Rapid adaptation of signaling networks in the fungal pathogen *Magnaporthe oryzae*

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**Abstract**

**Background:** One fundamental question in biology is how the evolution of eukaryotic signaling networks has taken place. “Loss of function” (lof) mutants from components of the high osmolarity glycerol (HOG) signaling pathway in the filamentous fungus *Magnaporthe oryzae* are viable, but impaired in osmoregulation.

**Results:** After long-term cultivation upon high osmolarity, stable individuals with reestablished osmoregulation capacity arise independently from each of the mutants with inactivated HOG pathway. This phenomenon is extremely reproducible and occurs only in osmosensitive mutants related to the HOG pathway – not in other osmosensitive *Magnaporthe* mutants. The major compatible solute produced by these adapted strains to cope with high osmolarity is glycerol, whereas it is arabitol in the wildtype strain. Genome and transcriptome analysis resulted in candidate genes related to glycerol metabolism, perhaps responsible for an epigenetic induced reestablishment of osmoregulation, since these genes do not show structural variations within the coding or promoter sequences.

**Conclusion:** This is the first report of a stable adaptation in eukaryotes by producing different metabolites and opens a door for the scientific community since the HOG pathway is worked on intensively in many eukaryotic model organisms.

**Keywords:** Rapid adaptation, HOG pathway, *Magnaporthe oryzae*, Rewiring, Reestablishment of osmoregulation, Epigenetics, Evolution of signaling networks, Suppressor

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**Background**

Adaptation is a central biological process that underlies diverse phenomena from the acquisition of antibiotic resistance by microbes to the evolution of niche specialization [1]. Most of these experimental analyses focused on bacterial systems studying the evolution of populations and genetic structures, the adaptive profit of new functions and the consequences of competition dynamics in large populations. The most prominent example to address molecular mechanisms of evolution is the *Escherichia coli* long-term evolution experiment [2]. It reveals complex dynamics, characterized by rapid adaptation, competition between beneficial mutations and extensive genetic parallelism in bacterial populations. However, until now, relatively little has been discovered about the mechanisms of molecular evolutionary adaptation in eukaryotes. One fundamental question in biology is how eukaryotic signaling pathways evolve to adapt towards changing environmental situations. The adaptation of molecular mechanisms from signaling networks and the question why the networks show a high level of complexity are of particular interest [3]. Over the last few years, the understanding of the molecular and biochemical principles of evolution have increased dramatically [4, 5]. Epigenetic modulation, such as DNA methylation, chromatin modification and noncoding RNAs, are found to be involved in the multigenerational transmission of phenotypes and transgenerational inheritance [6]. Nevertheless, there has been little detailed information about how phenotypic changes rest on the rate and the nature of the underlying genotypic or epigenetic changes [7]. Regulatory signaling networks represent the causality of developmental processes. The big challenges regarding regulatory networks generally remain in understanding how genetic variants generate phenotypes [8]. Genomic sequence variants influence the regulation of the expression of genes that encode proteins generating signaling patterns and execute...
differentiation or even adaptation to changing environments [9]. Microbial signal transduction pathways apply far more modular mechanisms to define their input-output interactions than classical metabolic pathways [10]. Thus, in addition to their core catalytic function, the proteins often contain multiple independently folding domains or motifs that mediate connectivity by interacting with other signaling elements [11]. These elements are found in different combinations with different signaling functions, suggesting insertion and recombination of modules may be a common mechanism of the evolution of new signaling networks [12]. Furthermore, protein domains are extensively recombined in order to facilitate functional innovations [5, 13]. Prominent examples of pathways with a modular architecture mediated by scaffold proteins are signaling pathways in yeast, including several mitogen-activated protein kinase (MAPK) cascades [12, 14]. Attachment of different scaffolds could, in principle, be sufficient to generate new signaling pathways from combinations of preexisting kinases [15]. Canonical MAPK cascades in fungi were reputed to be responsible for a large part of signal transduction. These MAPK pathways contain signal modules in which an activated MAPK kinase kinase (MAPKKK) activates a MAPK kinase (MAPKK), which then activates a MAPK (extracellular signal-regulated kinase: ERK). The MAPK targets comprise transcription factors, phosphatases, protein kinases and other classes of proteins. They regulate physiological processes, cellular morphology, cell cycle progression, metabolism and gene expression in response to various extra-cellular signals or environmental stresses [16]. Such MAPK pathways appear ideal for exploring the fundamental mechanisms of evolutionary adaptation. Three distinct MAPK genes – MoKSS1 (formerly MoPMK1), MoSLT2 (formerly MoMPS1), and MoHOG1 (formerly MoOSM1) [17] – have been identified in the genome of the filamentous rice blast fungus Magnaporthe oryzae (Pyricularia oryzae) and possess diverse roles in cellular signaling and pathogenesis-related development [18–20]. The high osmolarity glycerol (HOG) pathway is responsible for osmoregulation in fungi and seems to operate independently of the MoKSS1 and MoSLT2 pathways in M. oryzae by regulating the biosynthesis of arabinol and glycerol [20]. Upon high external osmolarity, cells have to quickly recover volume by water influx due to the accumulation of compatible solutes, i.e. mainly arabinol in the rice blast fungus. The HOG pathway in M. oryzae comprises the MoSln1p/MoHik1p-MoYpd1p-MoSsk1p phosphorelay system and the MAPK cascade MoSsk2p-MoPbs2p-MoHog1p (Fig. 1).

