sugar sensing or transport [186]. The existence of multiple low- and high-affinity Hxts allows cells to adjust their glucose uptake or metabolism in response to changing environmental conditions to adjust cellular physiology and growth [166]. All members of the HXT family contain 12 putative

transmembrane domains and some prominent examples are the sugar transporter-related genes SNF3, RGT2 or HXT1–17, [187]. This also highlights the central role of transport in the glucose-sensing process. Interestingly, Snf3p and Rgt2p, were found to be related to transporters but also function as sensors of extracellular glucose to regulate the expression of HXT genes [188]. That somehow distinguishes these sensors from other common Hxts. The use of transporter-like proteins as nutrient sensors may be a more common strategy in eukaryotic cells and is reviewed in reference [182].

9. Light Sensing

Light covers almost all above-ground areas on earth and represents one major driving force for adaptation and evolution. It can be both a negative and positive stimulus, since it has harmful effects, particularly at ultraviolet wavelengths, but also provides a signal to sense the environment [166]. An indisputable advantage to studying light signaling is that light behaves at light speed; the application is easy and quickly stopped, facilitating the study of stimulus-response relationships. Light sensing is conserved throughout the evolution of all the kingdoms of life, thereby, controlling important physiological and morphological responses [189]. Fungal light sensing is a good example of signal transduction in eukaryotes, and enables fundamental knowledge about the molecular basis of how cells respond and react to environmental stimuli [133]. Fungi use specialized proteins, so-called chromoproteins, to perceive blue, green, red, far-red and near-ultraviolet light. They 'see' multiple colors of light by means of different photoreceptors, for example phytochromes for red light, cryptochromes and the prominent White Collar proteins for blue light or opsins for green light [133]. The red light receptor phytochrome is found in the nucleus and cytoplasm and is linked to other signaling proteins [190,191], whereas the blue light photoreceptors reside in the nucleus, directly regulating the transcription of light-dependent genes [192]. The opsin photoreceptor for green light is a transmembrane protein, and it is still unclear how signaling takes place exactly [189,193].

Light signaling was found to be tightly linked to signal transduction pathways responsible for cellular differentiation, sporulation, primary metabolism, secondary metabolism or the production of hydrolytic enzymes [194–197]. In addition, light regulates developmental differentiation, such as spore germination, vegetative growth or the development of sexual reproductive structures [133].

The molecular mechanisms of fungal light signaling have been studied intensively in the filamentous fungus *N. crassa*, since White Collar-1 (WC-1) was identified as the first fungal photosensor in this fungus [166]. The WC-1 is part of the White Collar complex (WCC), which is composed of WC-1 and WC-2 and essential for light sensing in *N. crassa* [198]. Biochemical characterization of WC-1 revealed in the beginning that this protein is a blue light photoreceptor [199]. Subsequently, it was found that the blue light responses are the induction of sexual development and sporulation, the synthesis of carotenoids and the control of the circadian clock. All these responses are reliant on the products of WC-1 and WC-2. The WCC system has been intensively studied with focus on how the clock protein frequency (FRQ) and interacting factors are controlled and regulated by the WCC [200,201]. The WC-1 features a transactivation (TAD) domain, two classic Per-Arnt-Sim (PAS) domains (required for dimerization), a light-, oxygen- and voltage-sensing (LOV) domain (which was found to be dispensable for clock function), a DNA binding (DBD) domain and a zinc-finger (ZnF) domain required for DNA binding; WC-2 has PAS and ZnF DNA-binding domains [200,202]. A structural change occurs in the WC-1 protein upon blue light perception. In more detail, a connection between the flavin and a nearby cysteine on the molecular level leads to protein structure changes, resulting in photoreceptor activation [203]. The dimer, which is formed by WC-1 (the blue light photosensor) and WC-2 (the transcriptional activator), translocate into the nucleus and is recruited onto the promoter sequences of target genes (LRE: Light Response Elements) in order to activate their expression [204]. In short, WC-1 and WC-2 proteins dimerize at their PAS domains forming the WCC com-

plex [205,206], which, in turn, heterodimerizes upon light and mediates the light responses by starting the transcription of light-inducible genes [189,207] (Figure 10).

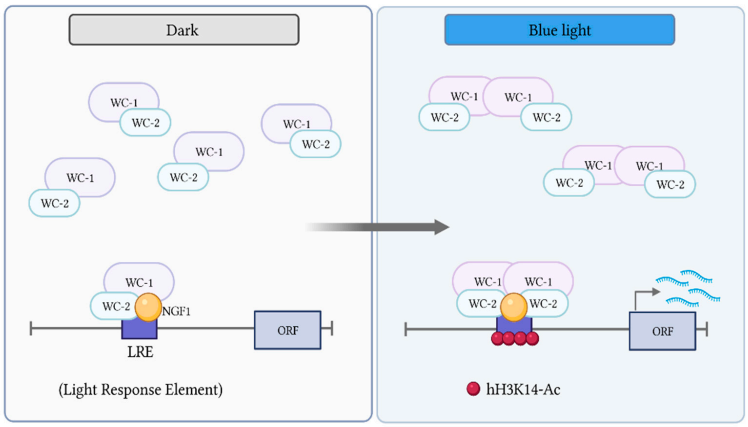

Figure 10. A simplified model for the activation of transcription by light and photoadaptation in *N. crassa*. In the dark, WC-1 and WC-2 are dimers. Light reception by the WC-1/WC-1 dimer should trigger the formation of a WCC-heterodimer, which in turn leads to chromatin remodeling through the histone acetyltransferase NGF-1 at the light response element, and the activation of gene transcription. The acetylated histones hH3K14-Ac are shown by red balls at the site of promoter binding.

Additionally, the DNA motif GATN repeats (N stands for any nucleotide) are known to be consensus sequences within the promoter regions of these light-dependent genes, but the molecular mechanism that controls the transcription during the light/dark transition it is still not understood completely. Chromatin modifications regulated by WCC in response to light are involved in the induction of these light inducible genes. Light induces the acetylation in the promoter region of histone H3-K14, which is essential for the induction of these genes [208]. This K14-acetylation is mediated by the histone acetyltransferase NGF-1. The latter interacts with WC-1 in the dark, and it was found that light promotes the activation of this WCC/NGF-1 complex, resulting in conformational changes in the WCC architecture (converting it to 'on'). Consequently, the acetyltransferase activity of NGF-1 increases [209].

Step by step, homologues of WC-1 have been identified in zygomycetes [210,211], basidiomycetes [212] and other ascomycetes [192,213]. This information extends the function of WC-1 homologues in light signaling across the fungal kingdom.

The question arises: why do fungi evolve so many different photoreceptors? Do fungi need to distinguish different wavelengths, having up to 11 photoreceptors, as described in *B. cinerea*, with 3 phytochromes, 6 blue light receptors and 2 opsins [214]? The major problem of answering this question may be our limited knowledge of the biology of fungi, since most experiments so far have been restricted to laboratory conditions [189]. To explain in more detail, light signals do not only mediate an 'on' or 'off' answer to initiate a biological response. The example of phytochromes illustrates that it is the ratio between the P_{red} and $P_{far\text{-}red}$ forms which is used to 'sense' the daytime. The use of different photoreceptors is important in different habitats and, thus, may be essential for competing in nature. It certainly makes sense, since the wavelengths responsible for green light, for example, dominate in forests, whereas these for red light penetrate soil deeper than these for blue light [189].

10. Concluding Remarks and Future Perspective

Signaling receptors and the related signaling pathways in fungi have been increasingly studied in the last years, and the more information was collected, the more questions have opened up. Thus, it is not surprising that actual knowledge does not yet allow us to present an exhaustive review encompassing all aspects of the molecular mechanisms of fungal signaling.

One question that comes up over and over again is about the relevance of the limited number of signaling proteins being present in one fungus with regard to the extremely high number of environmental signals to be processed. One major finding in the last years is functional pleiotropy of many molecular components in signaling networks. A lot of signaling proteins do not have just one function, but are involved in plenty of cellular processes, as explained for the p38 MAPK Hog1p in this review. Molecular mechanisms of fungal signaling should not be seen as a kind of limited 'on/off' mapping but rather integrate dynamic time- and intensity-based events to regulate and control cellular processes. Despite the fact that signaling pathways and signaling proteins are very important in the biology of fungi, many of them are still not characterized sufficiently. As a consequence, future research has to focus on this topic in order to close the gaps and lead to a better understanding of how signaling proteins encrypt information, coordinate different transmission routes and deploy response to various environmental stimuli. Apart from the molecular mechanisms of fungal signaling presented in this review, highly interesting signaling processes and (putative) new signaling molecules have been found in the last years. Among the most promising and scientific relevant candidates to be studied are chitosan-sensing [215], nitric oxide sensing [216] and iron–sulfur signaling [217].

In the end, for most if not all of the signaling pathways and components known so far, a dynamic time- and intensity-based comprehensive characterization will pave the way to answer one of the most important questions of cellular signaling: how can the huge number of different signals surrounding living cells be recognized, processed and transmitted by a limited number of signaling proteins?

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5. Directed experimental adaptive evolution of osmoregulation in the fungal pathogen *Magnaporthe oryzae* is independent of glycerol metabolism associated genes

Directed experimental adaptive evolution of osmoregulation in the fungal pathogen *Magnaporthe oryzae* is independent of glycerol metabolism associated genes

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Supplementary Materials: Annex of this document

This manuscript presents the first fundamental findings on the adaptive evolution of signaling networks in the fungal pathogen *Magnaporthe oryzae* using directed adaptive laboratory evolution (DALE), with results prepared for publication. Directed adaptive evolution in fungal pathogens remains largely unexplored, yet our study reveals that long-term cultivation under osmotic stress consistently drives the emergence of suppressor strains that restore osmoregulation in "loss of function" mutants of the HOG pathway. Two distinct types of suppressors emerged: reversible strains, which still struggle under osmotic stress, and irreversible strains, which adapt fully to stress conditions. Interestingly, both types of suppressors produce glycerol, not arabitol (as seen in wild-type strains), as a stress response, with irreversible strains generating nearly twice as much glycerol as reversible ones. This suggests a role for glycerol metabolism (gm) in adaptive evolution. However, by generating double mutants with deleted gm-related genes in HOG pathway lof mutants, we observed suppressor emergence under stress, ruling out gm-associated genes as direct drivers of adaptation. This leaves the molecular mechanisms behind this adaptation process still to be uncovered.

Abstract

Directed experimental adaptive evolution in fungal pathogens is largely unexplored. Long-term cultivation on osmotic stress was found to be a driving force absolutely reproducible leading to individuals arising as suppressor strains with reestablished osmoregulation out of osmosensitive "loss of function" mutants of the high osmolarity glycerol (HOG) pathway in the phytopathogenic fungus *Magnaporthe oryzae*. The underlying mechanisms are not known. Here, we found that two different types of suppressor strains emerged from mycelium parts of each *ΔMohik1, ΔMoypd1, ΔMossk1, ΔMossk2, ΔMopbs2* and *ΔMohog1*: reversible suppressors, which still struggle with osmotic stress, and irreversible suppressors, which can cope the same stress situations. This directed experimental adaptive evolution only takes place in lof mutants which are related to the HOG pathway and not in other osmosensitive mutants. Both suppressor types produce glycerol as stress response instead of arabitol as it is in the wildtype strain. Glycerol-production was found to be almost twice as high in the irreversible as compared to the reversible strains. Thus, glycerol metabolism (gm) was assumed to be involved in the molecular mechanism of this adaptive driven evolution. We generated a set of double mutant strains in which we deleted different gm-related genes within the HOG lof-mutants and observed still suppressors upon long term stress. . As a consequence, we could exclude gm-associated genes acting as drivers for adaptive driven evolution leaving the molecular mechanisms behind left to be discovered.

Keywords: Directed experimental adaptive evolution, , High osmolarity glycerol (HOG) pathway, *Magnaporthe oryzae*, osmoregulation, signaling networks, Suppressor, glycerol, arabitol

Introduction

Evolution is generally understood as an incredibly slow process acting over thousands of years, but in the last decades, research in the field of evolutionary dynamics or directed experimental adaptive evolution shed light in biological processes in microorganisms that are found to be much faster

(Selmecki et al., 2015). Yet, the molecular mechanisms causing these more rapid evolutionary adaptations are not well understood because they rely on rare to study often unpredictable spontaneous mutations. Research in this area is not only of academic significance; but of critical importance for understanding how organisms handle global challenges, such as changes in ecosystems, the climate change, the emergence of pathogens, the expansion of invasive organisms, and of course the (multi-) resistance to vaccines and drugs (Gladieux et al., 2014). Advantageous changes on genome-, transcriptome- or protein-level which evolve and persist by natural selection are processes termed as "adaptive walk" (Schoustra et al., 2009). One prominent example of an adaptive walk of high importance is the development of multi drug resistance in microbial organisms (Fieri et al. 2018). Consequently, being able to (rapidly) evolve is particularly for pathogenic microbes a prerequisite to survive (Naranjo-Ortiz and Gabaldón, 2020). However, rapid adaptation of microorganisms to changing environmental conditions can take place even within few generations, facilitating observations of adaptations, genetic evolution of populations, and competitive dynamics (Card et al., 2021). The majority of research efforts so far focused on bacterial systems (Good et al., 2017). Evolutionary adaptation of microorganisms is, among others, explained by the instability of chromosomes after duplication of the whole genome (Covo, 2020). Furthermore, transposable elements are discussed intensively as driving forces in the evolution of organisms (Fouché et al., 2020). Both of these examples lead to a diversification of species by re-structuring of the genome. It is also hypothesized that these processes lead to the emergence of disease resistance genes in plants. In contrast to resistance genes and their diversity, the evolution of avirulence genes (Avr genes) in plant/pathogen interaction is little studied (Figueroa et al., 2020). In one of the world's top ten most dangerous pathogens in crops, the rice blast pathogen *Magnaporthe oryzae* (syn. *Pyricularia oryzae*), the underlying mechanisms for "normal" evolution are documented so far in high variation in nucleotides, high substitution ratios, and frequent polymorphisms (Huang et al., 2014). Pathogenic organisms like *M. oryzae* are forced to constantly adapt to changing environmental stimuli and dynamic changes during host-pathogen interactions (Naranjo-Ortiz and Gabaldón, 2020). In addition to "long-term" adaptation

mediated by alterations in the genome or epigenetic changes, the facultative pathogenic lifestyle requires a rapid adaptive behavior enabling a fast regulation of cellular processes. For this, a dynamic alteration of protein structure or protein-protein interaction is facilitated by posttranslational modifications (PTMs) that are both known to be highly flexible and partially reversible (Wang et al., 2022). The genome of *M. oryzae* encodes a huge set of protein kinases and more than 60 peptidases, highlighting the biological importance of such PTMs. Except for the consideration of Avr genes as drivers for rapid evolutionary processes in microorganisms, almost nothing is known about evolutionary dynamics and the molecular mechanisms behind the modulation of signaling networks regulating physiological and biochemical systems/processes (Sutthuphai et al., 2021). Signaling pathways cannot be studied as single modules, but rather are complex networks of interactions between several pathways, transcriptional regulations, and biochemical mechanisms (Jacob et al., 2022). That complicates the search for single evolutionary events because a small change in just one molecule can affect an entire network of signaling pathways (Scheuerl et al., 2020). In this context Adaptive Laboratory Evolution (ALE) stands as a powerful methodology within evolutionary biology, enabling researchers to observe and manipulate the evolutionary trajectory of microorganisms under controlled laboratory conditions. Through ALE, microbial populations are subjected to specific environmental stresses or selective pressures over multiple generations, allowing for the rapid accumulation of beneficial mutations and the emergence of adaptive traits. By iteratively exposing organisms to challenging conditions and selecting for desired phenotypes, ALE facilitates the study of evolutionary dynamics in real-time, shedding light on the underlying genetic and physiological mechanisms driving adaptation. This approach not only provides valuable insights into the evolutionary processes occurring within microbial populations but also offers practical applications in diverse fields such as biotechnology, medicine, and environmental science.

In contrast to most of the classical metabolic pathways, many signal transduction pathways use several modular mechanisms to route and coordinate an input-output interaction (Jacob et al., 2022). One prominent example is the high osmolarity glycerol (HOG) pathway in the fungal pathogen *Magnaporthe*

oryzae (Jacob et al., 2015). The physiological role of the HOG pathway is to regulate the adaptation of the cells to increased osmolarity in the surrounding environment. The molecular architecture of the HOG pathway consists of a phosphorelay system composed of MoSln1p/MoHik1p-MoYpd1p-MoSsk1p and a downstream MAPK cascade (MoSsk2p-MoPbs2p-MoHog1p) (Bersching and Jacob, 2021). Targets of MAPK include generally plenty receivers like transcription factors, phosphatases, further MAPK-activated protein kinases, and other protein classes that regulate physiological processes, cell morphology, cell cycle progression, metabolism, and gene expression in response to various extracellular signals or environmental stresses (Hohmann, 2009). The HOG pathway in *M. oryzae* has already proven to be a suitable model system for fundamental research of physiological functions and the mode of action of agricultural fungicides (e.g. fludioxonil) (Bersching and Jacob., 2021). Initially, loss of function (lof) mutants of the HOG pathway were used to characterize the individual functional elements (Jacob et al., 2016). It is known that these lof mutants are not only osmosensitive, but also fail to produce important compatible solutes to cope with osmotic stress such as arabitol. Arabitol is produced by the *M. oryzae* wildtype strain as response to osmotic stress (Dixon et al., 1999). In 2019, Bohnert *et al.* cultivated the osmosensitive lof mutants *ΔMohog1, ΔMopbs2, ΔMossk2, ΔMossk1*, and *ΔMoypd1* several weeks under permanent osmotic stress and found stable individuals arising from the mycelium of each lof mutant. Interestingly, these rapidly "evolved" suppressor strains are not only able to handle osmotic stress again, but produce glycerol instead of arabitol in response to osmotic stress (Bohnert et al., 2019, Jacob and Bersching, 2021). In line with this, it was hypothesized in a previous study, that glycerol metabolism dependent genes may be responsible for the suppressor phenotype (Bohnert et al., 2019). A set of candidate genes which was found to be upregulated in both the salt stress samples of the *ΔMohog1(adapted)* and the wildtype strain, whereas these genes were not regulated in the lof mutant *ΔMohog1*. Among these candidates were the glycerol H+-symporter MoSlt1p (MGG_09852), the phosphoglycerate mutase (MGG_06642,), one glycerol-3-phosphate dehydrogenase (MGG_00067 (MoGpd1p)) and one phosphatidyl synthase (MGG_00099 (MoHad1p)).

In contrast to previous hypotheses, the inactivation of these candidate genes within the lof HOG mutants does not result in the absence of the suppressor phenotype As a consequence, the underlying molecular mechanisms are still to be discovered, what underscores the complexity of the evolutionary mechanisms of signaling networks in *M. oryzae*.

Material and Methods

Cultivation of *Magnaporthe oryzae*

The *Magnaporthe oryzae* strains used in this study were *M. oryzae* 70-15 (*MoWT*, Fungal Genetics Stock Center), the loss of function mutants *ΔMohik1* (MGG_11174), *ΔMoYpd1* (MGG_07173), *ΔMossk1* (MGG_02897), *ΔMossk2* (MGG_00183), *ΔMopbs2* (MGG_10268), *ΔMohog1* (MGG_01822) (Jacob *et al.*, 2015) and to all the lof mutants the corresponding suppressor strains (Bohnert *et al.*, 2019). The strains were grown at 26 °C on complete medium (CM). The CM at pH 6.5, 2% agar, contains per l iter 1 g casamino acids, 10 g glucose, 2 g peptone, 1 g yeast extract, 50 mL nitrate salt solution (contain ing per liter: 10.4 g KCl, 30.4 g KH_2PO_4, 10.4 g $MgSO_4 \times 7\ H_2O$, 120 g $NaNO_3$) and 1 mL of a trace element solution (containing per liter: 1.7 g $CoCl_2 \times 6\ H_2O$, 1.6 g $CuSO_4 \times 5\ H_2O$, 5 g $FeSO_4 \times 7\ H_2O$, 11 g H_3BO_3, 5 g $MnCl_2 \times 4\ H_2O$, 50 g Na_2EDTA, 1.5 g $Na_2MoO_4 \times 2\ H_2O$, 22 g $ZnSO_4 \times 7\ H_2O$, pH 6.5 adjusted by 1 M KOH). All chemicals used were p.a. quality unless stated otherwise.

Single spore isolation

Conidia of each strain (approximately 11 days old) were filtered through two layers of Miracloth tissue (Merck). The suspension was adjusted two $1x10^3$ conidia mL^{-1} in sterilized H_2O. About 50 – 100 µl of the conidia suspension was placed on a selection media and incubated at 26 °C for about 12- 24 h. After the incubation the single spores are visible, can be selected and placed on a new media containing the selection media for further incubation.