The HOG pathway in M. oryzae was previously found to be an attractive system to study the basics of physiological functions of signaling proteins and the mode of action of agricultural fungicides, such as fludioxonil [21–23]. M. oryzae-mutants lacking components of the HOG pathway are osmosensitive, since they showed a dramatically decreased growth at high osmolarity.

**Fig. 1** The Magnaporthe oryzae HOG signaling cascade. The two-component hybrid histidine kinases MoSln1p and MoHik1p are the sensors for environmental changes. Under normal environmental conditions, the phosphotransferase protein MoYpd1p transfers the phosphate to the response regulator MoSsk1p. Consequently, the phosphorelay system MoSln1p-MoHik1p-MoYpd1p-MoSsk1p is active and the MAPK cascade MoSsk2p-MoPbs2p-MoHog1p is inactive (left). High osmolarity or salt stress leads to dephosphorylation of the sensor kinases MoSln1p and/or MoHik1p resulting in a decrease of MoSsk1p-phosphorylation. Lower phosphorylation-levels at MoSsk2p activate the MAPK cascade MoSsk2p-MoPbs2p-MoHog1p, MoHog1p translocates into the nucleus and activates the cellular reactions.
reduced ability to accumulate arabitol in the mycelium [20]. In the study presented, osmosensitive “loss of function” (lof) mutants ΔMohog1, ΔMopbs2, ΔMossk2, ΔMossk1 and ΔMoypd1 were cultivated upon osmotic stress, resulting in stable individuals outgrowing from the mycelium of each lof mutant after four weeks of stress. These adapted individuals show a reestablished osmoregulation and produce glycerol and not arabitol as the major compatible solute upon osmotic shock. To the best of our knowledge, we present such an independent and stable adaptation of individuals outgrowing from lof mutants for the first time. Furthermore, we believe that this study will open the door for many research groups, since this rapid adaptation phenomenon will be easily addressable in a broad spectrum of eukaryotic model organisms.

Results

“Adapted” mutants arise out of independent “loss of function” strains upon long-term cultivation on stress

Apart from molecular biology techniques and next-generation sequencing approaches, classical vegetative growth assays were used to characterize potential functions of genes associated with osmoregulation to study the HOG pathway in M. oryzae. Therefore, we cultivated the previously generated lof mutants ΔMohog1, ΔMopbs2, ΔMossk2, ΔMossk1, ΔMoypd1, ΔMohik1 and ΔMosln1 [21] on different stress-inducing media to compare them with the wildtype strain. Mutants with an inactivated HOG pathway are sensitive to osmotic stress and resistant to the fungicide fluioxonil [21]. We noticed in the course of these assays that individual mycelium parts grew out of the sensitive lof mutants ΔMohog1, ΔMopbs2, ΔMossk2, ΔMossk1 and ΔMoypd1 after cultivation for at least four to six weeks under continuous salt stress (Fig. 2).

We isolated these individual mycelium parts in order to separate them as pure cultures ready for further investigations and named them ΔMohog1(adapted), ΔMopbs2(adapted), ΔMossk2(adapted), ΔMossk1(adapted) and ΔMoypd1(adapted). We have not been able to get adapted strains from the mutants ΔMosln1 and ΔMohik1 in the conditions tested so far. That underpins the hypothesis from our previous studies that the two-component hybrid histidine kinase (HK) MoHik1p and the HK MoSln1p can partially take over the function from each other [24] and, thus, the selection pressure may not sufficient for an adaptation event in ΔMosln1 and ΔMohik1. Furthermore, it was not possible for us to isolate adapted strains from HOG pathway-independent osmosensitive Magnaporthe-mutant strains, i.e. ΔMoss1 (transcription factor in cAMP/PKA signaling pathway, ΔMoypd1 (glycerol-3-phosphate dehydrogenase) or ΔMoskn7 (response regulator protein). Of course, we checked in the adapted strains whether the genes originally inactivated in the “parent” lof mutants were still inactivated in order to avoid any possibility of contaminations or confusions about mixed cultures. We did this for all adapted strains using ITS-sequencing and southern blot analyses (Additional file 2: Figure S1).

In empirical investigations, a combination of different vegetative growth assays was used to characterize the “rapid adaptation frequency” in the different lof mutants. Twenty mutant strains of each of ΔMohog1, ΔMopbs2, ΔMossk2, ΔMossk1, and ΔMoypd1 were grown continuously on solid complete medium (CM) including 1 M KCl or 1.5 M sorbitol as stress-inducing agents. Several adapted strains arose out of the lof mutants in each plate, being able to grow much faster under the stress condition. The adapted strains were then transferred onto CM without stressors for the following two weeks. Subsequently, we transferred the colonies onto repeated stress medium to investigate in which strains the “adaptation” is stable and identified the stably adapted strains (Additional file 3: Figure S2). Notably, we were able to identify more adapted strains from ΔMopbs2 and ΔMoypd1 under salt stress than under sorbitol stress. By contrast, we obtained more adapted strains under sorbitol stress than under salt stress from ΔMossk2 and ΔMossk1 (Additional file 3: Figure S2).

Osmoregulation is permanently restored in the adapted mutants

The strains ΔMohog1(adapted), ΔMopbs2(adapted), ΔMossk2(adapted), ΔMossk1(adapted) and ΔMoypd1(adapted)
displayed significant differences in growth speed on salt stress compared to their “parent-strains,” the lof mutants ΔMohog1, ΔMopbs2, ΔMossk2, ΔMossk1 and ΔMoypd1 (Fig. 3 [1]). All lof mutants were strongly sensitive towards 1 M KCl stress, whereas all the adapted strains were less sensitive, similar to the wildtype strain (Fig. 3 [1], lower row, colonies [A]). Particular attention has to be paid to the finding that adapted strains, which were pre-cultivated on normal CM (unstressed conditions) and then transferred back to the repeated salt stress, were found to grow as fast as those taken directly from stress conditions and, thus, being able to adapt to KCl stress immediately (Fig. 3 [1], colonies [C]). In conclusion, the mutations/alterations within ΔMohog1(adapted), ΔMopbs2(adapted), ΔMossk2(adapted), ΔMossk1(adapted) and ΔMoypd1(adapted) appear to be stable. Similar results to the growth assays on solid media could be observed in liquid cultures upon KCl stress (Fig. 3 [2]) and sorbitol stress (Additional file 4: Figure S3).

Glycerol is the major compatible solute produced by the adapted strains after salt shock

Intracellular production of compatible solutes was determined by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and compared to compatible solute production of the lof mutants and the wildtype strain to further investigate the phenomenon of restored osmoregulation in ΔMohog1(adapted), ΔMopbs2(adapted), ΔMossk2(adapted), ΔMossk1(adapted) and ΔMoypd1(adapted). Hyperosmotic shock was imposed by 0.5 M KCl stress towards the fungal strains, and the intracellular levels of the major osmolytes mannitol, trehalose, arabitol and glycerol were determined. No increases in the mannitol and trehalose levels were detected in the lof mutants and the adapted strains after osmotic shock (data not shown). A slight increase in the mannitol level was detected and no significant change for the trehalose level of the wildtype strain (data not shown). Similar to data from [20], arabitol was...
found to be the major intracellular compatible solute produced by the wildtype strain after osmotic shock (Fig. 4).

By contrast, the lof mutants ΔMohog1, ΔMopbs2, ΔMossk2, ΔMossk1 and ΔMoypd1 were not able to produce either arabitol or glycerol in significant amounts. Interestingly, it was found that all the adapted strains ΔMohog1(adapted), ΔMopbs2(adapted), ΔMossk2(adapted), ΔMossk1(adapted) and ΔMoypd1(adapted) responded to hyperosmotic stress by accumulating high amounts of glycerol rather than arabitol (Fig. 4). Based on these observations, we conclude that glycerol may somehow compensate for the lack of arabitol upon salt stress in the adapted strains.