Construct of vectors for genetic manipulation

The gDNA from *Magnaporthe oryzae* was isolated from mycelia of three-day-old liquid cultures (Jacob *et al.*, 2015). For the purification the GeneJET™ Plant Genomic DNA Purification Mini Kit (Thermo

Fisher Scientific, Waltham, MA, USA) was used according to the manual's user guide. The procedures of standard molecular cloning were based on Bohnert et al., 2019. All plasmids used in this study were generated by using the Gibson assembly cloning method (Gibson *et al.*, 2009). As a backbone the *Bgl*II/*Pst*I-restricted plasmid psj-basic was used (Bohnert et al., 2019). The exchange of the resistance cassette of the inactivation mutants was conducted via homologous recombination. Therefore, the flanks were chosen before the start codon and after the stop codon. The size of the flanks can be different between the mutants, because care was taken not to interrupt or unintentional modify other genes. The sizes of the flanks can vary between 250 and 3900 bp. Bigger flanks were chosen if genes are located nearby to further avoid unattentionally further incatication of genes nearby. The flank (flank 1/ flank 2) size for the re-integration of the original HOG genes were: *ΔMohik1*/*HIK1* (1000 bp/1000 bp), *ΔMoypd1*/*YPD1* (600 bp/ 330 bp), *ΔMossk1*/*SSK1* (500 bp/ 1950), *ΔMossk2*/*SSK2*, *ΔMopbs2*/*PBS2* (500 bp/ 3900 bp) *and ΔMohog1*/*HOG1* (1500 bp/ 998) and for the inactivation mutants: *ΔMostl1, ΔMogpd1* (700 bp/ 700 bp*),ΔMopga1* (841 bp/ 683 bp) and *ΔMohad1* (1000 bp/ 100 bp).The BAR resistance cassette was amplified from a modified bialaphos resistance gene, BAR) (Bersching and Jacob, 2021).

For bacterial transformation NEB® 10-β competent *Escherichia coli* strains (high efficiency) were used. The fungal transformation was performed via *Agrobacterium tumefaciens*-mediated transformation (de Groot *et al.*, 1998; Rho, Kang and Lee, 2001; Odenbach *et al.*, 2007), resulting in the mutant strains listed in the supplements S1-S6. The selection of transformants containing a resistant to glufosinat-ammonium was performed by using 100 μg mL^{-1} of the antibiotic in minimal medium and the successful replacement within the gDNA of the mutants was confirmed by southern blot analysis or specific PCR screens (Fig. S1-S13).

All oligonucleotides used in this study were generated by Eurofins Genomics (Ebersberg, Germany) and listed in the supplementary.

Plant infection assays

The plant infection assays were carried out as described previously (Jacob *et al.*, 2014) and performed with *MoWT*, the lof-mutants of the HOG pathway, the corresponding suppressor strains and the

generated complementation mutants. For the plant infection assay the conidia of the above mentioned strains were harvested from 11-day old cultures, which were cultivated on CM in order to assess virulence. The cultivated rice plants (CO39) were grown for 21 days using daily cycle of 16 h light and 8 h darkness at 28 °C. The conidial adjusted suspension (diluted in H_2O) containing 0.2 % gelatin were spray inoculated. After 5 das of incubation, the lesions were analyzed.

Vegetative growth assays

Agar blocks of approximately 0.8 cm diameter were cut from the cultures and placed onto freshly prepared CM or Minimal medium (MM) (MM, pH6.6, 2 % agar, contains per litre: 1g glucose, 50 mL nitrate salt solution, 0.25 mL biotin-solution (0.01 %), 1 mL thiamin dichloride solution (1 %) and 1 mL of trace elements) with different compounds to induce stress. The cultures were grown for 10 d at 26 °C.

Germination assay

The germination assay was carried out according to (Jacob, Schüffler and Thines, 2016).

HPAEC-PAD analysis to quantify compatible solute production

The osmolytes (sugar alcohols, monosaccharides and disaccharides) were detected by the HPAEC-PAD analysis as described previously (Grünewald, Bohnert and Jacob, 2021). Different stressors besides 0.5 M KCl were tested and are listed in the results.

Results

Two distinct types of suppressors were generated by directed experimental adaptive evolution

Magnaporthe oryzae lof mutants with an inactivated HOG pathway are sensitive to osmotic stress and in our previous study, it was found that under permanent osmotic stress individual suppressor strains with reestablished osmoregulation arise out of such lof mutants (Bohnert et al., 2019). In ongoing investigations of these suppressor strains (formerly identified and referred to as "adapted" strains, Bohnert et al., 2019), which have been grown out of *ΔMohik1*, *ΔMoypd1*, *ΔMossk1*, *ΔMossk2*, *ΔMopbs2* and *ΔMohog1*, we now identified at least two types (or two "stages") of suppressor strains and therefore hypothesized the mechanism of directed experimental adaptive (DEA) evolution even more dynamic than originally expected (Fig. 1 [A]).

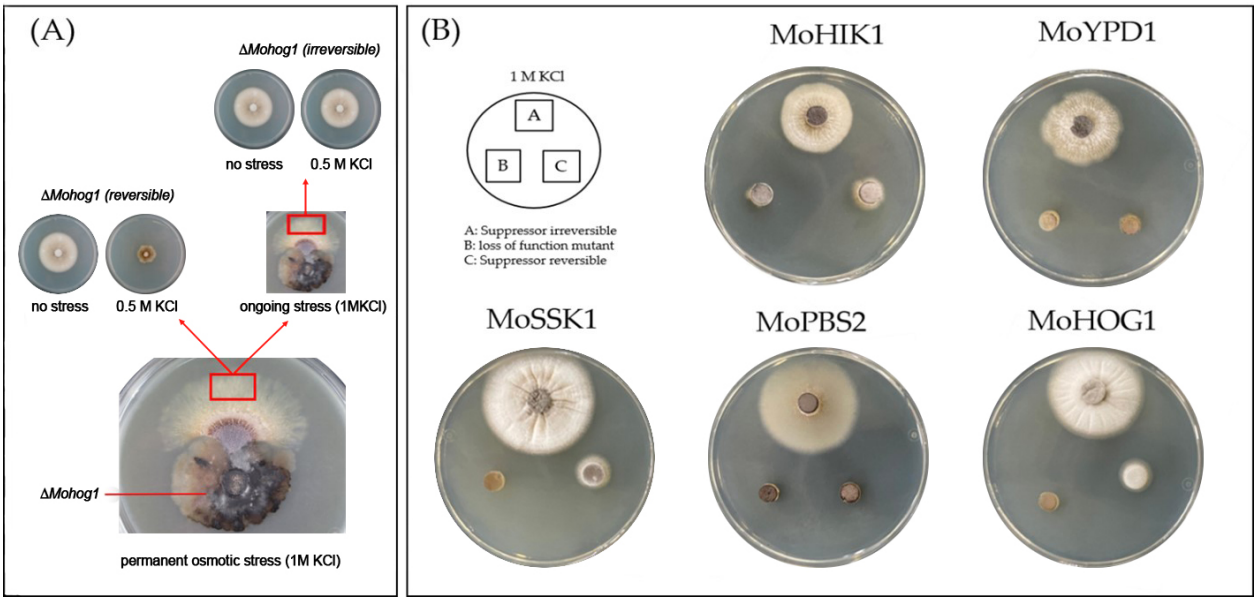

Fig. 1. Directed experimental adaptive (DEA) evolution in *Magnaporthe oryzae* upon osmotic stress. (A) Two types of DEA: reversible (left) and irreversible (right) suppressor mutants arise out of lof mutants with an inactivated HOG pathway upon long time cultivation of osmotic stress. (B) Overview of vegetative growth of the *Magnaporthe oryzae* lof-mutants and the suppressor strains (single spore isolates of the reversible and irreversible suppressors) for 5 days upon osmotic stress (1 M KCl).

Initially, individual mycelium parts were found outgrowing from the mycelium of each lof mutant *ΔMohik1*, *ΔMoypd1*, *ΔMossk1*, *ΔMossk2*, *ΔMopbs2* and *ΔMohog1* after about three to four weeks of cultivation on permanent osmotic stress. These individual mycelium parts were isolated, named as

"reversible". and subjected to further investigations. After two more weeks of constant stress, further pieces of mycelium grew out of the original cultures and these were named "irreversible" (Fig.1 [A]). After sub-cultivating under unstressed conditions on CM medium for 8-11 days, both suppressor types have been re-cultivated upon osmotic stress (e.g. KCL or Sorbitol). Surprisingly, not all suppressor mutants were able to cope with osmotic stress. We could clearly distinguish two different types of suppressor strains: (I) the reversible suppressor mutants, which are found to be impaired in the ability to cope with repeated osmotic stress and (II) the irreversible suppressor mutants, which are able to grow on osmotic stress (Fig 1 [A]). From each reversible and irreversible suppressor mutant of MoHOG1, MoPBS2, MoSSK2, MoSSK2, MoSSK1, MoYPD1 and MoHIK1, single spore isolation was performed in order to be safe to separate only pure cultures. The individually strains, which were obtained by single spore isolation, have been named *ΔMohik1(reversible)*, *ΔMoypd1(reversible)*, *ΔMossk1(reversible)*, *ΔMossk2(reversible)*, *ΔMopbs2(reversible)*, *ΔMohog1(reversible)*, and *ΔMohik1(irreversible)*, *ΔMoypd1(irreversible)*, *ΔMossk1(irreversible)*, *ΔMossk2(irreversible)*, *ΔMopbs2(irreversible)* and *ΔMohog1(irreversible)*, respectively.

These suppressor strains then were used in vegetative growth assays in order to compare their ability to cope with osmotic stress. Apart from KCl stress, NaCl as well as sorbitol were used as stress inducing agents (Fig.2).

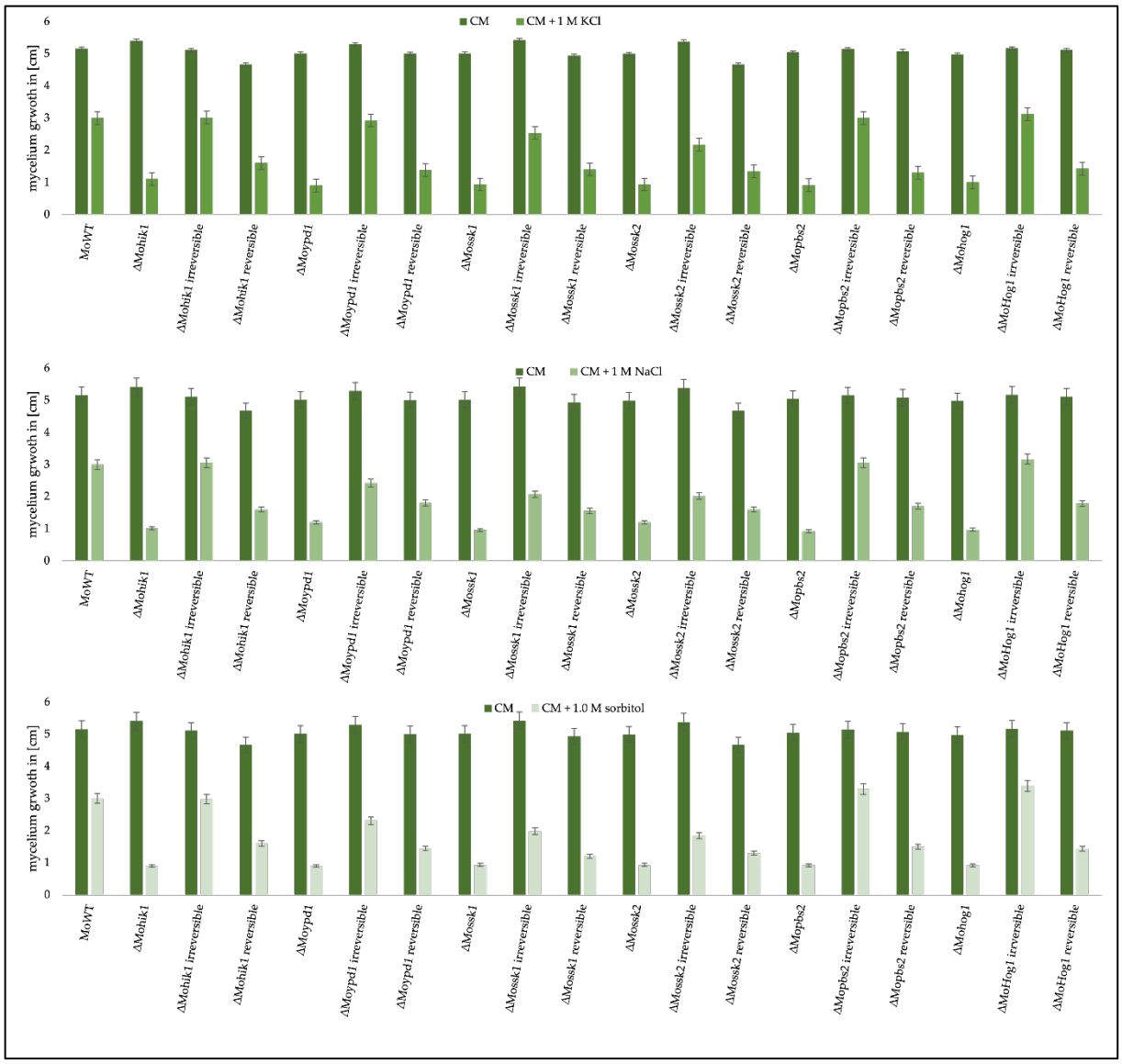

Fig. 2: Vegetative growth of the wildtype strain (MoWT), the original lof mutants and the suppressor strains (irreversible and reversible). Growth diameter of the colonies was measured after 7 days on CM, or CM supplemented with 1 M KCl, 1 M NaCl or 1 M sorbitol. The experiments were conducted in triplicates each.

It clearly has been documented, that all irreversible suppressor strains *ΔMohik1(irreversible)*, *ΔMoypd1(irreversible)*, *ΔMossk1(irreversible)*, *ΔMossk2(irreversible)*, *ΔMopbs2(irreversible)* and *ΔMohog1(irreversible)* are almost as vital as the wildtype strain upon the different stressed situations. In contrast, the growth of reversible suppressor strains *ΔMohik1(reversible)*, *ΔMoypd1(reversible)*, *ΔMossk1(reversible)*, *ΔMossk2(reversible)*, *ΔMopbs2(reversible)* and *ΔMohog1(reversible)* was strongly impaired as compared to the irreversible strains and the wildtype strain. But nevertheless, it has to be mentioned, that the reversible strains are not as sensitive as the lof strains (Fig.2).

The inactivated HOG pathway is the key location for directed experimental adaptive evolution

Expanding to our previous published observations (Bohnert et al., 2019), it was further confirmed that both types of DEA evolution occur only in lof-mutants with inactivated HOG pathway, namely *ΔMohik1, ΔMoypd1, ΔMossk1, ΔMossk2, ΔMopbs2* and *ΔMohog1*. Analysis of other inactivation mutants, which are osmosensitive but not related to the HOG pathway, such as (*ΔMoskn7* (MGG_03516), *ΔMostu1* (MGG_04185), *ΔMofluG* (MGG_16491*), ΔMompg1* (MGG_10315), *ΔMossp2* (MGG_00803), *ΔMonpr2* (MGG_00664) and *ΔMopmk1* (MGG_09565), revealed the DEA evolution is strictly limited to lof mutants of the HOG pathway. Here, we demonstrated, that no mycelium parts arose out of all non-HOG related osmosensitive mutant strains whereas the reversible as well as the irreversible suppressors grew out of the mutants with inactivated HOG pathway.

Then, we added empirical investigations of different parameters of stresses which were known to trigger the HOG pathway, like $CaCl_2$ (Ion-stress), $CoCl_2$ (hypoxia), and $NaNo_2$ (salt stress and hypoxia), fludioxonil (fungicide stress), temperature and light/dark rhythm in order to find different factors promoting or constraining DEA evolution (see material and methods, and Bersching and Jacob, 2021). Apart from osmotic stress (NaCl, KCl, sorbitol), additional agents like $CaCl_2$ (Ion-stress), $CoCl_2$ (hypoxia), and $NaNo_2$ (salt stress and hypoxia), different pH-values and fludioxonil (fungicide stress) were not successful in the generation or suppressor mutants. The lof-mutants *ΔMohik1, ΔMoypd1, ΔMossk1, ΔMossk2, ΔMopbs2* and *ΔMohog1* have been cultivated for more than 10 weeks on medium supplemented with sublethal concentrations of before mentioned stress inducing agents (Fig. 3)

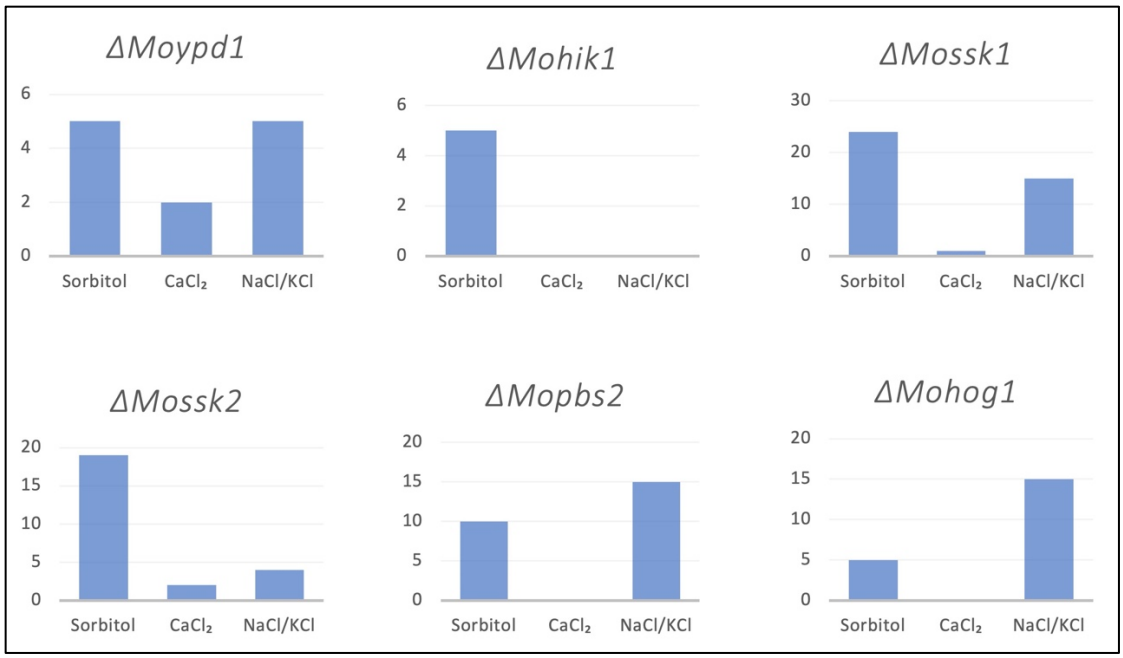

Fig. 3: Empirical investigation of the ALE for the adaptation process of the suppressor mutants. The number represent the number of generated suppressor mutants for each above mentioned HOG inactivation mutants (reversible and irreversible are counted together).

It could be observed, that none of all these stressors resulted in DEA evolution suppressor strains as it was found for NaCl, KCl and sorbitol.

Reintegration of HOG pathway-inactivated gene in the suppressor strains restores naturally biochemical and physiological phenotype

Since we know that the suppressor strains arose only out of lof mutants with inactivated HOG pathway, one fundamental question driving our research is, which impact has a genetic reconstruction of the functional HOG pathway on the phenotypic characteristics of the suppressor strains *ΔMohik1(reversible)*, *ΔMoypd1(reversible)*, *ΔMossk1(reversible)*, *ΔMossk2(reversible)*, *ΔMopbs2(reversible)* and *ΔMohog1(reversible)* as well as on *ΔMohik1(irreversible)*, *ΔMoypd1(irreversible)*, *ΔMossk1(irreversible)*, *ΔMossk2(irreversible)*, *ΔMopbs2(irreversible)* and *ΔMohog1(irreversible)*. In order to investigate the impact of a full functional HOG pathway within the reversible and irreversible suppressor strains, we reintegrated the original lof-gene into the genome of the respective lof mutants (classical complementation-experiment) and in each reversible and each irreversible suppressor strain. The

resulting strains were named *ΔMohik1/HIK1*, *ΔMoypd1/YPD1*, *ΔMossk1/SSK1*, *ΔMossk2/SSK2*, *ΔMopbs2/PBS2 and ΔMohog1/HOG1*, the complemented suppressors *ΔMohik1(reversible)/HIK1*, *ΔMoypd1(reversible)/YPD1*, *ΔMossk1(reversible)/SSK1*, *ΔMossk2(reversible)/SSK2*, *ΔMopbs2(reversible)/PBS2* and *ΔMohog1(reversible)/HOG1* as well as *ΔMohik1(irreversible)/HIK1*, *ΔMoypd1(irreversible)/YPD1*, *ΔMossk1(irreversible)/SSK1*, *ΔMossk2(irreversible)/SSK2*, *ΔMopbs2(irreversible)/PBS2* and *ΔMohog1(irreversible)*/HOG1. Then, we monitored vegetative growth upon stress, the production of compatible solutes as well as virulence of all the complemented suppressors and compared it to the lof mutant strains as well as to the wildtype strain. Initially, intracellular production of compatible solutes was determined by HPAEC-PAD and it was clearly demonstrated that arabitol was found to be the major component produced after stress in all the strains in which the HOG pathway was genetically reactivated, almost as high as it was detected in the wildtype strain (Fig. 4).