**Fludioxonil susceptibility is restored in the adapted mutants**

Vegetative growth assays were conducted using minimal medium (MM) and MM including 10 μg/ml fludioxonil to further investigate whether fludioxonil-susceptibility and not only osmoregulation is restored in ΔMohog1(adapted), ΔMopbs2(adapted), ΔMossk2(adapted), ΔMossk1(adapted) and ΔMoypd1(adapted) (Fig. 5).

Apart from the osmoregulation capacity, fludioxonil sensitivity was reconstituted in the adapted strains. The HOG pathway lof mutants were resistant towards the fungicide (Fig. 5, colonies [A], lower row), whereas all adapted strains were susceptible, but not quite as strongly as the wildtype strain (Fig. 5, colonies [B], lower row). Similar to the findings concerning salt stress presented previously in Fig. 3, the adapted strains, which were pre-cultivated in unstressed conditions for a long time, showed the same phenotype as the adapted strains taken directly from stress medium (Fig. 5, colonies [C], lower row).
Fludioxonil sensitivity in the adapted strains is not dependent on the production of arabitol or glycerol

Fludioxonil induces a hyperactivation of the HOG pathway and, thus, a continuous stress response towards high osmolarity. We found, by using HPAEC-PAD analytics, that fludioxonil treatment resulted in the production of arabitol and glycerol in the wildtype strain (Fig. 6). We conducted the experiments with the lof mutants and the adapted strains ΔMohog1(adapted), ΔMopbs2(adapted), ΔMossk2(adapted), ΔMossk1(adapted) and ΔMoypd1(adapted) to check whether fludioxonil-dependent production of the compatible solutes was altered in the adapted strain. The fungal cultures were grown in liquid CM (2% glucose) and stressed with 10 μg/ml fludioxonil. As
expected, we did not find increased compatible solute production upon fludioxonil-treatment in the lof mutants. Exemplarily, we present the data for ΔMohog1 (Fig. 6).

Interestingly, no increase of arabitol or glycerol production was detectable in the fludioxonil-susceptible adapted strains in the presence of the fungicide (Fig. 6). It has to be pointed out that the metabolic response of the adapted strains after fludioxonil treatment is different compared to the compatible solute production we observed upon KCl treatment (Fig. 4). All the adapted strains ΔMohog1(adapted), ΔMopbs2(adapted), ΔMossk2(adapted), ΔMossk1(adapted) and ΔMoypd1(adapted) responded to salt stress by accumulating high amounts of glycerol, whereas this is not the case under fludioxonil stress. In conclusion, fludioxonil sensitivity in the adapted strains does not appear to be dependent on compatible solute production.

Reestablished osmoregulation does not complement reduced virulence of the lof mutants

The lof mutants of the HOG pathway in M. oryzae were found to be reduced in virulence towards rice plants compared to the wildtype strain. Interestingly, ΔMohog1(adapted), ΔMopbs2(adapted), ΔMossk2(adapted) and ΔMossk1(adapted) were not found to be as virulent as the wildtype strain, and rather less virulent than the lof mutants (Additional file 5: Figure S4). We were not able to conduct the pathogenicity assays regarding ΔMoypd1(adapted), since the mutant failed to produce conidia exactly like ΔMoypd1 [21].

There are no relevant structural variations on DNA-level in the genomes of adapted strains