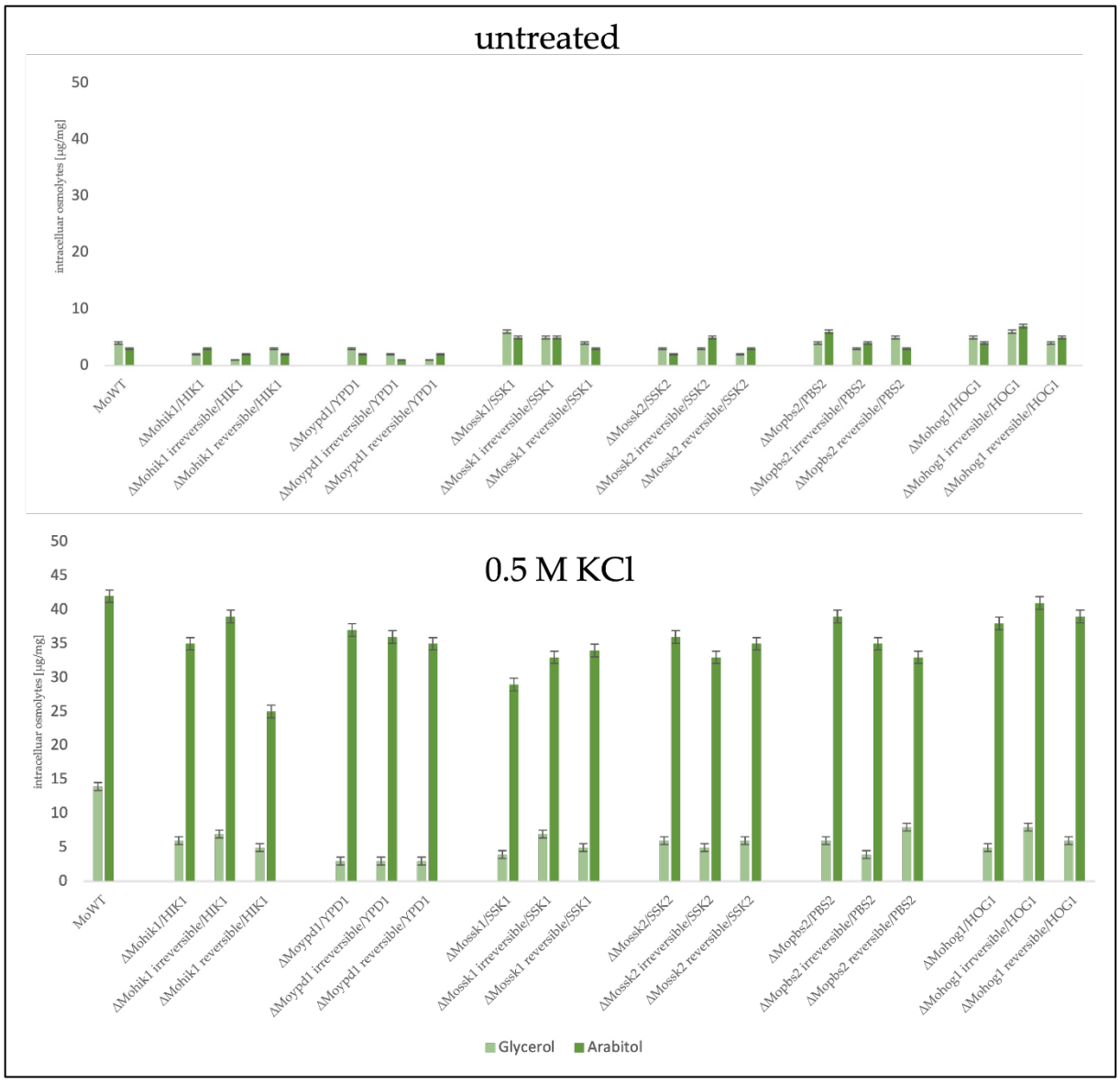

Fig 4: The production of compatible solutes as stress response in *Magnaporthe oryzae* wildtype strain (MoWT), and mutant strains with reintegrated lof-gene of the HOG pathway. The fungal strains were grown for 72 h in CM before being shocked with 0.5 M KCl. Carbohydrates were extracted after 7 h and quantified by HPAEC-PAD. Error bars represent the standard deviation of three biological replicates of each strain.

Furthermore, the genetic reconstruction and consequently a functional HOG pathway in the lof mutants as well as in the suppressor strains (irreversible and reversible) resulted not only to the production of arabitol as major stress response upon osmotic stress, but also in reestablished osmoregulation capacity in the lof mutants as well as in all the suppressor strains (Fig. 5).

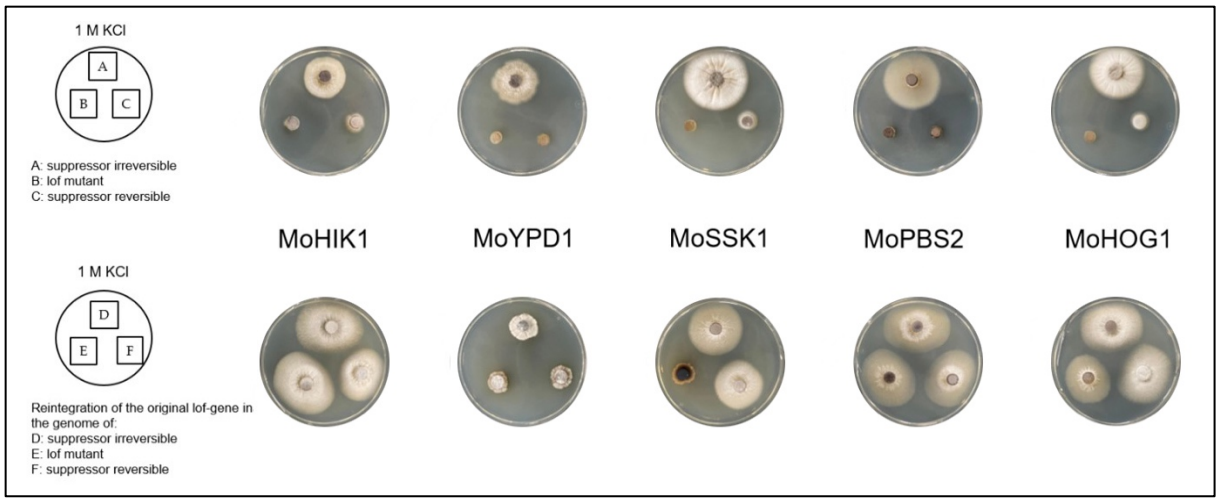

Fig 5: Vegetative growth of the *Magnaporthe oryzae* lof mutants, the suppressor mutants (irreversible and reversible) and the strains with the reintegration of the original lof-gene of the HOG pathway into the genome. The cultures were grown on media with osmotic stress (1 M KCl) for 7 days at 26°C.

Additionally, it is documented, that the lof mutants with inactivated HOG pathway *ΔMohik1*, *ΔMoypd1*, *ΔMossk1*, *ΔMossk2*, *ΔMopbs2* and *ΔMohog1* are reduced in virulence as compared to the wildtype strain (Jacob et al., 2015). Interestingly, the suppressor strains were shown to be even less virulent as compared to the lof mutants (Bohnert et al., 2019). In this study, we found, that virulence was not only reconstituted in the complemented original lof strains *ΔMohik1/HIK1*, *ΔMoypd1/YPD1*, *ΔMossk1/SSK1*, *ΔMossk2/SSK2*, *ΔMopbs2/PBS2 and ΔMohog1/HOG1*, but also in all the tested complemented suppressors *ΔMohik1(reversible)/HIK1*, *ΔMoypd1(reversible)/YPD1*, *ΔMossk1(reversible)/SSK1*, *ΔMossk2(reversible)/SSK2*, *ΔMopbs2(reversible)/PBS2* and *ΔMohog1(reversible)/HOG1* as well as in *ΔMohik1(irreversible)/HIK1*, *ΔMoypd1(irreversible)/YPD1*, *ΔMossk1(irreversible)/SSK1*, *ΔMossk2(irreversible)/SSK2*, *ΔMopbs2(irreversible)/PBS2* and *ΔMohog1(irreversible)/HOG1* (Fig.6).

Analysis / Organism	*MoWT*	*ΔMohik1*	*ΔMohik1/HIK1*	*ΔMohik1(irreversible)*	*ΔMohik1(irreversible)/HIK1*	*ΔMohik1(reversible)*	*ΔMohik1(reversible)/HIK1*
Osmosensitivity	no	yes	no	no	no	yes	no
Osmolyte after stress induction	arabitol	arabitol	arabitol	gycerol	arabitol	gycerol	arabitol
Virulence	yes	reduced	yes	strongly reduced	yes	strongly reduced	yes
Suppressor arises under stress	no	yes	no	no	no	no	no
Fludioxonil sensitivity	yes	no	yes	reduced	yes	reduced	yes

Analysis / Organism	*MoWT*	*ΔMoypd1*	*ΔMoypd1/YPD1*	*ΔMoypd1(irreversible)*	*ΔMoypd1(irreversible)/YPD1*	*ΔMoypd1(reversible)*	*ΔMoypd1(reversible)/YPD1*
Osmosensitive	no	yes	no	no	no	yes	no
Osmolyte after stress induction	arabitol	arabitol	arabitol	gycerol	arabitol	gycerol	arabitol
Virulence	yes	reduced	yes	strongly reduced	yes	strongly reduced	yes
Suppressor arises under stress	no	yes	no	no	no	no	no
Fludioxonil sensitivity	yes	no	yes	reduced	yes	reduced	yes

Analysis / Organism	*MoWT*	*ΔMossk1*	*ΔMossk1/SSK1*	*ΔMossk1(irreversible)*	*ΔMossk1(irreversible)/SSK1*	*ΔMossk1(reversible)*	*ΔMossk1(reversible)/SSK1*
Osmosensitive	no	yes	no	no	no	yes	no
Osmolyte after stress induction	arabitol	arabitol	arabitol	gycerol	arabitol	gycerol	arabitol
Virulence	yes	reduced	yes	strongly reduced	yes	strongly reduced	yes
Suppressor arises under stress	no	yes	no	no	no	no	no
Fludioxonil sensitivity	yes	no	yes	reduced	yes	reduced	yes

Analysis / Organism	*MoWT*	*ΔMopbs2*	*ΔMopbs2/PBS2*	*ΔMopbs2(irreversible)*	*ΔMopbs2(irreversible)/PBS2*	*ΔMopbs2(reversible)*	*ΔMopbs2(reversible)/PBS2*
Osmosensitive	no	yes	no	no	no	yes	no
Osmolyte after stress induction	arabitol	arabitol	arabitol	gycerol	arabitol	gycerol	arabitol
Virulence	yes	reduced	yes	strongly reduced	yes	strongly reduced	yes
Suppressor arises under stress	no	yes	no	No	no	no	no
Fludioxonil sensitivity	yes	no	yes	reduced	yes	reduced	yes

Analysis / Organism	*MoWT*	*ΔMohog1*	*ΔMohog1/HOG1*	*ΔMohog1(irreversible)*	*ΔMohog1(irreversible)/HOG1*	*ΔMohog1(reversible)*	*ΔMohog1(reversible)/HOG1*
Osmosensitive	no	yes	no	no	no	yes	no
Osmolyte after stress induction	arabitol	arabitol	arabitol	gycerol	arabitol	gycerol	arabitol
Virulence	yes	reduced	yes	strongly reduced	yes	strongly reduced	yes
Suppressor arises under stress	no	yes	no	no	no	no	no
Fludioxonil sensitivity	yes	no	yes	reduced	yes	reduced	yes

Fig 6: Overview of the most important phenotypes of the *Magnaporthe oryzae* lof mutants, the suppressor strains (reversible and irreversible) as well as the strains in which the HOG pathway is genetically reactivated.

Upon the different parameters tested in order to comprehensively characterize all generated strains in this study, fludioxonil susceptibility is an important characteristic feature which was also found to be reestablished in the complemented suppressor strains *ΔMohik1(reversible)/HIK1*, *ΔMoypd1(reversible)/YPD1*, *ΔMossk1(reversible)/SSK1*, *ΔMossk2(reversible)/SSK2*, *ΔMopbs2(reversible)/PBS2* and *ΔMohog1(reversible)/HOG1* as well as in *ΔMohik1(irreversible)/HIK1*, *ΔMoypd1(irreversible)/YPD1*, *ΔMossk1(irreversible)/SSK1*, *ΔMossk2(irreversible)/SSK2*, *ΔMopbs2(irreversible)/PBS2* and *ΔMohog1(irreversible)*/HOG1. Fludioxonil resistance was only observed for the lof mutants with a non-functional HOG pathway, whereas all the suppressor strains (reversible and irreversible) as well as all strains in which the HOG pathway was genetically reconstructed, were found to be as sensitive to the fungicide as the wildtype strain (Fig. 6). Additionally, the genetically reconstructed mutants with the possibly activated HOG pathway again are not able to generate further suppressor mutants via ALE.

The difference of the biochemical stress response in reversible and irreversible suppressor strains

The ability of the suppressor mutants to be able to cope with osmotic stress by producing glycerol as compatible solute in contrast to arabitol as it is in the wildtype strain was further analyzed for both types of suppressors Therefore, *the wildtype strain,* the lof mutants of the HOG pathway, *ΔMohik1(reversible), ΔMoypd1(reversible), ΔMossk1(reversible), ΔMossk2(reversible), ΔMopbs2(reversible)* and *ΔMohog1(reversible)* as well as *ΔMohik1(irreversible), ΔMoypd1(irreversible), ΔMossk1(irreversible), ΔMossk2(irreversible), ΔMopbs2(irreversible)* and *ΔMohog1(irreversible)* were grown upon osmotic stress followed by a HPAEC-PAD analysis of intracellular osmolyte content. Thereby, a significant difference was detected for osmolyte production between reversible and irreversible strains (Fig. 7).

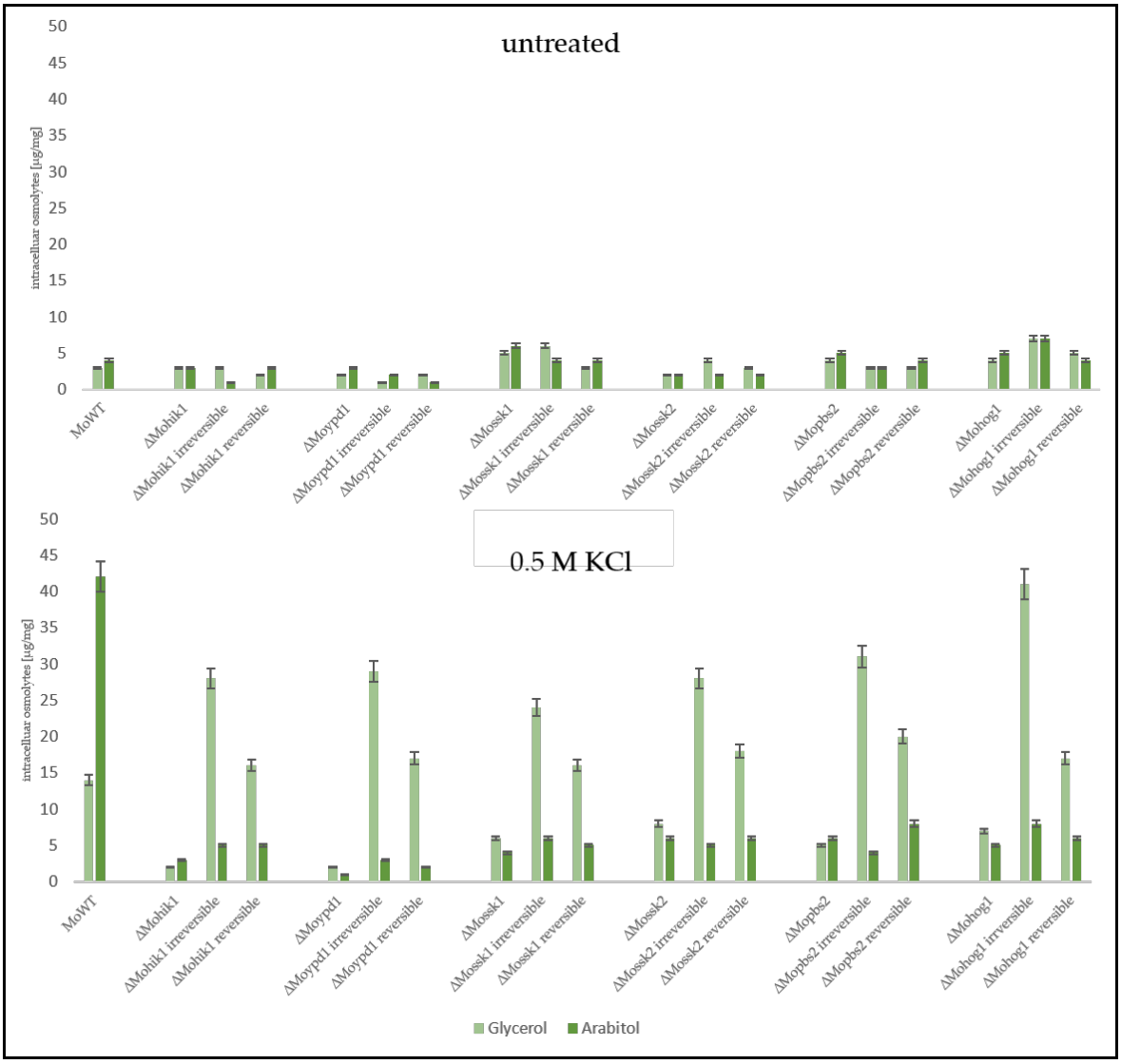

Fig 7: The production of compatible solutes as stress response in *Magnaporthe oryzae* wildtype strain (WT), the lof mutants of the HOG pathway, and the suppressor strains (irreversible and reversible). The fungal strains were grown for 72 h in CM (2% glucose) before being shocked with 0.5 M KCl. Carbohydrates were extracted after 7 h and quantified by HPAEC-PAD. Error bars represent the standard deviation of three biological replicates of each strain.

As expected, the wildtype strain produced intracellular arabitol as a major solute in order to compensate extracellular osmotic stress (0,5 M KCl) whereas it produced only small amounts of glycerol.

In contrast, the osmosensitive lof mutants *ΔMohog1, ΔMopbs2, ΔMossk2, ΔMossk1 and ΔMoypd1* were not able to produce either arabitol or glycerol in significant amounts after salt In contrast, both types of suppressor strains, reversible and irreversible, produced glycerol as main component in their biochemical stress response. Interestingly, the amount of glycerol was found to be strongly different between reversible and irreversible suppressors. Glycerol content in *ΔMohik1(irreversible),*

ΔMoypd1(irreversible), *ΔMossk1(irreversible)*, *ΔMossk2(irreversible)*, *ΔMopbs2(irreversible)* and *ΔMohog1(irreversible)* could be determined as almost twice as high as compared to *ΔMohik1(reversible)*, *ΔMoypd1(reversible)*, *ΔMossk1(reversible)*, *ΔMossk2(reversible)*, *ΔMopbs2(reversible)* and *ΔMohog1(reversible)* (Fig. 7).

Glycerol metabolism-associated genes are not the driving force for DEA evolution

Since the reversible and the irreversible suppressor mutants produce glycerol as a biochemical response in order to compensate high external osmolarity and furthermore, since we documented in a previous study that glycerin metabolism (gm)-associated genes have been upregulated in a suppressor strain *ΔMohog1*(suppressor) upon salt stress, we now checked the relationship of genes related to the production, metabolism or transport of glycerol to the DEA evolution in the lof mutants of the HOG pathway. To do that, we inctivated putative gm-related genes. In more detail, our a distinct set of gm-candidate genes were found to be upregulated in both, the salt stress samples of the suppressor strain *ΔMohog1*(suppressor) as well as in the wildtype strain, whereas exactly these genes were not significantly regulated in the lof mutant *ΔMohog1* (Bohnert et al., 2019). The most prominent candidates were the genes encoding the glycerol H+-symporter MoStl1p (MGG_09852), the phosphoglycerate mutase (MGG_06642; MpPga1p), the glycerol-3-phosphate dehydrogenase (MGG_00067; MoGpd1p) and the phosphatidyl synthase (MGG_00099; MoHad1p) (see supplementary Fig. S6-Fig.S9).

In order to evaluate, if these candidate genes have impact on the molecular mechanism of DEA evolution in the rice blast fungus and to point out, if there is a difference between the reversible and irreversible strains, we generated a set of so-called "double lof mutants". That means, we replaced the gm-associated candidate genes *MoSTL1*, *MoPGA1*, *MoGPD1* and *MoHAD1* in the genomes of the wildtype strain as well as in the genome of the lof mutant *ΔMohog1* with an antibiotik resistance marker gene (see supplementary Fig. S9-Fig.S13). The double mutants were named *ΔMohog1/Δstl1*, *ΔMohog1/Δpga1*, *ΔMohog1/Δgpd1*, and *ΔMohog1/Δhad1*. Then, we monitored, if these double-mutants were able to evolve into DEA evolution suppressor strains as it is for original*ΔMohog1*, checked their

osmosensitivity and analyzed their biochemical stress response by HPAEC-PAD. It could be documented, that molecular verified double mutants *ΔMohog1/Δstl1*, *ΔMohog1/Δgpd1*, *ΔMohog1/Δpga1* and *ΔMohog1/Δhad1* are still as osmosensitive as the lof mutant *ΔMohog1*. They did not produce either glycerol or arabitol in significant amounts as stress response (Fig.8).

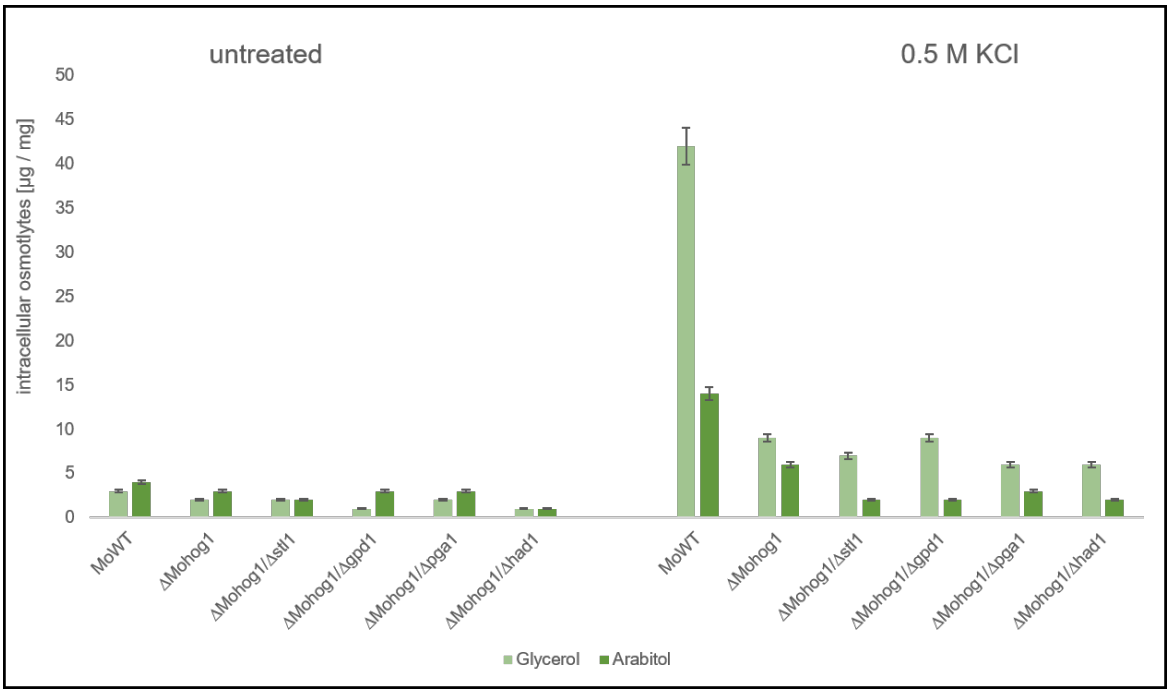

Fig 8: Production of compatible solutes as stress response in *Magnaporthe oryzae* wildtype strain (WT), the lof mutant *ΔMohog1* and the double mutant strains *ΔMohog1/Δstl1*, *ΔMohog1/Δpga1*, *ΔMohog1/Δgpd1*, and *ΔMohog1/Δhad1*. The fungal strains were grown for 72 h in CM before being shocked with 0.5 M KCl. Carbohydrates were extracted after 7 h and quantified by HPAEC-PAD. Error bars represent the standard deviation of three biological replicates of each strain.

Discussion/ Future research directions

One hallmark in biology is to understand the genetic and genomic processes behind evolutionary adaptation (Olson-Mannig et al., 2012; Alfoeldi et al., 2013). The knowledge about the evolutionary dynamics in adaptation processes is still limited in eukaryotic organisms. Particularly the rapid evolutionary adaptation of signaling pathways has not been subjected to intensive research so far. One central pillar on the road to unravel evolutionary dynamics is to better understand the molecular

mechanisms how signaling pathways can be modified respectively rewired in order to generate altered biochemical reactions or novel physiological properties (Pires-da Silva et al., 2003). How have signaling networks evolved and what are the molecular are existing signaling pathways and how evolve the pathways themselves? It is of great interest to better understand how evolutionary changes can promote or constrain signaling networks of pathogenic microorganisms enabling them to live beneficially within their host or their environment. Insights into the molecular basis of evolutionary dynamics can help to better understand the biology of pathogenic fungi and as a consequence will help to search for new disease control strategies. It is of high importance to understand, when and how cross-talk between signaling pathways evolve or how rewiring of signaling pathways take place in order to rearrange or modify them generating i.e. antibiotic resistance (multi-drug-resistance) (Pires-daSilva and Sommer, 2003 ; Naranjo-Ortiz and Gabaldon, 2020). The function of the HOG pathway in fungi is to regulate cellular homeostasis and therefore the adaptation to rapidly changing osmolarity in the environment (Hohmann, 2009). The lof mutants *ΔMohog1, ΔMopbs2, ΔMossk2, ΔMossk1 and ΔMoypd1* of the phytopathogenic fungus *Magnaporthe oryzae* are impaired in osmoregulation and resistant to the fungicide fludioxonil (Jacob et al., 2015). Previously, it was discovered that long-term cultivation of osmosensitive HOG pathway lof mutants upon high osmolarity for several weeks resulted in so-called suppressor strains (formerly known as "adapted" strains) being restored in osmoregulation outgrowing from each of the lof mutants *ΔMohog1, ΔMopbs2, ΔMossk2, ΔMossk1* and *ΔMoypd1* (Bohnert et al., 2019). However, this is completely different from processes already known as "experimental evolution". Experimental evolution studies of evolving microbial populations (not individuals) nowadays form the fundamentals of the theory of evolution (McDonald, 2019). The experiments begin with a culture, the microbial cells are cultivated in liquid medium and grown to a high population density. Then, parts of the culture are transferred to new medium or the culture gets diluted with fresh medium to allow continued growth. This process can be continued ever on, and new generations accumulate under natural selection (Lenski, 2017). The most popular and longest running experimental evolution experiment is the "long-term evolution experiment" (LTEE) (Lenski et al., 1991; Lenski, 2017). 12

replicate populations of *E. coli* have been cultivated continuously since 1987 and reaches meanwhile over 68,000 generations. All these evolution experiments use "populations" whereas in this report, we describe a rapid evolutionary suppressor phenomenon which occurs extremely reproducible in single individuals with unfunctional HOG pathway growing on solid medium upon high osmolarity. Interestingly, the major compatible solute produced by the *M. oryzae* suppressor strains to cope with osmotic stress was glycerol, whereas it is arabitol in the wildtype strain. By studying the rapidly evolved suppressor strains in more detail, it was found that the suppression is at least a two-step-process. In step one, suppressor strains with temporary (reversible) reestablished osmoregulation arise from the osmosensitive lof-mutants upon several weeks of cultivation on permanent osmotic stress. Further maintenance of these suppressor strains on high osmolarity led to a second dynamic step of rapid adaptation. The reversible suppressor strains then transform into irreversible strains able to cope with high osmolarity due to a permanently reestablished osmoregulation (Fig.1). It could be clearly demonstrated for the first time that the course of rapid evolution of the osmoregulation in *M. oryzae* is a highly reproducible process that takes place in several steps, making it very suitable for investigation of evolutionary dynamics. While Adaptive Laboratory Evolution (ALE) stands as a valuable tool in elucidating evolutionary dynamics, its application may not be suitable in the context described. The phenomenon under investigation involves the emergence of suppressor strains from osmosensitive mutants of the high osmolarity glycerol (HOG) pathway in Magnaporthe oryzae, with a notable shift in osmoregulatory responses favoring glycerol over arabitol production. In this scenario, the iterative process of ALE, which involves subjecting microbial populations to specific environmental stresses over multiple generations, may not offer substantial insight. The ability to replicate the observed phenomenon with different mutants and on various media suggests that the emergence of suppressor strains is reproducible under controlled laboratory conditions without the need for prolonged evolutionary experiments facilitated by ALE. Instead, the focus may shift towards targeted genetic and physiological analyses to unravel the underlying mechanisms driving the observed adaptations. In

order to address this phenomenon, biochemical analysis, genetic manipulation and proteome- as well as phospho-proteome-profiling was performed.

A reintegration of the original lof-genes in the genomes of the suppressor mutants resulted in complemented strains producing arabitol as the main compatible solute upon osmotic stress in contrast to glycerol in the suppressor strains. This leads to the assumption that a functional HOG pathway is preferred by the organism as the osmoregulatory system in contrast to the "new" evolved suppression mechanism. In the era of next generation sequencing, bioinformatics has become a major discipline in biology for analyzing evolutionary processes (Kanehisa, et al., 2019). In order to study the putative role in evolutionary dynamics of the genes which were found to be upregulated in NGS datasets of the reversible and irreversible suppressor strains (Bohnert et al. 2019), lof mutants of the genes encoding the glycerol H+-symporter MoStl1p (MGG_09852), the phosphoglycerate mutase (MGG_06642; MpPga1p), the glycerol-3-phosphate dehydrogenase (MGG_00067; MoGpd1p) and the phosphatidyl synthase (MGG_00099; MoHad1p) have been generated in the lof mutant *ΔMohog1* and characterized. We observed, that these candidate genes were not involved in the two-step-process of reversible and irreversible suppression, since the double mutant strains *ΔMohog1/Δstl1, ΔMohog1/Δgpd1, ΔMohog1/Δpga1* and *ΔMohog1/Δhad1* were still able to transform to suppressors upon permanent osmotic stress. De Vries et al. 2003 and Lowe et al. 2008 revealed that in order to generate glycerol the NADP+ dependent glycerol dehydrogenase is required and not the glycerol 3-phosphate dehydrogenase as believed so far. Even though the possible candidate has now been noticeable in the different analyses so far, the NADP+ dependent glycerol dehydrogenase should be the focus of further research in order to find the possible connection between the metabolic switch. The Mitochondrial glycerol-3-phosphate shuttle consists of two components: a cytoplasmic glycerol-3-phosphate dehydrogenase 1 (MoGdp1_MGG00067) and a mitochondrial glycerol-3-phosphate dehydrogenase 2 (MoGdp2, MGG_03147). The inactivation mutant of the cytoplasmic glycerol 3-phosphate dehydrogenase could not efficiently utilize glycerol as a carbon source, suggesting the involvement of *GPD2* in glycerol utilization in *M. oryzae* ((Shi *et al.*, 2018). In the end, we have achieved significant

progress in the characterization of the suppressor phenome in *M. oryzae*. We were able to clearly demonstrate for the first time that this rapid evolution is at least a two-step process and to show the differences between reversible and irreversible suppressor strains. This provides a strong basis for future studies to elucidate the exact molecular mechanism behind this dynamic evolution.

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Author contributions

KB,CG and SJ designed research, KB, CG and JK performed experiments, KB,CG and SJ analyzed the data, KB and SJ wrote the manuscript.

6. Results and Discussion

6.1. Navigating Stress Responses in *Magnaporthe oryzae* Through the High Osmolarity Glycerol Pathway

The fungal pathogen *Magnaporthe oryzae*, commonly known as the rice blast fungus, poses a significant threat to one of humanity's staple crops, rice, and extends its impact to various other cereal grains like wheat (Wang *et al.*, 2022). To understand the role of *M. oryzae* as a significant threat, it is necessary to investigate the mechanisms of resistance, its use as a model organism in laboratories, the study of signaling pathways, the analysis of glycerol production and its adaptability under different stress factors. With rice being a primary food source for over half of the world's population, any threat to its cultivation poses a severe risk to food stability (Meng *et al.*, 2019). The economic harm by crop damage, subsequent yield losses and growing resistance is increasing in cultivation areas (Baldrich and San Segundo, 2016). The battle against the pathogen *M. oryzae* underscores the permanent struggle and is only one example against evolving pathogens. Efforts to fight its devastating effects have led to the development of resistant rice varieties through traditional breeding methods and modern biotechnological approaches (Chandran *et al.*, 2019). However, the fungus has shown a remarkable ability to adapt by quickly evolving resistance mechanisms, so research must continue to find new targets for intervention. This fungal pathogen's ability to overcome plant defenses and adapt to varying environmental conditions underscores the urgent need for continued research into its biology and interaction with host plants (Devanna *et al.*, 2022). One key to its adaptability lies in the High Osmolarity Glycerol (HOG) pathway, which is crucial for managing osmotic stress.

The HOG pathway in *M. oryzae* represents an efficient signaling pathway through which the fungus manages to overcomes osmotic stress. This signaling pathway is not just crucial for survival in fluctuating external osmolarities but is also for the pathogen's virulence and adaptation to host environments (Thines, Weber and Talbot, 2000b; Foster *et al.*, 2017).
In addition to defense reactions, such as the hypersensitive reaction through the formation of H_2O_2 (ROS, reactive oxygen species; Wojtaszek, 1997), the pathogen has to deal above all with increasing concentrations of salts and other osmolytes, which arise through degradation of the plant tissue (Jacob *et al.*, 2015). The HOG pathway is divided into a phosphorelay system (MoSln1p, MoHik1p, MoYpd1p and MoSkk1p) and the downstream MAPK (MoSsk2p, MoPbs2p and MoHog1p) (de Nadal, Alepuz and Posas, 2002). Under normal conditions the

phosphorelay system is phosphorylated and inhibits the activation of the downstream MAPK cascade. Under salt or sugar stress, the phosphorelay system is dephosphorylated and leads to an activation of the MAPK cascade. Salt stress (NaCl) is detected by MoSln1p, and sorbitol stress by MoHik1p. The dephosphorylation of MoSsk1p activates the MAPK cascade, leading to the response reaction. An inactivation of the individual genes of the HOG pathway in *M. oryzae* led to mutants with significant defects in osmotic stress response (Jacob *et al.*, 2015). Once activated, MoHog1p translocates into the nucleus, where it initiates a transcriptional response to enhance the expression of genes crucial for coping with osmotic stress. Among these are genes involved in the biosynthesis and regulation of glycerol, the primary compatible solute that helps in balancing the internal osmotic pressure with the external environment. Enhanced glycerol synthesis via enzymes such as glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase ensures the fungus retains water, stabilizes its cellular structures, and maintains metabolic activities under stress conditions (Foster *et al.*, 2017).

M. oryzae's significance extends beyond agricultural fields, as it serves as a valuable model organism in laboratory settings. Its amenability to genetic manipulation, well-characterized pathogenicity, and conserved molecular pathways make it an invaluable tool for studying fundamental biological processes. Insights gained from studying the rice blast fungus have far-reaching implications, aiding research in plant-pathogen interactions, host defense mechanisms, and fungal signaling transduction. Moreover, the lessons learned from *M. oryzae* transcend disciplinary boundaries, offering valuable insights into broader areas of microbiology and molecular biology (Wilson and Talbot, 2009).

6.2 The Role of Directive Adaptive Laboratory Evolution in Unraveling Fungal HOG Pathway Adaptation to Environmental Stresses

Adaptive laboratory evolution (ALE) is an important tool for studying evolutionary dynamics and unravelling adaptive mechanisms in various biological systems, including fungi (Nam, Conrad and Lewis, 2011). While the application of ALE in fungal systems is less extensive studied compared to bacteria or yeast, it offers profound insights into fungal adaptation to diverse environmental stresses and selective pressures. Experimental protocols in ALE studies with fungi typically involve subjecting fungal populations to controlled environmental conditions mimicking specific ecological niches or host environments. Such conditions may encompass alterations in temperature, pH, nutrient availability, exposure to antifungal agents, or other relevant stressors pertinent to fungal ecology or pathogenicity (Li, Zhang and Dang, 2016). Central to ALE experimentation is the principle of serial passaging, whereby a fraction

of the fungal population is successively transferred to fresh growth medium across generations. This method makes it possible to observe phenotypic and genotypic changes over time, providing an insight into the evolutionary dynamics and adaptation mechanisms in organisms (Dragosits and Mattanovich, 2013). Phenotypic characterization throughout ALE experiments involves monitoring growth kinetics, morphological alterations, stress tolerance, virulence attributes, or other relevant phenotypic traits reflective of adaptive responses to selective conditions. A significant application of ALE in fungal research can be illustrated by the study on the HOG pathway mutants of *M. oryzae*. Long-term laboratory cultivation of these mutants under osmotic stress conditions, such as exposure to salt or sugar, led to the experimental evolution of osmoregulation, with the exception of repeating the adaptation process several time.

This study investigates the directive adaptive laboratory evolution (DALE) from osmosensitive "loss of function" (lof) mutants, which are unable to cope with osmotic stress to suppressor strains, which are again able to cope with osmotic stress comparable to the wildtype. One other main difference of the these suppressor strains compared to the wildtype and the “lof-mutants” is the compatible solute which is being produced upon osmotic stress. Whereas wildtype strains produced arabitol, the suppressor mutants produced glycerol as a primary metabolite under stress conditions (Bohnert *et al.*, 2019; Jacob and Bersching, 2021). The adaptation experiment has been conducted more than 100 times with an similar outcome. This phenomenon has so far only been possible with inactivation mutants related to the HOG pathway (Δ*Mohik1,* Δ*Moypd1,* Δ*Mossk1,* Δ*Mossk2,* Δ*Mopbs2* and Δ*Mohog1)* and no other osmosensitive mutant. The only exception is that the adaptation process has not been possible with the inactivation mutant of the two component hybrid histidine kinase MoSln1p (Δ*Mosln1).* This is the first finding of DALE in filamentous fungi, as the experiment was conducted several times with the same result, which is a change in the primary metabolite upon osmotic stress (Jacob and Bersching, 2021). The main reasons for a possible metabolic switch are nutrient availability, environmental stress, development stress and interactions with other organisms. While development stress and interaction with other organisms can be excluded as main drive for the organisms to adapt towards the permanent osmotic stress, nutrient availability and environmental stress are in the focus of the conducted DALE.

Metabolic switches, the ability of organisms to alter their metabolic pathways in response to environmental changes, nutrient availability, or developmental stages, have been observed across a wide range of species. These switches play crucial roles in adaptation and survival. In *Saccharomyces cerevisiae* one of the most well-known metabolic switches is the diauxic shift. Yeast cells initially metabolize glucose through glycolysis and fermentation, producing ethanol. When glucose becomes scarce, they switch to utilizing ethanol through the tricarboxylic acid (TCA) cycle and oxidative phosphorylation, a more efficient energy

production process (Cha *et al.*, 2021). Another example is a similar adaptability in *Escherichia coli.* They can switch between different carbon sources based on availability. For instance, in the presence of both glucose and lactose, *E. coli* will preferentially consume glucose first. Once glucose is depleted, the bacteria activate the lac operon to metabolize lactose, showcasing a regulatory mechanism for optimal energy utilization (Inada, Kimata and Aiba, 1996).

Two other examples are within the human body and are related to cancer and muscle cells during intense exercise:

(I) Cancer cells exhibit a notable metabolic switch known as the Warburg effect, where they prefer aerobic glycolysis over oxidative phosphorylation, even in the presence of sufficient oxygen. This switch allows cancer cells to rapidly produce energy and support the synthesis of biomolecules necessary for quick cell division and growth (Hsu and Sabatini, 2008).

(II) In muscle cells, a metabolic switch occurs during intense exercise. These cells shift from aerobic respiration to anaerobic glycolysis to quickly meet the increased energy demands, resulting in the production of lactate. This switch is vital for sustaining high levels of activity (Gladden, 2004). Although a metabolic switch has already been demonstrated in several organisms, this type of switch from arabitol production to glycerol production in *M. oryzae* is the first observation of its kind. Additionally, it is important to emphasize that this DALE citation can be repeated only within the HOG pathway.

Next to the change of the primary metabolites from arabtitol to glycerol upon osmotic stress, the reversible as well as the irreversible mutants have a more reduced virulence compared to the parental lof HOG pathway mutants (Δ*Mohik1,* Δ*Moypd1,* Δ*Mossk1,* Δ*Mossk2,* Δ*Mopbs2* and Δ*Mohog1).* This observation was made with all related reversible and irreversible mutants. Compared to the wildtype, it can be concluded that the irreversible mutants as well as the reversible mutans are not only strongly impaired on virulence but have additionally also a reduced fludioxonil sensitivity. Not only the adapted mutants but also the lof-HOG mutans are reduced in virulence. This gives the information, that the organism are not able to focus/produce as much glycerol as needed for the turgor in the appressorium, which accumulates very high glycerol concentrations within the cell, of 8.0 MPa to infect the plants (Foster *et al.*, 2017) and is independent on the production of glycerol or arabitol upon osmostic stress. The optimization of pathway usage in ALE is often caused by adaptations on processes which enhance fitness for one specific environment (Nam, Conrad and Lewis, 2011), as is the case with permanent osmotic stress for *M. oryzae* and not for the turgor in appressoria for the penetration peg.

After the analysis of the suppressor strains, the next research question was: What will happen if the HOG pathway is functional and restored again in the suppressor mutants of *M. oryzae*?

The genetic reconstruction of the HOG pathway was conducted with the reintegration of the original lof-genes into the genome of the respective reversible and irreversible suppressor strains. The complemented suppressor strains Δ*Mohik1(reversible)/HIK1,* Δ*Moypd1(reversible)/YPD1,* Δ*Mossk1(reversible)/SSK1,* Δ*Mossk2(reversible)/SSK2,* Δ*Mopbs2(reversible)/PBS2* and Δ*Mohog1(reversible)/HOG1* as well as Δ*Mohik1(irreversible)/HIK1,* Δ*Moypd1(irreversible)/YPD1,* Δ*Mossk1(irreversible)/SSK1,* Δ*Mossk2(irreversible)/SSK2,* Δ*Mopbs2(irreversible)/PBS2* and Δ*Mohog1(irreversible)/*HOG1 demonstrated that the compatible solute being produced upon salt stress was arabitol, like the wildtype of *M. oryzae*, again and not glycerol anymore. As well as the virulence was reconstituted after the integration of the original lof-genes, the fludioxonil sensitivity was comparable to the wildtype again. The metabolic changes during the adaption process of the lof HOG mutants with the adapted suppressor strains (reversible as well as irreversible), which improved fitness under toxic conditions (permanent high osmolarity) is the way of the fungus to protect the integrity of the genome, macromolecules, the membrane and in order to survive (Nam, Conrad and Lewis, 2011). The subsequent activation of the HOG pathway in the suppressor mutants leads to an usage of the HOG pathway again. This might be also the most energy efficient pathway in order to survive high osmolarities compared the pathway which was used on order to survive for the suppressor mutants (DALE) of *M. oryzae*.

The findings from DALE studies have profound implications for fungal biology. They not only enhance our understanding of fungal pathogenicity and resistance mechanisms but also assist in engineering fungal strains for biotechnological applications. DALE enables the development of novel antifungal strategies and optimization of industrial fungal strains. The reduced fludioxonil sensitivity of the reversible and irreversible suppressor mutants is an example of the possible usage of DALE for the optimization of antifungal strategies. Furthermore, it advances our knowledge in fungal ecology and evolution, providing essential insights into the fitness landscapes and evolutionary trajectories of fungal populations under various ecological pressures or pathogenic conditions (Li, Zhang, and Dang, 2016; Roper et al., 2011).