We had a deeper look at the genomes of ΔMohog1 compared to ΔMohog1(adapted) in order to find out the cause of that rapid adaptation in the adapted strains. We also added data from the genome sequencing of ΔMopbs2(adapted) to strengthen the analysis and narrow down the outcome of putative candidate genes showing structural variations in the adapted strains. Single nucleotide variations (SNVs) and short indels were detected for ΔMohog1(adapted), ΔMopbs2(adapted) and ΔMohog1 in comparison with the reference sequence of the wildtype strain. The resulting variants were further annotated based on their chromosomal location and biological effects, such as synonymous/non-synonymous single-nucleotide polymorphisms (SNPs), upstream/downstream, untranslated regions (UTRs) and intergenic regions (Additional file 1: SVs summary). The ratio of transition and transversion was also calculated for single nucleotide variation. Over 80% of all SNPs detected were found to be located outside exons and a significant enrichment in regions adjacent to exons and UTRs was detected. Furthermore, in silico protein modelling suggested that several non-synonymous SNPs are probably direct targets of selection, as they lead to amino acid replacements in functionally important sites of proteins. Hence, the structural variation discovery analysis of small-scale (< 20 bp) and large-scale variations (> 20 bp) such as frameshift, stop codon insertion resulted in a list of three genes (five putative gene variants) in the overlap of ΔMohog1(adapted) and ΔMopbs2(adapted), but none of them leads to a protein effect. The variations are only transitions not changing the amino acid composition of the corresponding proteins. Furthermore, we checked the homologous gene loci of the known yeast suppressor mutation genes RSG1 (RHB1) [25], SOO1 [26], SGDi [27] and PMKI (KSSI) [28] intensively. Within all these loci, we could not identify structural variations in the genome of the adapted strains ΔMohog1(adapted) and ΔMopbs2(adapted).

Since regulatory gene elements, such as promoters, are of prior interest regarding their direct influence on gene expression alteration, we decided to investigate the presence of genetic structural variations in the putative promoter regions of all annotated genes in the M. oryzae genome. The promoter region was defined as the region on the genomic DNA 1500 bp upstream of each annotated gene start codon. We performed a structural variation discovery analysis of small-scale (< 20 bp) and large-scale variations (> 20 bp) resulting again in no significant variations in the overlap of the promoter regions of ΔMohog1(adapted) and ΔMopbs2(adapted) (Additional file 6: Figure S5 [A]). We considered here only insertions and deletions as a “polymorphism type” and insertions, truncations and frame-shifts as a “protein effect.”

Differential transcriptomic profiles in the adapted strains in response to osmotic stress

Next-generation sequencing analysis of RNA samples from the wildtype strain, ΔMohog1 and ΔMohog1(adapted) before and after 25 min salt stress (0.5 M KCl) should present insights into transcriptional changes which may be responsible for the adaptation phenomenon observed (methods for cultivation before RNA-isolation, see [29]). A principal component analysis was performed to characterize the relationship between the strains analyzed. The processed transcriptome data of ΔMohog1 (untreated), ΔMohog1 (25 min 0.5 M KCl-stress), ΔMohog1(adapted) (untreated) and ΔMohog1(adapted) (25 min 0.5 M KCl-stress) appears to form distinct clusters within each sample group of strain samples investigated (Fig. 7a).

Since the principal component analysis revealed distinct differences on transcriptomes of corresponding strains, we decided to follow up by clustering the genes according to their expression followed by construction of a co-expression network. In the clustering analysis, represented by a heat map visualization, the fact that the
transcript value is strongly different in $\Delta$Mohog1(adapted) compared to the WT 70–15 and $\Delta$Mohog1, even in untreated conditions, is clearly visible (Fig. 7b). Cluster 6 is significantly exclusively upregulated and cluster 1 is harboring exclusively down-regulated genes in $\Delta$Mohog1(adapted), whereas exclusively up-regulated genes in the WT 70–15 could be found in cluster 2 (Fig. 7b).

**Glycerol metabolism-associated genes are affected in the adapted strains**

To investigate, whether the groups of differentially expressed genes (DEGs) are functionally related, we performed a gene ontology (GO) enrichment analysis to determine the biological functions associated to them. Generally, this approach helps to highlight groups of genes with coherent biological functions that are presumably acting in coordination in response to salt stress. The clustering analysis was employed over the whole gene co-expression network and in a selected subset or cluster of interest. The result indicated that there were several significantly enriched terms of DEGs, from which the most representative are “lipid biosynthetic process,” “glutamine family amino acid metabolic process,” “carbohydrate transport,” “dephosphorylation” and “carboxylic acid biosynthetic process” (Fig. 8).

This enrichment analysis suggests that the highly connected genes from the largest cluster in the network are involved in the regulation of complementary processes triggered by salt stress.

In order to follow up the results of glycerol-production in the adapted strains, we further investigated most of the genes potentially contributing to the production, metabolism or transport of glycerol. However, we checked these genes presented resulting in a list of homologous genes potentially related to the production, metabolism or transport of glycerol from a database- and literature-based approach (Table 1).