6.3. Glycerol

The function of glycerol is similar in both eukaryotes and prokaryotes, but the specific metabolic pathways and regulatory mechanism can vary between them. Glycerol is utilized as a sole carbon and energy source for both bacteria and fungi, indicating its significance as a versatile substrate (Xiao *et al.*, 2022). Similarities in the function are the energy production, the membrane structure and the osmotic regulation (Lamitina *et al.*, 2004). Research into the metabolic routes of glycerol breakdown has been primarily carried out using mutant strains of

the model organism *S. cerevisiae*. However such studies are less common in other yeast species and filamentous fungi (Klein *et al.*, 2017). Glycerol serves as a key osmoprotectant in microbial cells, including bacteria (e.g. *E. coli*) and yeast (e.g. *S. cerevisiae*), enabling them to maintain cellular homeostasis and viability under osmotically challenging conditions (Tilloy, Ortiz-Julien and Dequin, 2014; Klein *et al.*, 2017). While *E. coli* and yeast are phylogenetically distinct microorganisms with divergent cellular structures and metabolic pathways, they share common principles in their adaptive response to osmotic stress, particularly in glycerol production and its regulation (Hohmann, 2015).
Fungi, including pathogenic species, frequently encounter various environmental stresses, such as osmotic stress, during their lifecycle.

In *M. oryzae*, glycerol plays diverse roles, especially during infection and host tissue colonization. Osmotic stress presents a significant challenge to *M. oryzae* during spore germination, hyphal growth, and host tissue penetration. The accumulation of glycerol helps the fungus maintain cellular integrity and survive in osmotically stressful environments encountered during pathogenesis. Additionally, glycerol is crucial for regulating turgor pressure, which is essential for the penetration peg to exert the mechanical force necessary to breach the host cuticle during infection. Glycerol also serves as a vital carbon and energy source, supporting the metabolic processes and growth of *M. oryzae* throughout its lifecycle. To investigate the importance of glycerol in the adapted suppressor mutants of *M. oryzae (∆Mohik1(reversible)/(irreversible), ∆Moypd1(reversible)/(irreversible), ∆Mossk1(reversible)/(irreversible), ∆Mossk2(reversible)/(irreversible), ∆Mopbs2(reversible)/(irreversible)* and *∆Mohog1(reversible)/(irreversible))*, candidate genes encoding enzymes involved in glycerol metabolism and transport were identified. Proteins of *M. oryzae suppressor mutants* with high expression levels in RNA analyses were selected to elucidate their potential roles in the metabolic response to stress in suppressor mutants. This research aims to enhance our understanding of the mechanisms by which *M. oryzae* adapts to osmotic stress and to identify targets for controlling fungal pathogenicity. The most promising candidate genes were Mogpd1p (MGG_00067) and Mostl1p.

6.3.1 Targeting the Glycerol Biosynthesis Glycerol-3-Phosphate for Glycerol production upon osmotic stress

Glycerol is apart from arabitol a key metabolite synthesized by *M. oryzae* in response to osmotic stress, serves as a critical component of its adaptive arsenal. By accumulating glycerol, the fungus maintains cellular turgor pressure, counteracting the deleterious effects of hyperosmotic conditions and ensuring cellular homeostasis. The enzymatic machinery

involved in glycerol biosynthesis represents an attractive target for antifungal agents, offering a promising avenue for developing novel fungicides with enhanced efficacy. Furthermore, deciphering the regulatory mechanisms governing glycerol production sheds light on the broader metabolic adaptations employed by fungi to thrive in challenging environments.

The production of glycerol in *M. oryzae* involves the enzymatic conversion of glycerol-3-phosphate (G3P) from glycolysis to glycerol through the action of glycerol-3-phosphatase. Glycerol-3-phosphate dehydrogenase, encoded by GPD1 (MGG_00067), catalyzes the reduction of dihydroxyacetone phosphate (DHAP) to G3P, a crucial intermediate in glycerol synthesis. Glycerol metabolism can have a significant importance in fungal physiology, particularly in the context of adaptation to environmental stresses, including osmotic stress, and the regulation of pathogenicity. Fungi, including pathogenic species, encounter various environmental challenges during their lifecycle, necessitating mechanisms for osmoregulation and stress adaptation. Glycerol serves as a critical osmoprotectant in fungi and bacteria, facilitating the maintenance of cellular homeostasis, turgor pressure, and viability under adverse conditions (Sangappillai and Nadarajah, 2020).
Glycerol is synthesized through a pathway involving several enzymatic reactions that convert precursor molecules into glycerol-3-phosphate (G3P), which is then dephosphorylated to form glycerol (Cronwright, Rohwer and Prior, 2002), can be generated through lipids or from dihydroxy-acetone-phosphate which concerts to dihydrocy-acetone which is then catalyzed to glycerol.

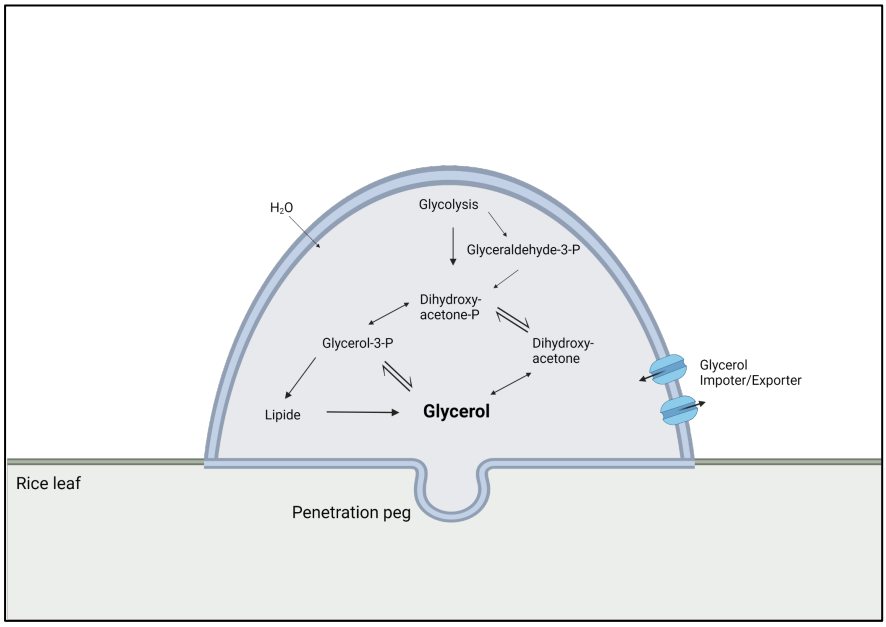

Figure 3: Possible metabolic pathways for glycerol production and the relevant enzymes thought to exist in Magnaporthe oryzae *appressoria. The appressorium builds pressure to break through the rice cuticle with a penetration peg, as illustrated. Lipids serve as one source of glycerol, released by abundant triacylglycerol lipase activities within the appressoria. In addition, two distinct glycolytic pathways for glycerol synthesis are present in* M oryzae*, shown here. A mitochondrial enzyme, glycerol-3-phosphate dehydrogenase, likely exists as well, although not depicted, facilitating redox balance by oxidizing glycerol-3-phosphate into dihydroxyacetone phosphate.(Based on (Foster* et al.*, 2017)). Created with BioRender.com.*

This pathway plays a crucial role in fungal metabolism, enabling fungi to adapt to changing environmental conditions and thrive in diverse ecological niches. Glycerol serves as a critical metabolite involved in various cellular processes, including energy metabolism, osmoregulation, and stress response (Foster *et al.*, 2017). GPD1 and GPD2 are key enzymes in the glycerol biosynthesis pathway, catalyzing the conversion of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G3P) in a NADH-dependent manner (Cronwright, Rohwer and Prior, 2002). This enzymatic reaction is a crucial step in glycerol production, as it provides the organism with a way to alleviate osmotic stress, regulate cellular redox balance, and serve as a carbon and energy source under certain conditions.
In yeast, for example, two isoforms of NAD+-dependent glycerol-3-phosphate dehydrogenase exist, GPD1p and GPD2p (Hubmann, Guillouet and Nevoigt, 2011). These are localized in the cytoplasm and mitochondria, respectively, and perform different functions. For instance, only GPD1p is involved in polyol biosynthesis, catalyzing the rate-limiting step and its encoding gene is activated under hyperosmotic stress conditions. Additionally, this isoform seems to be responsible for a component of the glycerol-3-phosphate ($NADH/FADH_2$) shuttle between the cytosol and mitochondria. GPD2, on the other hand, cannot substitute for this function in deletion mutants of GPD1 due to their different cellular localization; instead, it is presumed to function under anaerobic conditions, as the gene GPD2 is activated in such conditions (Hubmann, Guillouet and Nevoigt, 2011).

While MoGpd1p was one of the first genes which were addressed within this work in order to understand the adaptation mechanism of the suppressor mutants, but it was found that the gene was not responsible for the mechanisms. An inactivation of the gene revealed that the production of primary metabolites upon osmotic stress (e.g. KCL) was mainly arabitol and not glycerol in the inactivation mutant Δ*Mogpd1*. Additionally, the mutant with an inactivation of the HOG pathway and Mogpd1p, Δ*Mohog1/*Δ*Mogpd1* was able to adapt under permanent osmotic stress of 6-8 weeks. The DALE was possible within the mutant Δ*Mohog1/*Δ*Mogpd1*. In order to fully understand the glycerol production and usage of glycerol further research must be conducted and focus on the glycerol pathways. In the absence of glycerol-3-phosphate dehydrogenase activity, glycerol uptake followed by its phosphorylation via glycerol kinase might serves as an alternative pathway for glycerol-3-phosphate production, maintaining cellular functions dependent on this metabolite.
Future research should explore the pathway for glycerol-3-phosphate formation in absence of glycerol-3-phosphate dehydrogenase activity. It can be hypothesized within this work that glycerol-3-phosphate can be synthesized through an alternative route involving the uptake of glycerol, which is subsequently converted to glycerol-3-phosphate by glycerol kinase.

Investigating this pathway could provide new insights into metabolic flexibility and regulatory mechanisms in fungi under varying environmental conditions.

6.3.2 Glycerol/H+ symporter in fungi

A response of microorganisms to increased osmolarity in the environment is the production or uptake of compatible solutes, such as glycerol, which accumulate in the cytosol in order to compensate the osmotic imbalance (Brown, 1978). Glycerol and arabitol are the most important compatible solutes related to osmotic stress response in fungi. The solutes serve to protect proteins from high ionic strength and thus preserve the hydration shell and native confirmations (Kempf and Bremer, 1998). One example for glycerol uptake is the transporter STL1. The transporter STL1, also known as "*Saccharomyces cerevisiae* Transporter Like 1", was first described to be a sugar transporter protein found in the yeast *S. cerevisiae* (Zhao *et al.*, 1994) and due to further research and knowledge is later to be changed to a glycerol/H^+ symporter (Ferreira *et al.*, 2005). It is also known as the uniporter-antiporter family, as they are secondary transporters that transport small molecules e.g. polyols or ions across the membranes using concentration gradients (Ferreira *et al.*, 2005). It belongs to the glycerol facilitator (Fps1) family of aquaglyceroporins. STL1 plays a crucial role in the uptake of sugars, particularly glucose and fructose, into yeast cells. The expression of STL1 is regulated by various environmental cues and nutritional signals (Ferreira *et al.*, 2005). It is induced under conditions of glucose limitation, osmotic stress, and during the diauxic shift, a metabolic transition that occurs when glucose is depleted from the growth medium, and yeast cells switch to utilizing alternative carbon sources such as ethanol. STL1 facilitates the transport of sugars across the plasma membrane, allowing yeast cells to acquire essential nutrients for energy production and metabolic processes. Its activity is essential for yeast growth, metabolism, and survival under changing environmental conditions (de Nadal, Alepuz and Posas, 2002) . Sugar transporters are intimately linked to fungal virulence and pathogenicity, particularly in plant-pathogenic fungi like *M. oryzae*. Sugar transporters contribute to fungal pathogenesis by facilitating nutrient uptake and metabolic adaptation, thereby enhancing fungal virulence and ensuring the survival and propagation of the pathogen within the host environment.

The glycerol/H^+ symporter in fungi, notably *M. oryzae*, serves as an important component in the uptake of sugars, such as glycerol, from the environment, facilitating essential metabolic processes, energy production, and growth. *M. oryzae* heavily relies on glucose as a primary carbon source to fuel energy production and biomass synthesis, for growth and pathogenicity. Additionally glycerol is known to be synthesized for membrane lipids (Klein *et al.*, 2017), plays a role in the redox balance between cytosol and mitochondria (So *et al.*, 2019)(Valadi *et al.*, 2004), in intracellular pH regulation, in heatshock and in protection against oxidative stress

(Klein *et al.*, 2017). STL1, the sugar transporter found in *S. cerevisiae*, is not directly involved in metabolite production of arabitol or glycerol in the fungus *M. oryzae*. In *M. oryzae*, the HOG pathway plays a critical role in the response to osmotic stress and regulates the production of glycerol, which serves as a compatible solute to maintain cellular osmotic balance. Conditions such as glucose limitation, osmotic stress, and host colonization induce the expression of sugar/glycerol transporters, enabling fungi to adapt their metabolic pathways to fluctuating environmental conditions. Such regulatory mechanisms ensure the efficient utilization of available resources and enhance fungal fitness in dynamic ecological niches (Busti *et al.*, 2010). The questions towards this research topic is: Is the sugar transporter MoStl1p the key for the DALE process of *M. oryzae* under permanent osmotic stress and the metabolic switch in the suppressor mutants Δ*Mohik1(reversible)/(irreversible),* Δ*Moypd1(reversible)/(irreversible),* Δ*Mossk1(reversible)/(irreversible),* Δ*Mossk2(reversible)/(irreversible),* Δ*Mopbs2(reversible)/(irreversible)* a*nd* Δ*Mohog1(reversible)/(irreversible)*?

Within this work, the direct connection between the process of DALE and Mostl1p can be ruled out. It was possible to show that inactivation of the protein does not lead to increased glycerol production (no noticeable change in the production of primary metabolites upon stress compared to the WT). In addition, the mutant Δ*Mostl1* is osmosensitive but was not able to adapt and therefore no targeted DALE could be performed, which is so far only possible with inactivation mutants related directly to the HOG pathway (Phosphorelay system or MAPK-cascade). While there may be other sugar transporters or glycerol symporters in *M. oryzae* involved in the uptake of sugars and glycerol from the environment, specific transporters analogous to STL1 in *S. cerevisiae* have not been characterized so far. Therefore, the direct involvement of STL1-like transporters in sugar uptake and subsequent metabolite production, such as arabitol or glycerol, in *M. oryzae* remains unclear and would require further investigation.

While in *S. cerevisiae* the Scstl1p is responsible for the update of glycerol and an inactivation of the symporter leads to no growth on media with glycerol as the only C-source, the glycerol/H^+ Symporter is not alone responsible for the glycerol update in *M. oryzae*. It seems, that the function of STL1p in yeast is at least divided into more than one protein in the filamentous fungi. For further analysis of the glycerol uptake the proteins Mofps1p and Mofps2p, which are coding for aquaglyceroporins should be further investigated. Mofps1p and Mofps2p are crucial proteins in fungi and plants that regulate glycerol transport, osmoregulation, and isoprenoid biosynthesis, highlighting their importance in various cellular processes and fungal as well as plant physiology. FPS1 encodes a glycerol channel protein that mediates the uptake of substances like arsenite and antimonite in *S. cerevisiae*. The FPS1

gene is part of the major intrinsic protein (MIP) family and functions as the major facilitator of glycerol transport in response to changes in extracellular osmolarity (Beese, Negishi and Levin, 2009). FPS1 is an aquaglyceroporin that facilitates the passive transport of glycerol out of the cell (Beese-Sims, Lee and Levin, 2011). Additionally, FPS1 is regulated by the mitogen-activated protein kinase Slt2, which modulates arsenite transport through the aquaglyceroporin Fps1 (Ahmadpour *et al.*, 2016).

In *S. cerevisiae* an mutated ScFps1 and therefore constitutively open protein fail to accumulate glycerol and are not able to grow under high osmolarity (Hohmann, 2002). Additionally, the phosphorylation of ScRgc2, which is a identified regulator of FScps1 channel activity during stress, is partly regulated by ScHog1, influencing the efflux of glycerol (de Nadal and Posas, 2022; Muir *et al.*, 2015). ScFsp1p closes upon hyperosmotic stress (also in ∆*hog1* cells) and ScFsp1p was identified to be in in relation to the TOR pathway which might be involved in the cellular response upon osmotic shock (Muir *et al.*, 2015).

6.4. Pathways to Resilience: *Magnaporthe oryzae*'s Adaptive Strategies

In fungi, the crosstalk between signaling pathways represents a sophisticated network of interactions that enables these organisms to adapt and respond effectively to their ever-changing environments. This complex communication system among different cellular pathways allows fungi to integrate various external and internal signals, ultimately orchestrating a coordinated response that optimizes their growth, development, and survival (Fuchs and Mylonakis, 2009). Signaling crosstalk involves multiple layers of regulation, from transcriptional control to post-translational modifications, ensuring that fungi can adjust their metabolic processes, stress responses, and reproductive cycles according to the specific demands of their surroundings (Wang *et al.*, 2022).

Crosstalk between the HOG pathway and other MAPK pathways, such as the pheromone response pathway, the invasive growth (IG) and the cell wall integrity pathway (CWI), has been observed in filamentous fungi (Zhang *et al.*, 2021). These interactions allow for coordinated responses to different types of stress and environmental cues.

Furthermore, the HOG pathway in *M. oryzae* intricately interacts with other signaling pathways, modulating its pathogenicity traits. It regulates not only stress responses but also influences developmental processes critical for effective colonization and infection of the host tissue. The crosstalk of the HOG pathway with other pathways could be a lead for the understanding of the adaptation process of the suppressor mutants ∆*Mohik1(reversible)/(irreversible),* ∆*Moypd1(reversible)/(irreversible),* ∆*Mossk1(reversible)/(irreversible),* ∆*Mossk2(reversible)/(irreversible),* ∆*Mopbs2(reversible)/(irreversible)* and ∆*Moho*

g1(reversible)/(irreversible). While the inactivation of the genes, which were predicted to be involved in the adapation process were not the key to solve the puzzle, the crosstalk between signaling pathways should be additionally considered.

In organisms such as *M. oryzae*, which predominantly rely on HOG pathway to cope with osmotic stress, the utilization of alternative pathways offers manifold advantages, even in instances where the HOG pathway is reinstated (Jacob, Bühring and Bersching, 2022). In dynamic environmental contexts, the presence of multiple pathways ensures the organism's adeptness in adapting to diverse stressors, even when one pathway is compromised or overwhelmed (Fuchs and Mylonakis, 2009). Alternative pathways afford varying degrees of efficiency and adaptability under specific stress conditions. Depending on stress intensity and nature, certain pathways may facilitate rapid adaptation or ensure long-term survival, enabling the organism to optimize its response according to prevailing environmental cues. Diversifying stress response mechanisms allows for efficient resource allocation (Zhang *et al.*, 2021). Different pathways may necessitate distinct sets of resources or regulatory factors, and strategic utilization of alternative pathways minimizes resource competition, enhancing overall fitness in challenging environments. Furthermore, the maintenance of functional redundancy and flexibility in stress response pathways confers evolutionary advantages. Populations equipped with diverse stress response mechanisms exhibit heightened adaptability and evolution in response to changing environmental conditions, enabling them to thrive across diverse ecological niches over evolutionary timescales (Nikolaou *et al.*, 2009). Additionally, in the realm of pathogenic fungi like *M. oryzae*, employing alternative pathways may facilitate evasion of host defense mechanisms and adaptation to specific host environments (Feng *et al.*, 2021). Hosts may deploy strategies to disrupt or counteract particular stress response pathways, necessitating the activation of alternative pathways for successful colonization and infection.

6.4.1 TOR pathway as an alternative to overcome osmotic stress?

Crosstalk between the HOG pathway and nutrient-sensing pathways, such as the Target of Rapamycin (TOR) pathway, has been observed in fungi (So *et al.*, 2019; Muir *et al.*, 2015). These interactions help coordinate cellular responses to changes in nutrient availability and metabolic status, ensuring proper growth and adaptation under varying conditions.

The TOR (Target of Rapamycin) pathway is a highly conserved signaling pathway found in eukaryotic organisms, playing a central role in the regulation of cellular growth, proliferation, metabolism, and response to environmental cues (So *et al.*, 2019). The TOR pathway is initiated by various extracellular and intracellular signals, including growth factors, nutrients

(such as amino acids and glucose), energy status (ATP levels), oxygen levels, and stress conditions (Betz and Hall, 2013). These signals are sensed by upstream regulators of the TOR pathway, such as cell surface receptors, intracellular sensors, and signaling molecules. In response to favorable conditions, TOR forms two distinct protein complexes known as TORC1 and TORC2 (So *et al.*, 2019).

TORC1 is primarily sensitive to nutrients, especially amino acids, and regulates processes such as protein synthesis, ribosome biogenesis, and autophagy. TORC2, on the other hand, responds to growth factors and regulates cytoskeletal organization, cell survival, and metabolism. Once activated, TOR complexes phosphorylate and regulate a wide array of downstream effectors, including kinases, transcription factors, and regulatory proteins. These effectors mediate the cellular responses orchestrated by the TOR pathway, influencing processes such as mRNA translation, ribosomal protein synthesis, lipid metabolism, cell cycle progression, and autophagy (Hu *et al.*, 2015). The TOR pathway serves as a central hub for integrating signals from various sources, allowing cells to coordinate their growth and metabolic activities in response to changing environmental conditions. By modulating the activity of key regulators and effectors, the TOR pathway helps cells adapt to nutrient availability, energy status, stress, and other stimuli, thereby promoting cell survival and homeostasis. Dysregulation of the TOR pathway has been implicated in a wide range of human diseases, including cancer, metabolic disorders, neurodegenerative diseases, and aging-related conditions (Mentlak *et al.*, 2012). Excessive TOR activity can lead to uncontrolled cell growth and proliferation, while insufficient TOR activity can impair cellular functions and contribute to cellular senescence and degenerative processes (Wullschleger, Loewith and Hall, 2006).

In *S. cereviaise* hypertonic conditions activate the HOG pathway and intracellular glycerol accumulates to overcome the osmotic imbalance. To prevent glycerol efflux, the aquaglyceroporine Fsp1p closes. As this closure of Fsp1p has additionally been observed in a inactivated Hog1p mutants in *S. cerevisiae*, the crosstalk between the HOG pathway and the TOR pathway has been made in in *S. cerevisiae* (Muir *et al.*, 2015). In an unstressed environment the TORC2 signaling pathway phosphorylates Fsp1p, which leads to an opening of the channel which results in an glycerol efflux (Figure 4).

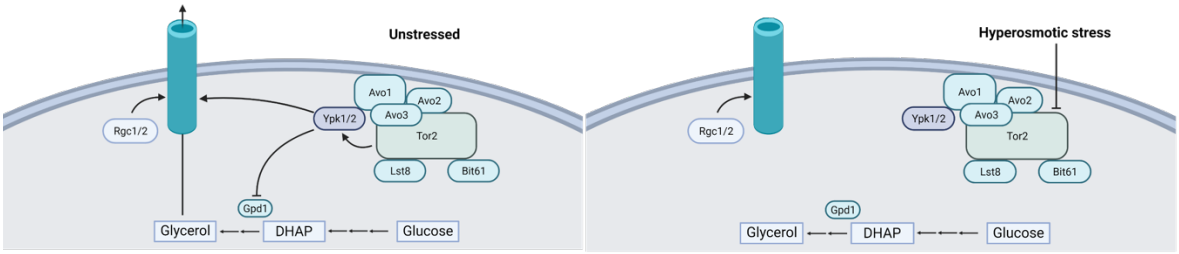

Figure 4: In unstressesd conditions within Saccharomyces cerevisiae *(left), active TORC2-Ypk1 keeps intracellular glycerol levels low by suppressing Gpd1 and holding the Fps1 channel open through Ypk1 phosphorylation. When exposed to hyperosmotic stress (right), TORC2-driven Ypk1 phosphorylation is quickly diminished. This reduction releases Gpd1 from inhibition, boosting glycerol production. Simultaneously, the loss of Ypk1 phosphorylation closes the Fps1 channel, despite the presence of Rgc1 and Rgc2, thereby encouraging glycerol accumulation to counter the high external osmolarity. (Based on (Muir* et al.*, 2015)). Created with BioRender.com.*

An osmotic environment leas to an inhibition of the phosphorylation of Fsp1p. The aquaglyceroporine Fsp1p is closed and the intracellular glycerol accumulation increases (Muir *et al.*, 2015). The organism is able to compensate the osmotic imbalance and is able to survive. Compared to the yeast fungi *S. cerevisiae* the function of the TOR signaling is not well understood phytopathogens like *M. oryzae.* In *M. oryzae* only a single TOR homolog to *S. cerevisiae* was identified (Shertz *et al.*, 2010).The TOR pathway is known to be involved in the inhibition of appressoria formation (Marroquin-Guzman and Wilson, 2015) and the inhibition of the cAMP/PKA signaling (Marroquin-Guzman and Wilson, 2015) as well as an evidence for root colonization has been presented (Franceschetti *et al.*, 2011). In fungal pathogens *Candida albicans* and *Cryptococcus neoformans* is the TOR pathway important in virulence and as a possible antifungal drug target (So *et al.*, 2019). While the multifaceted functions of the TOR pathway seem to be evolutionarily preserved across fungi, the regulatory mechanisms of this pathway and its interactions with other signaling pathways exhibit considerable variation among different fungal species (So *et al.*, 2019). For these reasons, the TOR signaling pathway should be explored in more detail regarding the adaptation process. It cannot yet be ruled out that the TORC signaling pathway, as in the case of *S. cerevisiae*, might also be responsible for the accumulation of glycerol within the cell.

6.4.2 cAMP pathway

The cyclic adenosine 3’5’ monophosphatase (cAMP) pathway in fungi is a crucial signaling mechanism that mediates a variety of physiological processes, including growth, development, and response to environmental stimuli (Sun *et al.*, 2022). cAMP is being regulates by two enzymes, adenylate cyclase (AC) and phosphodiesterade (PDE). The central enzyme is the adenylyl cyclase, which catalizes the conversion of ATP to cyclic AMP (cAMP). The activity of

adenylyl cyclase can be stimulated or inhibited by various external stimuli, such as nutrients, pheromones, and stress signals In the cAMP signaling pathway, the transduction of extracellular signals involves several steps: extracellular cues interact with G-protein coupled receptors (GPCRs) on the cell surface, which then activate heterotrimeric G-proteins. These G-proteins, in turn, activate adenylyl cyclase (AC), catalyzing the production of cyclic AMP (cAMP) (Huang *et al.*, 2019). The accumulation of cAMP activates protein kinase A (PKA), which then phosphorylates various downstream proteins, including transcription factors. This phosphorylation cascade ultimately influences a range of biological processes by altering gene expression activities within the cell.

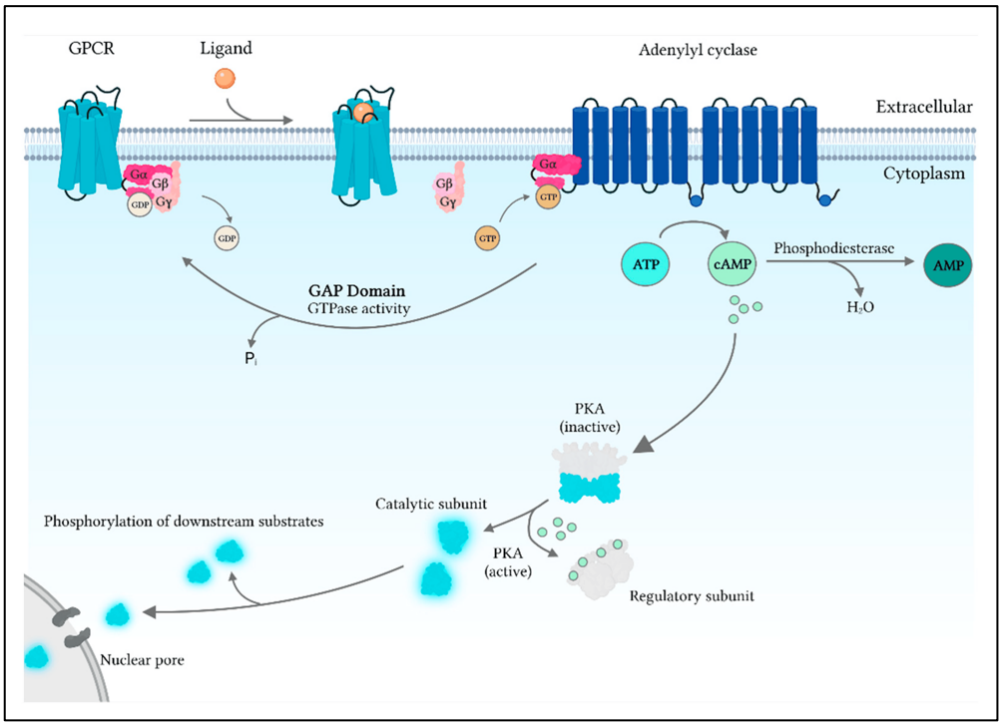

Figure 5: This pathway diagram illustrates the cAMP and PKA signaling process in Saccharomyces cerevisiae*. Upon ligand binding to the G-protein-coupled receptor (GPCR), adenylyl cyclase becomes active. This activation engages GTPase-activating proteins (GAPs) that facilitate GTP hydrolysis, resulting in the GDP-bound inactive form and the release of inorganic phosphate (Pi). Additionally, adenylyl cyclase converts ATP into cAMP. When cAMP attaches to the regulatory subunits of inactive PKA, the catalytic subunits are freed, enabling phosphorylation of cytosolic targets or entry into the nucleus to act on other substrates. (Jacob, Bühring and Bersching, 2022).*

How can the cAMP signaling pathway can be involved in the metabolic switch or the production of glycerol? The cAMP pathway is crucial for the metabolic adaptation of fungi to different environmental nutrients and conditions (Sun *et al.*, 2022). This pathway can influence the switch between different metabolic states, such as from fermentative to respiratory metabolism depending on nutrient availability. For example, in *S. cerevisiae* the availability of glucose triggers a signaling cascade through the cAMP pathway that promotes fermentative growth over respiratory growth when glucose is abundant (van den Brink *et al.*, 2008). This switch is mediated by the effect of cAMP on protein kinase A (PKA), which then alters the activity of key metabolic enzymes and regulatory proteins, thus reprogramming cellular metabolism.

The cAMP pathway could also influence the production of glycerol, particularly under conditions where intracellular water balance needs to be maintained, such as during osmotic stress. For instance, in the *S. cerevisiae*, the PKA activity is a determent of osmotic shock tolerance (Norbeck and Blomberg, 2000). In fungi, when the cAMP pathway is activated, it is possible to lead to an activation of enzymes involved in glycerol synthesis. In *C. neoformans* cells with mutations in components of the cAMP signaling pathway, including the GPA1 (Gα subunit), CAC1 (adenylyl cyclase), and PKA1 (protein kinase A), can have their normal function restored by a deletion of the hog1 gene. This observation suggests that the HOG pathway suppresses the cAMP signaling pathway (Bahn, 2008) and can be involved in the glycerol production as well and the behavior of the suppressor (irreversible and reversible) mutants.

6.5. Limitations of Phospho and Proteom Analysis

Phosphoproteomics and proteomics are essential for unraveling the complexities of cellular signaling pathways in fungi. Despite their importance, phosphoproteomics and proteomics face several significant challenges that complicate their application. The levels of protein within the cells can vary widely (González-Fernández, Prats and Jorrín-Novo, 2010). While some proteins are easily detectably, others are rare and found only in very small quantities. This range makes detecting and quantifying all proteins within a sample challenging. Additionally, proteins undergo numerous post-translational modifications, which can alter their function, stability, and location (Cohen and Walt, 2019). Phosphorylation, one such modification, is particularly prevalent in signaling pathways and requires precise methods to detect and analyze. However, the utility is limited by constraints related to dynamic range, post-translational modifications, temporal and spatial resolution, data analysis, sample preparation, quantification accuracy and incomplete coverage. Recognizing and overcoming these challenges are essential for using phospho- and proteomic analysis to elucidate the complexity of cellular signaling networks (Cox *et al.*, 2014). Furthermore, the analysis of proteomics data is complex and data-intensive. The interpretation of large datasets generated by proteomic analyses demands advanced bioinformatics tools and expertise. Sample preparation and quantification accuracy are also critical, as errors in these steps can lead to misleading conclusions. Despite advances in technology, current methods still often fail to cover the entire proteome. This incomplete coverage means that some potentially crucial proteins may not be detected in a study, limiting our understanding of cellular processes (Wu *et al.*, 2011). Addressing these challenges is crucial for fully leveraging phosphoproteomics and proteomics in fungal research. By improving methodologies and technologies, researchers can enhance

the accuracy and depth of their analyses, paving the way for breakthroughs in understanding fungal biology and developing new therapeutic strategies (Bersching *et al.*, 2023).

6.5.1 Setting New Standards in Phospho-Proteomics Through Advanced Acquisition Methods

The intricate dynamics of signaling networks in cellular processes underscore the significance of reversible protein phosphorylation, prompting the advancement of analytical methods like quantitative phospho-proteomics (Rigbolt and Blagoev, 2012). These techniques have evolved from specialized approaches to versatile platforms for comprehensively analyzing phosphorylation profiles in living organisms. Despite considerable advancements in instrumentation and bioinformatics, the persistence of a high number of missing values due to experimental procedures remains a significant challenge (Wu *et al.*, 2011). These missing values stem from either random phospho-peptide enrichment selectivity or borderline signal intensities, both of which result in exclusion from fragmentation, particularly in the commonly used data-dependent acquisition (DDA) mode (Zhang *et al.*, 2009). Consequently, incomplete datasets undermine confidence in subsequent statistical bioinformatic analyses. In this study, we addressed these challenges by employing data-independent acquisition (DIA) in the filamentous fungus *M. oryzae* as a model organism. Our findings demonstrate that while maintaining data quality, including phosphosite and peptide sequence confidence, the completeness of the dataset increases dramatically. By reducing LC-MS/MS analysis time from 3 hours to 1 hour and significantly enhancing the number of identified phosphosites, up to 10-fold compared to published studies in *M. oryzae*, we present a refined methodology and an extensive resource for investigating signaling processes in filamentous fungi. Furthermore, our approach outperforms previous methodologies, such as the chromatography method used by W.L. Franck et al. in 2015, which required a 3-hour runtime. Our LC method, featuring a 45-minute gradient and 60-minute total runtime, offers improved efficiency. While the application of DIA presents promising opportunities for comprehensive phospho-proteomics datasets, it necessitates resource-intensive and time-consuming bioinformatic processing, alongside challenges in intuitive spectra visualization. Proprietary software solutions like Spectronaut offer intuitive visualization of extracted ion chromatograms (XICs) of precursors and fragments. Nonetheless, DIA-NN, an open-source tool employing neuronal networks, demonstrates superior identification performance. Despite the absence of a direct phospho-peptide identification benchmark between these software types, our study suggests an intriguing starting point for further bioinformatics research. Our study not only provides a refined methodology for phospho-peptide analysis in filamentous fungi but also furnishes a substantial

dataset that serves as a valuable resource for advancing signaling research in *M. oryzae* and beyond (Bersching *et al.*, 2023).

To elucidate the molecular underpinnings of evolutionary dynamics in the filamentous fungus *M. oryzae*, a combination of methodologies is imperative. In addition to biochemical stress response analysis via HPAEC-PAD, proteomics, and phospho-proteomic analyses offer valuable insights into whether suppressor strains, emerging individually and autonomously, follow similar evolutionary trajectories, resulting in akin phenotypes, or diverge due to unique adaptations. A recent establishment of a pipeline for proteomic and phospho-proteomic sample analysis in *M. oryzae* has facilitated the study of evolutionary dynamics. By assessing the variability of phenotypes acquired by reversible and irreversible suppressor strains compared to the wild type, overlaps in the putative "events" driving rapid evolutionary suppression were explored. High overlap in proteome profiles of suppressor strains enabled the selection of a representative strain for deeper investigation.

6.5.2 Understanding the Proteomic Response to Salt Stress in *Magnaporthe oryzae* with Emphasis on Glycerol Metabolism and Osmotic Stress Adaptation

Within the focus of the production of primary metabolites and the production of glycerol upon osmotic stress a detailed time-course proteome profiling under salt stress was conducted in ∆*Mohog1(irreversible)* strain compared to the wild type at multiple timepoints. Notably, while the proteomic landscape in ∆*Mohog1(irreversible)* resembled that of the wild type up to 4 hours post salt stress, significant alterations were observed 24 hours post stress induction, with 399 downregulated and 475 upregulated proteins compared to wild type. Further analysis via k-means clustering and GO term enrichment unveiled enrichment in glycan degradation, peroxisomal fatty acid degradation, carbohydrate catabolic processes and glycerolipid metabolism, with Dihydroxyacetone kinase (MGG_04014) emerging as a key candidate associated with glycerol production, a primary osmolyte in the suppressor strains (Michna, 2023).

In bacteria, yeast, and plants, DHAK plays critical roles in energy metabolism and the response to osmotic stress. Interestingly in mammals, the enzyme's function is more related to glycerol turnover and lipid metabolism. In filamentous fungi, Dihydroxyacetone kinase (DHAK) is likely involved in glycerol metabolism pathways (Zhang *et al.*, 2018), which play essential roles in cellular energy production, carbon utilization, and osmoregulation. Glycerol is a common metabolite produced during various cellular processes and serves as a key intermediate in the metabolism of carbohydrates and lipids (Klein *et al.*, 2017). In order to study the evolutionary

dynamics in *M. oryzae* the long-term adaptation to osmotic stressors in fungi is hypothesized to involve changes in the expression and regulation of various genes and metabolic pathways, including those related to glycerol metabolism, in which DHAK plays a critical role. DHAK is an enzyme found in filamentous fungi, as well as in other microorganisms and some plants. Its primary function is to catalyze the phosphorylation of dihydroxyacetone (DHA) to produce dihydroxyacetone phosphate (DHAP) using ATP as a phosphate donor (Klein *et al.*, 2017). This reaction is a crucial step in the metabolism of glycerol, which is utilized by organisms as a carbon and energy source (Siderius *et al.*, 2000; Zhang *et al.*, 2018).

The reversible transformation of glycerol into sn-glycerol 3-phosphate is facilitated by glycerol kinase (GlcA) and glycerol 1-phosphatase (GPP). The subsequent conversion of sn-glycerol 3-phosphate to dihydroxyacetone phosphate, which is also an intermediate in glycolysis, is catalyzed by two different glycerol 3-phosphate dehydrogenases (GFD). Additionally, glycerol can be transformed into dihydroxyacetone and then into dihydroxyacetone phosphate by glycerol dehydrogenase (GldB) and dihydroxyacetone kinase (DakA) (Li *et al.*, 2022).

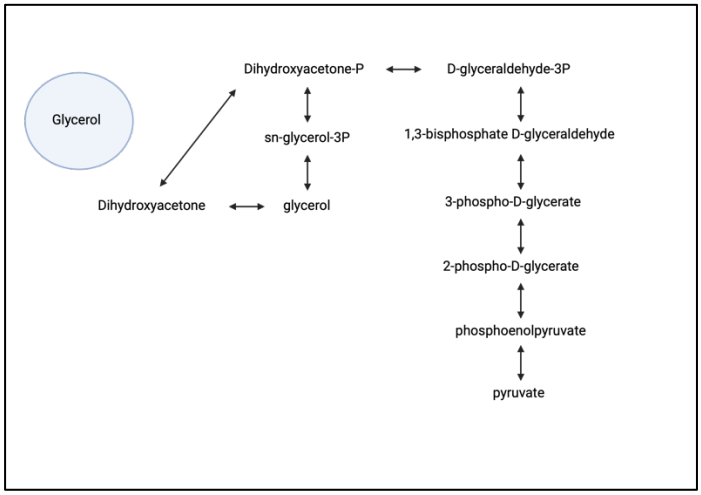

Figure 6: Sugar metabolic network (Glycerol – description top left) of Aspergillus niger. *The EC number is: 1.1.1.156. Based on Li et al.*, 2022.

This places DHAK at a crucial intersection between glycerol catabolism and the glycolysis/gluconeogenesis pathway, as DHAP is an intermediate in both.

The activity of dihydroxyacetone kinase (DHAK), an enzyme crucial in metabolic processes, is modulated through a complex web of regulatory mechanisms that span transcriptional, post-transcriptional, and allosteric levels. This multifaceted regulation underscores the enzyme's adaptability and its integral role in cellular metabolism as well as adaptation towards stress (Molin, Norbeck and Blomberg, 2003). The local environment's nutrient composition plays a critical role in the regulation of DHAK. Specially, the availability of glycerol or dihydroxyacetone acts as a signal to the cell, triggering an upregulation in the expression of DHAK genes. This regulatory mechanism ensures that cells can rapidly respond to changes in nutrient availability,

increasing their capacity to metabolize these compounds when they become available (Klein *et al.*, 2017). By adjusting the expression levels of DHAK, cells optimize their metabolic efficiency, prioritizing the breakdown and utilization of glycerol or dihydroxyacetone for energy production or biosynthesis whenever they are present (Nomura, Aoki and Inoue, 2018). At the molecular level, the activity and efficiency of DHAK and dihydroxyacetone phosphate (DHAP) are further refined by post-translational modifications, such as phosphorylation. These chemical alterations can significantly impact the enzyme's functionality, affecting its catalytic efficiency or altering its substrate affinity (Klein *et al.*, 2017).

For instance, the addition of a phosphate group to certain amino acids within DHAK might induce conformational changes, enhancing or inhibiting its interaction with dihydroxyacetone or ATP. Through these modifications, the cell can fine-tune DHAK activity, ensuring that its function is precisely modulated according to immediate cellular needs and environmental conditions. DHAK typically phosphorylates dihydroxyacetone (DHA) to dihydroxyacetone phosphate (DHAP), an intermediate in glycolysis and gluconeogenesis. If DHAK is inactivated, DHA might accumulate because it cannot be efficiently converted to DHAP. Similarly, the inactivation of DHAK might impact gluconeogenesis, potentially altering glucose production rates from non-carbohydrate sources, including glycerol. However, excessively high concentrations of glycerol and unmetabolized DHA could become cytotoxic, potentially leading to cellular damage or dysfunction if not properly regulated (Nomura, Aoki and Inoue, 2018). DHAK and its associated pathways can vary significantly across different organisms.

The role of DHAK is yet not well known in the model organism *M. oryzae* and could be involved in the adaptation process of the suppressor mutants.