The analysis resulted in a set of candidate genes which were found to be upregulated in both the salt stress samples of the $\Delta$Mohog1(adapted) and the wild-type strain, whereas these genes were not regulated in the lof mutant $\Delta$Mohog1 (Table 1, yellow marked). Among these candidates, we identified genes encoding the glycerol H+-symporter MoSlt1p (MGG_09852), one phosphoglycerate mutase (MGG_06642), one glycerol-3-phosphate dehydrogenase (MGG_00067...
(MoGpd1p)) and one phosphatidyl synthase (MGG_00099 (MoHad1p)).

The HOG pathway is not responsible for adaptation in ΔMohog1(adapted)

We searched for possible interactions between MoHog1p and other osmotic stress responsive or associative gene products using the SMART website to find links between putative interaction partners of the MAPK MoHog1p in the adapted strains. The genes identified were finally used to inspect their expression patterns within our set of DE genes (STRING analysis, Fig. 9a).

As expected, the transcript level of the responsive genes belonging to HOG pathway were found to be highly upregulated in case of the wildtype strain in response to osmotic stress (Fig. 9b). Thus, among the genes with the most abundant transcripts were MGG_01822, MGG_08212 and MGG_08547 encoding HOG1, a BZIP transcription factor and CAMKI kinase, respectively (Fig. 9b, c. Meanwhile, none of the HOG1-associated genes, except for MGG_06759 encoding a heat shock protein, were transcriptionally active in ΔMohog1 and in ΔMohog1(adapted) (Fig. 9b). These results have been validated by means of qPCR (Additional file 7: Figure S6). That leads to the conclusion that other mechanisms operating independently/outside of the HOG pathway may be responsible for the phenotype observed in the adapted strains.

Discussion

A central focus in biology is evolution and understanding the processes facilitating or preventing it. Research in this field has to integrate information at the organism level with the sequence level, transcriptional control, epigenetic modulation or cellular differentiation [3]. Despite their complexity, biological networks have an inherent similarity: They are modular with a design that can be separated into single units which perform almost independently. Little is known about the evolutionary origin of these properties. It is of great interest to better
understand how signaling pathways can be modified to generate new biochemical reactions or new morphologies [30]. How are new components integrated into existing signaling pathways and how evolve the pathways themselves? For example, a genome sequence analysis in yeast has identified healthy individuals even though carrying disease-related mutations. A second genomic mutation that can compensate for the detrimental effects of the disease allele may serve as possible explanation, that phenomenon is known as suppression [31]. Most of such suppression interactions are reported between genes that are annotated to the same biological process and thus have related functions [32–34]. This means that suppression appears to be caused by mutations in genes that are functionally similar to the query [8]. Suppression interactions are generally subdivided into two classes: Genomic suppressors and dosage suppressors. The first ones are mutations in the genome that bypass a mutant phenotype, and in the second ones, alteration of gene expression such as overexpression of a suppressor gene rescues a mutant phenotype [31]. Within this study, we found a suitable system to study rapid evolutionary adaptation processes in eukaryotic microorganisms, more exactly in fungal individuals. The physiological role of the HOG pathway is to orchestrate the adaptation of cells to increased osmolarity of the surrounding medium [35]. The lof mutants ΔMohog1, ΔMopbs2, ΔMossk2, ΔMossk1 and ΔMopd1 in M. oryzae are strongly impaired in osmoregulation and are resistant to the fungicide fluodioxonil [21]. We present that long-term cultivation upon high osmolality resulted in stable individuals being restored in osmoregulation outgrowing from each of these lof mutants. Interestingly, the major compatible solute produced by these adapted strains upon salt stress is glycerol, whereas it is known to be arabitol in the wildtype strain (Fig. 4). In order to unravel this phenomenon, DNA- and RNA-Seq analysis was performed in the adapted strains compared to their lof-parent strains. The structural variation discovery analysis leading to protein effect (such as frameshift, stop codon insertion) resulted in only transitions not changing the amino acid composition of the corresponding proteins. Structural variations observed on the genomic landscape may generally have an impact (negative or positive) on the gene expression, which, in turn, may explain the adaptive phenotype observed. We have to distinguish between structural genomic variations seen in exons or CDS and those located in the regulatory elements of the genes (such as promoters, enhancers and terminators). The latter may cause variations in the gene expression, which necessitates the comparative analysis of SNV analysis data with RNA-Seq data (DEGs). On the other hand, the SNV located in CDS and exons is not expected to be reflected in the DEG. The encoded products may have an impact on the secondary and tertiary structure, caused by amino acid replacements, which may lead to reduced or, in some cases, enhanced functionality of the enzyme/protein. We have not been able to identify structural genomic variations in the regulatory elements of the genes so far (Additional file 6: Figure S5 [A]). Of course, we have to increase the number of adapted strains from which we will sequence the genomes to get a higher amount of reliable data to analyze genomic changes in more detail. On
transcriptome-level of the adapted strains, the interaction network conducted by SMART analysis revealed that none of the \( HOG1 \)-associated genes, except for one heat shock protein, were transcriptionally active after salt stress in \( \Delta Mohog1 \) and \( \Delta Mohog1(\text{adapted}) \) in contrast to the \( MoWT \) (Fig. 9). This leads to the conclusion that other mechanisms operating independently of the HOG pathway may be responsible for the phenotype observed in the adapted strains. We found that set of candidate genes potentially contributing to the production, metabolism or transport of glycerol, which is significantly upregulated after salt stress in \( \Delta Mohog1(\text{adapted}) \) and the wildtype strain, whereas these genes were not regulated in the lof mutant \( \Delta Mohog1 \) (Table 1). These results fit well to the increased production of the compatible solute glycerol in the adapted strains in response to salt stress (Fig. 4). The phenomenon of the adapted strains \( \Delta Mohog1(\text{adapted}), \Delta Mopbs2(\text{adapted}), \Delta Mossk2(\text{adapted}), \Delta Mossk1(\text{adapted}) \) and \( \Delta Mopd1(\text{adapted}) \) is different to the yeast suppressor mutants regarding the genes \( RSG1 \) (\( RHB1 \)) [25], \( SOO1 \) [26], \( SGD1 \) [27] and \( PMK1 \) (\( KSS1 \)) [28]. Exemplarily, some Hog1p-dependent osmotic stress-induced gene expression patterns in \( S.\ \text{cerevisiae} \ \Delta hog1 \) mutants could be synthetically rewired under the control of Fus3p/Kss1p MAPKs (PMK1 homologues) [36]. Furthermore, alteration of only one amino acid in Kss1p suppresses the osmosensitive growth phenotype of the \( \Delta hog1 \) deletion mutant [28]. Moreover, it is known that overexpression of the \( GPD1 \) gene partly rescued the osmosensitive phenotype [37] and the gene \( SOO1 \) complements the temperature-dependent osmosensitivity of \( \Delta hog1 \) [26]. Overexpression of \( SGD1 \) partially suppresses the osmosensitivity of \( \Delta pbs2 \) and \( \Delta hog1 \) mutants [27]. In conclusion, the loss of one gene may be compensated by another gene with overlapping functions and expression patterns, as reported for several mutants in a range of model organisms [38]. Thus, disorder of a particular gene’s function in a network may alter the expression of other genes within the same network, thereby maintaining cellular homeostasis [9, 39]. Gene deletion in yeast may result in mutations in one or more genes modulating the pathway.
affected, thereby, partially or fully rescuing the final outcome [40]. However, all of the statements above are based on results found by using the tractable yeast knockout collection and analysis thereof [40], and all findings have only been made in mapping and interpreting the growth rate as a proxy for fitness [8]. By contrast, the current study has the potential to open the door for many research groups worldwide working on the HOG pathway in different eukaryotic model organisms to study epigenetic and evolutionary adaptation mechanisms from a different point of view. It is of the highest study epigenetic and evolutionary adaptation mechanisms in different eukaryotic model organisms to many research groups worldwide working on the HOG pathway.

**Methods**

All data generated or analyzed during this study are included in this published article or deposited at the NCBI GenBank SRA database under accession number PRJNA559166 and Bioproject number 559166.

**Strains, culture/growth conditions**

The fungal strain used in this study was *Magnaporthe oryzae* (*M. oryzae* 70–15 strain (MoWT), Fungal Genetics Stock Center (FGSC)) and the lof mutants of the HOG pathway generated previously [21]. The strains were grown at 26 °C on CM (pH 6.5, 2% agar, containing per liter: 10 g glucose, 1 g yeast extract, 2 g peptone, 1 g casamino acids, 50 mL nitrate salt solution (containing per liter: 120 g NaNO₃, 10.4 g KCl, 30.4 g KH₂PO₄, 10.4 g MgSO₄ × 7 H₂O) and 1 mL of a trace element solution (containing per liter: 22 g ZnSO₄ × 7 H₂O, 11 g H₃BO₃, 5 g MnCl₂ × 4 H₂O, 5 g FeSO₄ × 7 H₂O, 1.7 g CoCl₂ × 6 H₂O, 1.6 g CuSO₄ × 5 H₂O, 1.5 g Na₂MoO₄ × 2 H₂O, 50 g Na₂EDTA, pH 6.5 adjusted by 1 M KOH). The MM (pH 6.5) contains per liter: 1 g glucose, 0.25 mL of a 0.01% biotin solution, 50 mL nitrate salt solution, 1 mL of a trace element solution and 1 mL of a 1% thiamine dichloride solution.