While the glycerol production increases as a cellular response towards high osmotic stress and the HOG pathway is inactivated the mutants are in need to use another pathway for the production of primary metabolites to keep the osmotic balance towards the outer osmotic stress. While the suppressor mutants produce mainly glycerol and the DHAK is possibly involved in the intracellular glycerol production, this gene should be further analyzed. The involvement of the DHAK is also in line with the production of arabitol after the re-integration of the original lof-genes into the suppressor mutants. It seem to be possible, that the HOG pathway is nevertheless the most energy sufficient pathway in order to compensate osmotic imbalance by producing intracellular arabitol and not mainly glycerol.

6.5.3 Insight into Early Phosphorylation Events and Proteomic Alterations under Salt Stress - Highlighting MAPK Signaling and Regulatory Mechanisms

A phosphopeptide profiling complemented these findings, revealing early phosphorylation changes, including the absence of phosphorylated MoHog1p at 60 minutes post stress induction in Δ*Mohog1(irreversible*) (Michna, 2023). In contrast to the protein response, we were able to observe significant differences between the wildtype strain and Δ*Mohog1(irreversible)* already 60 min after salt stress. Clustering analyses highlighted pathways such as MAPK signaling, carbohydrate metabolic processes, and hydrolase activity regulation, indicating distinct responses compared to wild type. Proteins implicated in MAPK signaling include MoflbAp (MGG_14517) and MoPmp1p (MGG_15140), being a phosphatase known for its involvement in appressorium formation through Pmk1 regulation (Michna, 2023). We also observed histone modification processes, which encompass the transcription initiation factor TFIID subunit 1 (MGG_01207). Notably, histone modification processes and Ras protein and GTPase activity were also identified, suggesting intricate regulatory mechanisms at play. Comparative analysis of Gene Ontology terms related to osmotic stress response between wild type and adapted hog1 mutant revealed a 44% overlap. While several common pathways were identified, differences in specific protein entries underscored the challenge of explaining the adapted mutant's phenotype (Michna, 2023).

The protein response to salt stress in reversible and irreversible suppressor strains was compared with that of the wild-type strain (Michna, 2023). Analysis revealed that glycerol metabolism and carbohydrate catabolic processes are promising candidate clusters involved in the rapid evolutionary adaptation of osmoregulation in *M. oryzae* (Michna, 2023). The function of proteins involved in carbohydrate metabolism is closely linked to enzymatic functions in glycerol metabolism, consistent with glycerol production observed in both reversible and irreversible suppressor strains. Phospho-peptide analysis highlighted significant alterations in carbohydrate metabolic processes following salt stress in the suppressor strains. This process involves not only proteins from the MAPK signaling pathway but also includes phosphatases, which are crucial for regulating appressorium formation and glycerol accumulation necessary for turgor generation (Thines, Weber and Talbot, 2000b; Oh *et al.*, 2008). Interestingly, a histone modification involving the transcription initiation factor TFIID subunit 1 (MGG_01207) was found to be strongly regulated, suggesting it as a candidate for further investigation (Michna, 2023). This is particularly relevant because interactions between the transcription factor TFIID subunit 1 and Ubp3 in *S. cerevisiae* have demonstrated that Ubp3 is a substrate for Hog1 and is essential for the osmotic stress response in yeast (Auty *et al.*, 2004; Solé *et al.*, 2011).

When comparing the osmostress-sensitive phenotypes of both non-adapted (lof mutants) and reversibly adapted states, a notable distinction lies in the preserved cell integrity observed in the reversibly adapted phenotype (Michna, 2023). While the cellular proteome response in the reversibly adapted state mirrors that of the wild type, it appears largely stochastic in the non-adapted phenotype. Notably, the phosphopeptide response exhibits similar numerical trends and gene ontology enrichments in both osmosensitive phenotypes. Despite the identification of MAPK signaling, it fails to exhibit the characteristics of the adapted phenotype. It is conceivable that the presented proteomics data (Michna, 2023) may not effectively elucidate ongoing processes due to continual nonspecific protease activity and unknown cellular responses triggered by osmotic stress, potentially obscuring the pertinent response, particularly in the reversibly adapted phenotype. Consequently, the mechanisms underlying the reversal of adaptation remain ambiguous. In such instances, shotgun proteomics may not be the most suitable method to address these inquiries. Beyond protein phosphorylation, numerous post-translational modifications (PTMs) govern cellular processes. As a potential solution, we propose exploring epigenetic processes within the nucleus, such as analyzing histone modifications, to shed light on these questions.

6.7 Epigenetic Approaches to understand the adaptation of *Magnaporthe oryzae*

The focus is on how non-genetic changes influencing gene expression can drive evolutionary adaptations, enabling the fungus to survive harsh conditions. Epigenetics has been demonstrated to significantly influence the persistence of plant pathogens across various host environment (Poças-Fonseca, Cabral and Manfrão-Netto, 2020). Considering the critical role of *M. oryzae* as a fungal plant pathogen and its status as a model organism in fundamental research, it is essential to highlight the impact of epigenetic regulation on fungal development and pathogenesis. Many aspects of epigenetics remain unexplored, which could potentially enhance our understanding of the persistence and success of *M. oryzae* and other fungal pathogens. So far the focus of epigenetic mechanisms reported in *M. oryzae* are DNA methylation and modifications of the histones (Jeon *et al.*, 2015) . Epigenetic manipulation offers a straightforward approach to activating silent or cryptic gene clusters without the need for complex molecular biological techniques. Methods such as adding chemical epigenetic modifiers, using co-culture approaches, altering cultivation periods, and varying fermentation conditions can stimulate the production of uncharacterized fungal metabolites (Serrano *et al.*, 2021). Although this strategy may lack predictable outcomes, it remains a promising and easily implemented method in laboratories for discovering new fungal compound.

DNA methylation is an epigenetic mechanism that regulates gene expression through the chemical modification of DNA. It influences gene activity by either changing the binding affinity of transcriptional machinery to DNA or by attracting proteins involved in gene repression. This modification is crucial for various biological processes, including genetic imprinting, X-chromosome inactivation, cell differentiation, and gene silencing (Reik and Lewis, 2005; Bond and Baulcombe, 2015). The significance of the role of DNA methylation in regulating the pathogenicity of *M. oryzae* has yet to be established (Jeon *et al.*, 2015). Conducting a comprehensive analysis of the fungal methylome across various isolates and life cycle stages could shed light on the evolutionary significance of DNA methylation in fungal pathogenicity. Such insights could be instrumental in developing molecular tools to combat fungal infections effectively (Dubey and Jeon, 2017). Bisulfite sequencing a tool which reveals methylation sites in the genome.

While DNA methylation is a key epigenetic mechanism that regulates gene expression through chemical modifications of the DNA itself, histone modifications represent another crucial layer of epigenetic regulation. Histone modifications, such as methylation and acetylation, influence the structural configuration of chromatin, thereby impacting gene accessibility and transcription. Together, these processes orchestrate the complex regulation of gene activity, with DNA methylation providing a stable, long-term regulatory mark, and histone modifications offering more dynamic and reversible control over chromatin structure and function (Dubey and Jeon, 2017). Histone methylation is linked to both gene activation and repression. Using chromatin immunoprecipitation sequencing (ChIP-seq), it has been observed that histone methylation can switch from promoting gene activation to causing repression under varying environmental conditions (Pham *et al.*, 2015; Van Vu *et al.*, 2021). This finding underscores the connection between external factors and the dynamic nature of histone methylation.

The primary research objective is to delineate the role of epigenetic modifications in the adaptation of *M. oryzae* to osmotic stress. This involves comparing suppressor strains, which have reestablished osmoregulation, to control strains that include both the wild type and non-suppressor loss-of-function (lof) mutants. The study of epigenetics is structured to capture both the immediate and long-term epigenetic changes by utilizing suppressor strains alongside control strains at multiple time points after osmotic stress induction. Techniques such as bisulfite sequencing will analyze DNA methylation patterns, while ChIP-seq will examine histone modifications near genes associated with glycerol metabolism—the major solute used by adapted strains to cope with stress. Further approaches include ATAC-seq to identify open chromatin regions suggesting active regulatory domains and RNA-seq to correlate epigenetic changes with shifts in gene expression. Together, these methods aim to uncover the specific

epigenetic landscapes facilitating the suppressor strains' survival under osmotic stress. The above mentioned methods should be considered to be used while studying the model organism *M. oryzae* with the focus of DALE and the metabolic switch from arabitol to glycerol within the suppressor mutants.

6.7.1 The Impact of Bisulfite Sequencing on Understanding DNA Methylation

Epigenetic modifications, regarded as the link between genotype, phenotype, and environment in many eukaryotes, have been proposed as a crucial control mechanism utilized by fungi to regulate the transcription of genes associated with metabolite production (Liu *et al.*, 2012). Bisulfite sequencing is a technique used primarily to determine the methylation pattern of DNA, which is a key epigenetic modification and has emerged as an important tool in various branches of biological research. DNA methylation typically involves the addition of a methyl group to the cytosine or adenine DNA nucleotides, and it plays a crucial role in regulating gene expression without altering the actual DNA sequence (Maunakea *et al.*, 2010). Additionally it is involved guiding development mechanisms as well as influencing disease mechanisms. The DNA sample is treated with sodium bisulfite, which converts cytosine residues to uracil, but leaves 5-methylcytosine (a methylated form of cytosine) unchanged. This treatment effectively differentiates methylated from unmethylated cytosines. Following bisulfite treatment, the DNA is then subjected to PCR (polymerase chain reaction) amplification. During PCR, the uracil residues (originally cytosines) are amplified as thymine, whereas the 5-methylcytosines are amplified as cytosines (Wreczycka *et al.*, 2017).

In the field of epigenetic research, bisulfite sequencing is crucial for understanding how methylation affects gene regulation. By identifying specific sites of methylation within the genome, researchers can link these epigenetic marks to changes in gene activity during both normal development and pathological conditions. Cancer research has particularly benefited from advances in bisulfite sequencing (Maunakea *et al.*, 2010). Aberrant DNA methylation patterns are a hallmark of many cancers, and identifying these patterns can lead to better diagnostic, prognostic, and therapeutic strategies. Bisulfite sequencing enables the detection of methylation changes that serve as biomarkers, helping clinicians and researchers predict disease progression and response to treatments (Liang *et al.*, 2019). In developmental biology, bisulfite sequencing provides insights into the dynamic changes in DNA methylation as organisms grow and develop. This technique helps scientists decode how epigenetic modifications influence developmental pathways, shedding light on everything from embryonic development to aging processes (Dmitrijeva *et al.*, 2018). Plant genetics also relies on bisulfite

sequencing to explore how DNA methylation contributes to plant responses to environmental stress and the regulation of transposable elements, which are segments of DNA that can move around the genome (Dowen *et al.*, 2012). Understanding these mechanisms is crucial for improving plant resilience and productivity. This technique also quantitatively measures the methylation level at each cytosine site across the genome, facilitating a thorough analysis of epigenetic modifications (Masser *et al.*, 2018). However, there are challenges associated with bisulfite sequencing that researchers must consider. One significant issue is DNA degradation; the bisulfite treatment necessary for the technique can degrade DNA, complicating the analysis of very small or degraded samples. Additionally, the data analysis from bisulfite sequencing is complex and requires sophisticated bioinformatics tools to correctly interpret the methylation status across the genome, particularly in regions with intricate genomic structures (Wreczycka *et al.*, 2017).

In summary, bisulfite sequencing is a applicable method in the study of DNA methylation, offering detailed insights into one of the most crucial epigenetic modifications influencing gene expression. Its application spans various fields of biology, highlighting its importance in both fundamental research and clinical studies. Bisulfite sequencing would not only enhance our understanding of how osmotic stress impacts the fungal epigenome but also illuminate the metabolic switch in ALE suppressor mutants of the HOG signaling pathway in *M. oryzae*. This technique would allow the identification of changes in DNA methylation associated with the switch from arabitol to glycerol under osmotic stress. Understanding these changes is crucial, as they affect the expression of genes involved in the metabolic pathways of arabitol and glycerol. Specifically, bisulfite sequencing can be used to investigate the methylation status of genes encoding key enzymes in these pathways, such as arabitol dehydrogenase and glycerol-3-phosphate dehydrogenase. Determining if these genes are differentially methylated in response to osmotic stress could suggest an epigenetic mechanism for their regulation. Additionally, identifying differentially methylated regions (DMRs) in promoter regions, enhancers, and other regulatory elements of genes associated with arabitol and glycerol metabolism could indicate their role in gene expression regulation under these conditions. By correlating these epigenetic changes with gene expression data, we can gain a deeper understanding of the regulatory mechanisms driving this metabolic adaptation. This comprehensive approach provides valuable insights into how epigenetic modifications contribute to the fungal response to environmental stress.

6.7.2 Advancements in ChIP-Seq Analysis for Studying Filamentous Fungi

Chromatin Immunoprecipitation followed by sequencing (ChIP-Seq) studies the interactions between proteins and DNA within the genome. It helps to understand how gene expression is regulated by identifying the specific locations where proteins, such as transcription factors and histones, bind to DNA (Wu, Won and Li, 2015). ChIP-Seq is widely used to map global DNA binding sites for these proteins systematically, which is crucial for unraveling the complexities of gene regulation, epigenetic modifications, and more (Ma and Zhang, 2020).

ChIP-Seq can be and has been successfully used with filamentous fungi, including important model organisms and pathogens. This technique is valuable for studying gene regulation and chromatin organization in these organisms, allowing researchers to uncover the dynamics of transcription factor binding sites, histone modifications, and other DNA-associated proteins across the fungal genome. In *Neurospora crassa*, ChIP-Seq has been used to map histone modifications and to study the regulation of circadian rhythms (Sasaki *et al.*, 2014). Similarly, in plant-pathogenic fungi like *M. oryzae*, ChIP-Seq helps elucidate how transcription factors regulate genes involved in infection processes (Zhou *et al.*, 2020).

Chromatin immunoprecipitation (ChIP) assesses the in vivo binding of proteins to chromatin. This process begins with the crosslinking of proteins to chromatin, typically using formaldehyde, followed by the isolation of a specific protein (such as a transcription factor) along with any associated DNA, using either a protein-specific or epitope-specific antibody if the protein is epitope-tagged. The extent of protein binding to DNA can be determined using various downstream techniques. For a limited number of predetermined or suspected targets, the DNA isolated can be analyzed using PCR or quantitative real-time PCR (Tan and Wong, 2019). ChIP, ChIP-chip, and ChIP-seq are widely utilized in the study of various biological processes occurring within chromatin, such as transcription, chromatin remodeling, and DNA repair. These methods are considered highly effective in the fields of transcription and chromatin analysis.

By applying ChIP-chip or ChIP-seq techniques to RNA polymerase II (PolII), it is possible identify actively transcribing genes and measure the transcriptional activity of each gene by quantifying the PolII occupancy on the gene, which indicates the amount of DNA associated with PolII. Similar to RNA-seq, the readouts from PolII ChIP-seq or ChIP-chip provide quantitative data on gene expression, although these measurements may be slightly less precise for genes influenced by transcript stability mechanisms. A key advantage of using formaldehyde in ChIP-seq is its ability to rapidly crosslink proteins to DNA, thus providing an immediate snapshot of transcriptional activity at the moment of crosslinking. This attribute of ChIP-based methods is especially valuable for documenting transient transcriptional changes in time-course studies, enabling observations over brief periods as short as a few minutes

(Mason and Struhl 2005; Proft et al. 2006; Wong and Struhl 2011; Wong et al. 2014). This level of temporal resolution is not possible with techniques that measure steady-state mRNA levels. RNA-seq is a widely utilized technique for analyzing genome-wide gene expression. This technique measure the steady-state levels of mRNA, which result from the rates of mRNA synthesis and degradation. Although these methods provide estimates of gene expression, they do not accurately depict the actual transcriptional activity and are limited in capturing transient transcriptional changes, particularly over brief time interval (Tan and Wong, 2019). This limitation stems from the variability in mRNA degradation rates across different genes and even among different isoforms of the same gene (Geisberg *et al.*, 2014; Gupta *et al.*, 2014). Additionally, mRNA stability can change under various physiological conditions (Maekawa *et al.*, 2015; Miller, Brandt and Gresham, 2018), impacting the levels of steady-state mRNA. Consequently, traditional profiling methods like RNA-seq present significant limitations for detailed studies of fungal transcriptional dynamics. ChIP-seq is a critical technique widely used in the study of numerous biological processes occurring on chromatin, such as transcription, chromatin remodeling, and DNA repair. These methods are considered among the most effective in researching transcription and chromatin dynamics (Veri *et al.*, 2018; Tan and Wong, 2019).

With the research question in mind, ChIP-Seq. would be of interest to identify transcription factors and other regulatory proteins that bind to promoter regions of genes involved in arabitol or glycerol metabolism. The antibodies would be chosen to bind against key transcription factors or histone modifications associated with active transcription.

The specificity of antibodies is crucial for precipitating fungal proteins effectively; while antibodies for histone modifications are generally available due to their conservation across eukaryotes, those targeting specific fungal transcription factors may need to be specially developed. Additionally, fungal samples may yield lower amounts of DNA compared to mammalian cells, depending on the species and growth conditions, necessitating the use of more starting material or amplification techniques to obtain sufficient DNA for sequencing purposes. Due to the high demand over the last years the amount of commercial available antibodies for PolII ChIp and ChIp-Seq have increased and are available for model organism like *S. cerevisiae*, *Schizosaccharomyces pombe*, *C. albicans*, and *Aspergillus nidulans* (Tan and Wong, 2019) and is useful tool for various key fungal function (Veri *et al.*, 2018).

6.7.3 Advancing Fungal Genomic Research with ATAC-Sequencing Techniques

Chromatin serves as the structural framework that regulates gene accessibility and expression. Research gains insight into the complex regulatory networks that control gene expression as

well as cellular function (Elías-Villalobos, Barrales and Ibeas, 2019). Chromatin modifications in fungi are regulated by intrinsic cellular mechanisms and influenced by external environmental factors. These modifications are crucial for maintaining cellular integrity, guiding development and differentiation, enabling environmental adaptation, and regulating metabolic processe. Understanding how these dynamic changes in chromatin structure and function contribute to various biological processes is essential for advancing our knowledge of fungal biology and its responses to environmental cues. An assay for Transposase-Accessible Chromatin with high-throughput sequencing or ATAC-Sequencing, is a technique designed to determine chromatin accessibility across the genome. This method provides insights into the regulatory architecture of the genome by identifying regions of open chromatin that are accessible to transcription factors and other DNA-binding proteins.

Histone acetylation is a dynamic and flexible process, tightly regulated by the opposing actions of two enzyme groups. Histone acetyltransferases (HATs) catalyze the transfer of acetyl groups from acetyl-CoA to lysine residues on histone proteins, leading to a more relaxed chromatin structure and increased gene expression (Yang and Seto, 2007; Barneda-Zahonero and Parra, 2012). Numerous fungal plant pathogens employ histone acetyltransferases (HATs) during their infectious stages, including the corn smut fungus *Ustilago maydis*, the banana pathogen *Fusarium oxysporum*, and the wheat pathogen *Fusarium graminearum*. In these fungi, deleting genes encoding HAT family members has been shown to impact both fungal virulence and lifestyle transitions. For instance, in *U. maydis*, the histone deacetylase (HDAC) Sir2 is implicated in pathogenic development, although its role in deacetylation activity remains uncertain (Kramer *et al.*, 2023).

Conversely, histone deacetylases (HDACs) remove these acetyl groups, resulting in chromatin condensation and transcriptional repression. HDACs are crucial for chromatin remodeling and play a significant role in the epigenetic regulation of gene expression, various aspects of growth, development and pathogenesis in pathogenic fungi like *M. oryzae* (Lin *et al.*, 2021). In *N. crassa* histone modifications are important in for example temperature and pH sensitivity and therefore necessary in the response to environmental changes (Poças-Fonseca, Cabral and Manfrão-Netto, 2020).

Understanding how genes are turned on or off by examining which regions of the genome are open to transcription factor, tracking changes in chromatin accessibility during development to understand how gene regulation changes over time and identifying disruptions in chromatin accessibility linked to diseases, particularly genetic disorders and cancers.

In ATAC-Sequencing, a hyperactive Tn5 transposase is used. This enzyme simultaneously cuts DNA and inserts sequencing adapters into open and accessible regions of the chromatin. These regions are typically free from nucleosomes (the basic units of DNA packaging in

eukaryotes, consisting of DNA wrapped around histone proteins) or are associated with active regulatory elements like promoters, enhancers, and insulators. Once the transposase has inserted the adapters, the marked fragments are amplified using PCR, which makes them suitable for high-throughput next-generation sequencing (Grandi *et al.*, 2022). When applying ATAC-Sequencing to filamentous fungi, there are unique considerations and potential adaptations needed due to differences in fungal chromatin structure and genome organization compared to those of more commonly studied model organisms like mammals or yeast (Grandi *et al.*, 2022). Filamentous fungi have cell walls that require specific enzymatic or mechanical treatment to expose the chromatin without damaging it. Additionally may have the unique chromatin structures influence the efficiency of Tn5 transposase accessibility and integration. Another problem which might be that many filamentous fungi have complex genomes with high GC content or repetitive elements, which can complicate sequencing and data interpretation. But beneath the possible problems in sample preparation ATAC-Seq can help identify regulatory elements involved in the expression of genes critical for pathogenicity and virulence in plant or human pathogens and can analyze how fungi respond to environmental stresses by altering their chromatin structure, thereby regulating gene expression in response to external stimuli. An interesting topic to focus is on exploring how chromatin accessibility changes during the fungal life cycle, including sporulation or hyphal growth.

ATAC sequencing (ATAC-seq) would be highly beneficial for studying the metabolic switch from arabitol to glycerol in fungi under osmotic stress by providing detailed insights into chromatin accessibility and regulatory element activity. By mapping open chromatin regions across the fungal genome, ATAC-seq can identify changes in the accessibility of promoters, enhancers, and other regulatory elements associated with genes involved in the metabolic pathways of arabitol and glycerol. This technique allows us to pinpoint which regions of the genome become more or less accessible under osmotic stress, thereby revealing potential regulatory mechanisms that facilitate the metabolic switch. Furthermore, by integrating ATAC-seq data with RNA sequencing (RNA-seq) data, it is possible to correlate changes in chromatin accessibility with gene expression levels, providing a comprehensive understanding of how transcriptional regulation is altered during the metabolic adaptation. This combined approach can uncover key regulatory networks and transcription factors involved in the response to osmotic stress, enhancing our understanding of the epigenetic and transcriptional dynamics driving the metabolic switch from arabitol to glycerol in fungi

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9. Annex

Supplements 1 of Chapter 5

Southern blot analysis and schematic presentation of the transformation process by homologous recombination and verification of the inactivation mutants, suppressor mutants (irreversible and reversible) within the *Magnaporthe oryzae* genome. The genomic DNA of the different mutants (inactivation mutants, suppressor mutants (irreversible and reversibe))was isolated and restricted by restriction enzymes.

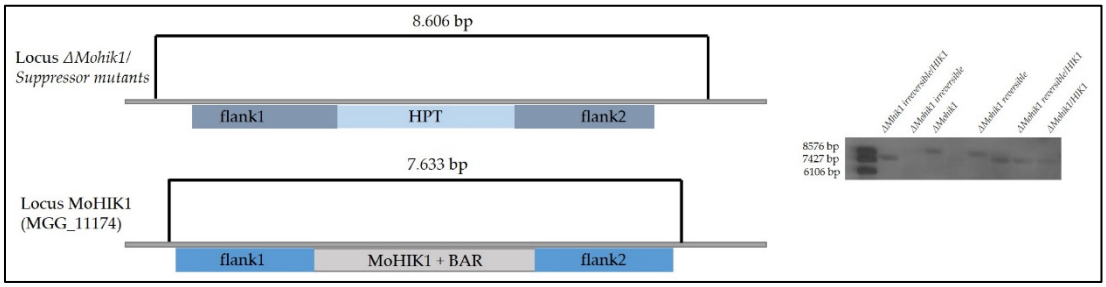

Fig. S1: Schematic representation of the genomic DNA of the *Magnaporthe oryzae* HOG inactivation mutants, suppressor mutants (irreversible and reversible) (expected fragment size of 8606 bp) of MoHik1 and the mutants with the re-integration of the inactivated MoHIK1-gene with the different hybridization sizes (expected fragment size 7663 bp). The sizes next to the images of the X-ray films indicate the respective fragment size of the hybridization signals.

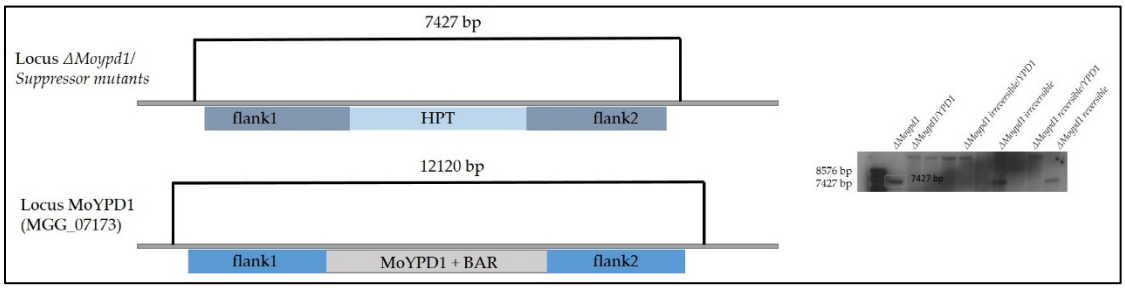

Fig. S2: Schematic representation of the genomic DNA of the *Magnaporthe oryzae* HOG inactivation mutants, suppressor mutants (irreversible and reversible) (expected fragment size of 7427 bp) of MoYpd1 and the mutants with the re-integration of the inactivated MoYPD1-gene with the different hybridization sizes (expected fragment size 12120 bp). The sizes next to the images of the X-ray films indicate the respective fragment size of the hybridization signals.

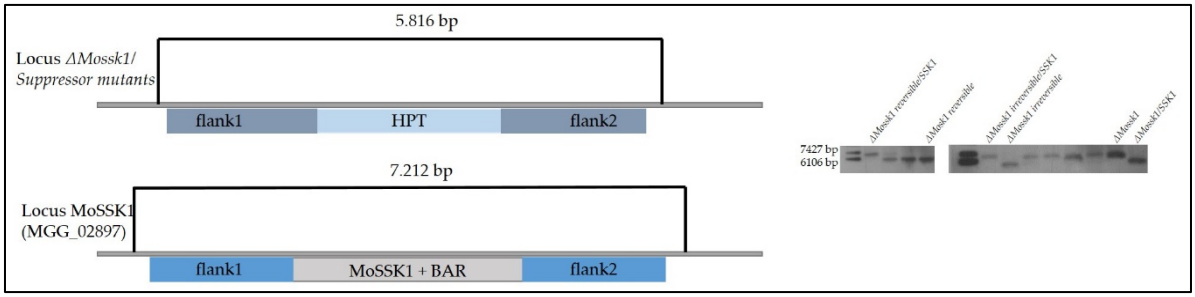

Fig. S3: Schematic representation of the genomic DNA of the *Magnaporthe oryzae* HOG inactivation mutants, suppressor mutants (irreversible and reversible) (expected fragment size of 5816 bp) of MoSsk11 and the mutants with the re-integration of the inactivated MoSSK1-gene with the different hybridization sizes (expected fragment size 7212 bp). The sizes next to the images of the X-ray films indicate the respective fragment size of the hybridization signals.

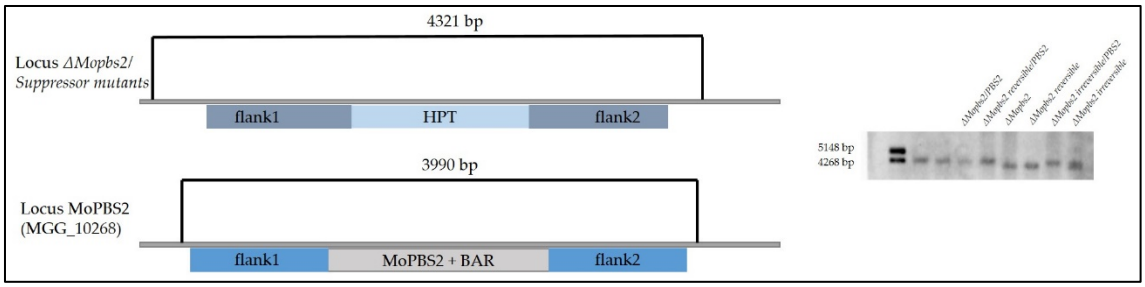

Fig. S4: Schematic representation of the genomic DNA of the *Magnaporthe oryzae* HOG inactivation mutants, suppressor mutants (irreversible and reversible) (expected fragment size of 4321 bp) of MoSsk2 and the mutants with the re-integration of the inactivated MoPBS2-gene with the different hybridization sizes (expected fragment size 3990 bp). The sizes next to the images of the X-ray films indicate the respective fragment size of the hybridization signals.

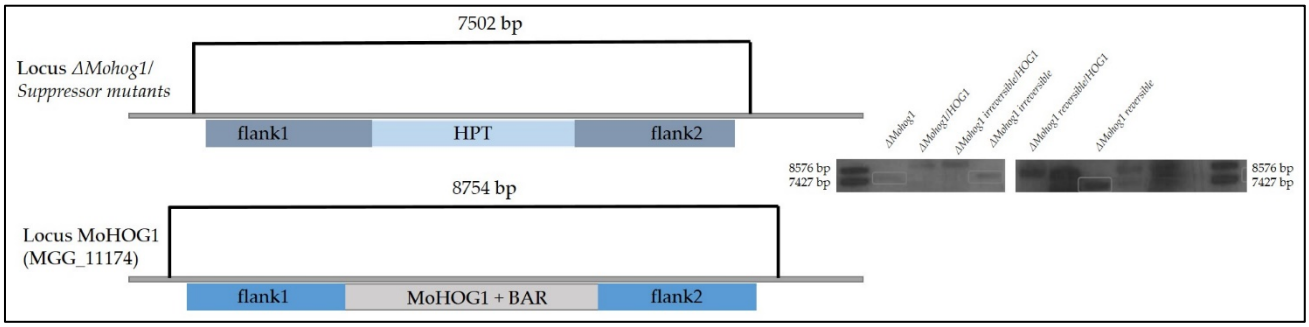

Fig. S5: Schematic representation of the genomic DNA of the *Magnaporthe oryzae* HOG inactivation mutants, suppressor mutants (irreversible and reversible) (expected fragment size of 7502 bp) of MoPbs2 and the mutants with the re-integration of the inactivated MoHOG1-gene with the different hybridization sizes (expected fragment size 8754 bp). The sizes next to the images of the X-ray films indicate the respective fragment size of the hybridization signals.

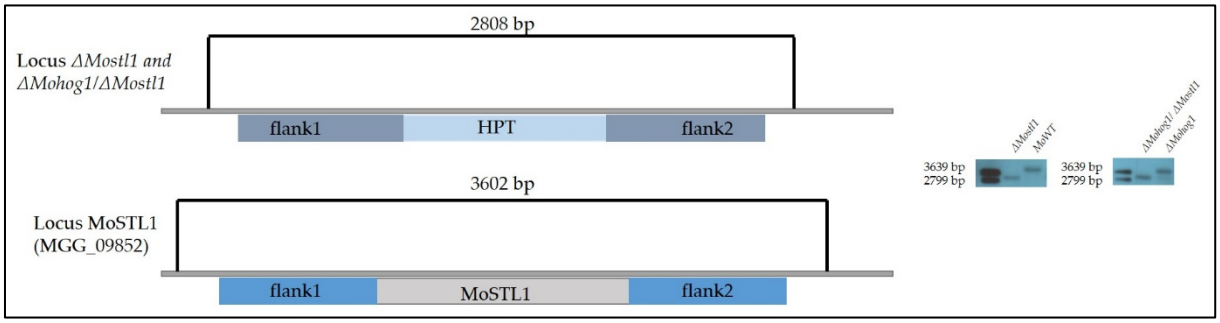

Fig. S6: Schematic representation of the genomic DNA of the *Magnaporthe oryzae* inactivation mutant with the different hybridization sizes and the inactivation in Δ*Mohog1*, (expected fragment size of 2808 bp) of MoStl1p and the wildtype and Δ*Mohog1* (expected fragment size 3602 bp). The sizes next to the images of the X-ray films indicate the respective fragment size of the hybridization signals.

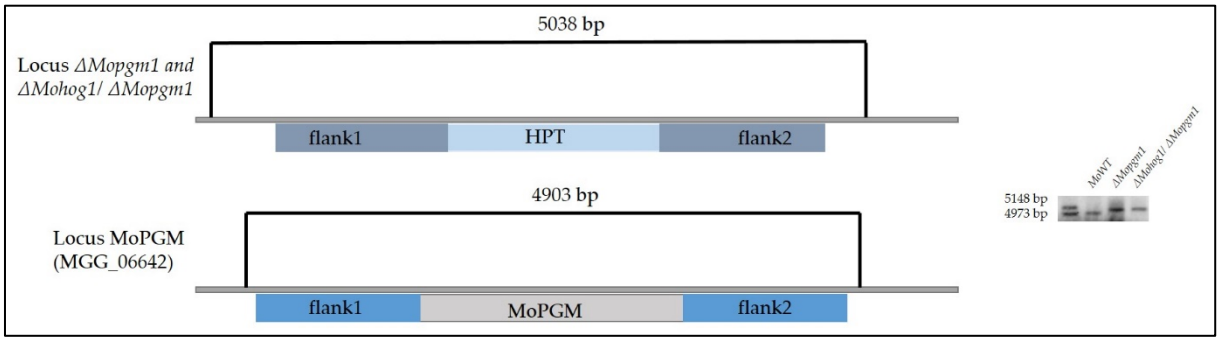

Fig. S7: Schematic representation of the genomic DNA of the *Magnaporthe oryzae* inactivation mutant with the different hybridization sizes and the inactivation in Δ*Mohog1*, (expected fragment size of 5038 bp) of MoPgm1p and the wildtype and Δ*Mohog1* (expected fragment size 4903 bp). The sizes next to the images of the X-ray films indicate the respective fragment size of the hybridization signals.

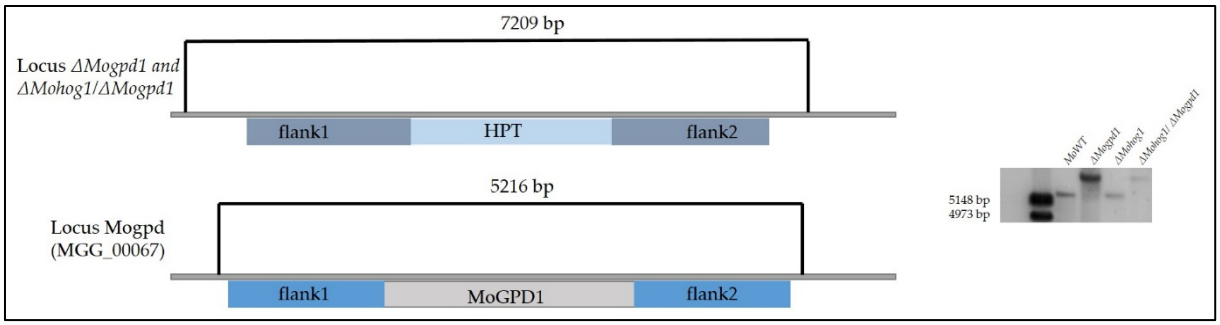

Fig. S8: Schematic representation of the genomic DNA of the *Magnaporthe oryzae* inactivation mutant with the different hybridization sizes and the inactivation in Δ*Mohog1*, (expected fragment size of 7209 bp) of MoGpd1p and the wildtype and Δ*Mohog1* sizes (expected fragment size 5216 bp). The sizes next to the images of the X-ray films indicate the respective fragment size of the hybridization signals.

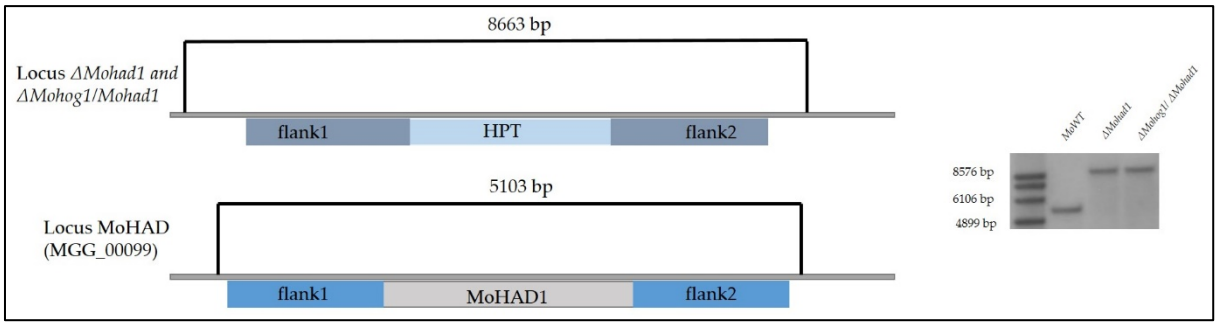

Fig. S9: Schematic representation of the genomic DNA of the *Magnaporthe oryzae* inactivation mutant with the different hybridization sizes and the inactivation in Δ*Mohog1*, (expected fragment size of 8663 bp) of MoHad1p and the wildtype and Δ*Mohog1* sizes (expected fragment size 5103 bp). The sizes next to the images of the X-ray films indicate the respective fragment size of the hybridization signals.

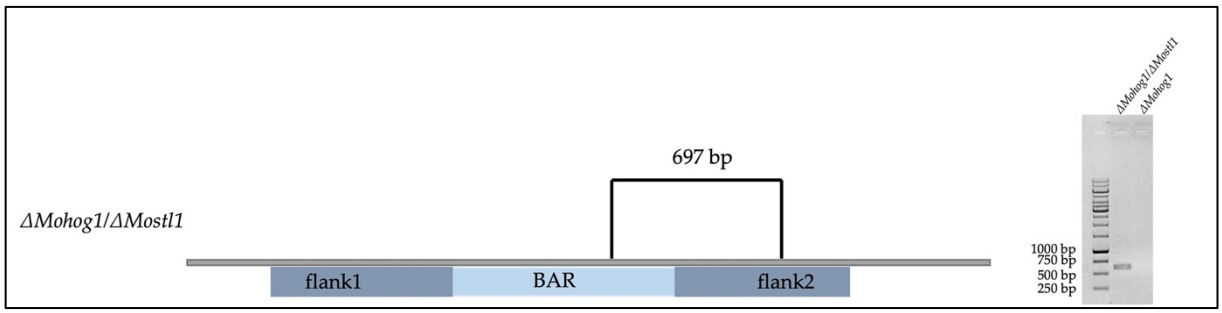

Fig. S10: Schematic representation of the genomic DNA of the inactivation in Δ*Mohog1*/Δ*Mostl1*, (expected fragment size of 697 bp) and Δ*Mohog1* sizes (no expected fragment).

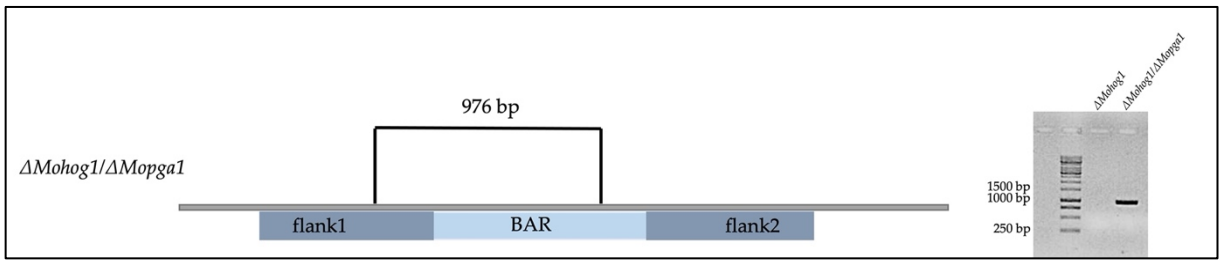

Fig. S11: Schematic representation of the genomic DNA of the inactivation in Δ*Mohog1*/Δ*Mopga1*, (expected fragment size of 976 bp) and Δ*Mohog1* sizes (no expected fragment).

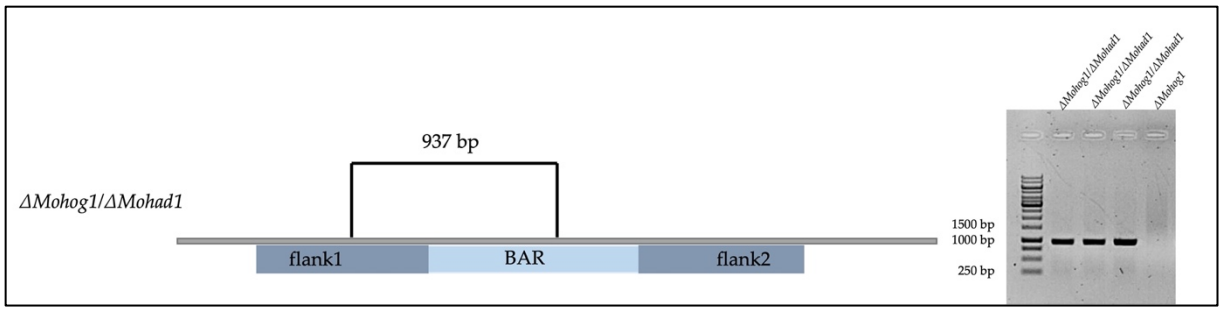

Fig. S12: Schematic representation of the genomic DNA of the inactivation in Δ*Mohog1*/Δ*Mohad1*, (expected fragment size of 937 bp) and Δ*Mohog1* sizes (no expected fragment).

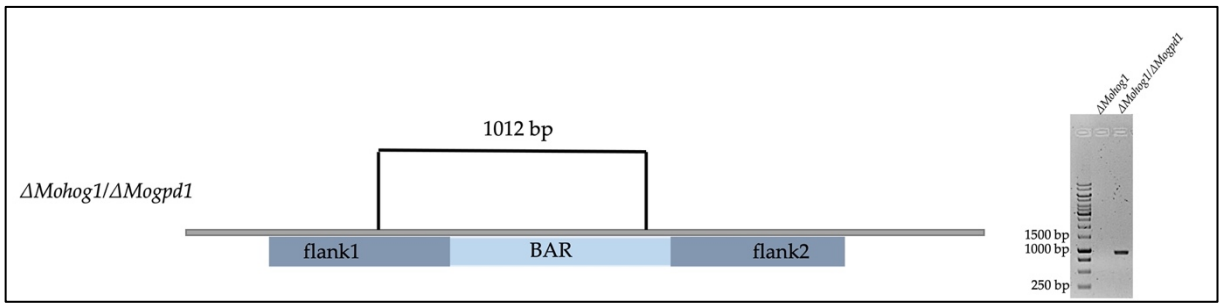

Fig. S13: Schematic representation of the genomic DNA of the inactivation in Δ*Mohog1*/Δ*Mogpd1*, (expected fragment size of 1012 bp) and Δ*Mohog1* sizes (no expected fragment).