All chemicals used were *p.a.* quality unless stated otherwise.

**Vegetative growth assays**

The antifungal activity and stress tolerance of vegetative growth on agar plates were conducted according to [24]. In order to verify the results, we determined the mycelium dry weight of the fungal colonies grown in liquid culture. The fungal colonies were grown in 250 mL CM including sorbitol or KCl for 6 d at 26 °C and 120 rpm.

**Empirical investigation of the “adaptation” frequency**

In order to determine the adaptation in *M. oryzae*, we conducted a combination of different vegetative growth assays to generate adapted mutant strains to characterize the “adaptation frequency.” We used a panel of 11-day-old lof mutant strains of each of ΔMohog1, ΔMophs2, ΔMossk2, ΔMossk1, ΔMoypd1, ΔMohik1 and ΔMosln1 to inoculate solid CM including 1 M KCl or 1.5 M sorbitol as stress-inducers. The most suitable concentration ranges of the individually selected stressors was chosen based on our previous work in the osmoregulation pathway [21, 24]: The mutants should be clearly restricted in growth but still being able to grow. All fungal cultures have been incubated at 26 °C and a light/dark rhythm of 16/8 h and have been regularly transferred to fresh medium. After 8–12 weeks, we selected all outgrowing mycelium parts (individuals) from this experimental setup and put them on normal CM without stressors for the following two weeks. We then put the colonies on repeated stress medium to investigate in which strains the “adaptation” is stable.

**Plant infection assays**

The plant infection assays were carried out as described previously [24].
HPAEC-PAD analysis to quantify compatible solute production

Osmolytes (sugar alcohols, monosaccharides and disaccharides) were analyzed using HPAEC. They were separated as anions under high alkaline conditions (pH > 12), coupled with PAD. Dried cell extracts were solved (sonicated) in 1 ml sterilized, double-distilled water and centrifuged (4°C, 13,000 rpm). All the samples were filtrated through a 0.2 μm filter. The injection volume was 10 μl. The HPAEC-PAD analysis was performed on a Shimadzu LC system equipped with two LC-10Ai pumps, a DGU-20A degassing unit, a SIL-10Ai autosampler, a CBM-20A controller and a CTO-20AC column oven. An analysis anion-exchange column of CarboPac MA1 (4 × 250 mm) in combination with a guard column of CarboPac MA1 (4 × 50 mm) at 20°C was used. A Dionex ED40 Electrochemical detector was used for the detection of carbohydrates and sugar alcohols in pulsed amperometric mode through standard quadruple waveform (t = 0–0.40 s, p = 1.00 V; t = 0.41–0.42 s, p = −2.00 V; t = 0.43 s, p = 6.00 V; t = 0.44–0.50s, p = −1.00 V). The eluent was prepared as a mobile phase which consisted of a 480 mM NaOH solution. Isocratic elution was performed at 0.4 ml/min. Aqueous sodium hydroxide (50%, w/w) was obtained from Merck, Germany. Standards were purchased from Sigma-Aldrich (Germany) and Carl Roth (Germany). Solutions were prepared in double-distilled water with a concentration of 10 mg/L.

Bioinformatics and data visualization

The DEBrowser recently published was used for data assessment. Firstly, the filtering of the genes was performed. The maximum count for each gene across all samples less than a threshold set to 10 was chosen as the filtering criterium. The application provides a rich and interactive web-based graphical user interface built on R’s shiny infrastructure. The novel method, called “Median Ratio Normalization,” which provides the lower number of false discoveries was used for the normalization of data prior to processing to heat maps and other plots.

The interactive heat map was generated using Clustergrammer [51] which is freely available at http://amp.pharm.mssm.edu/clustergrammer/. Clustergrammer is a web-based tool for visualizing and analyzing high-dimensional data as interactive and hierarchically clustered heat maps. It is commonly used to explore the similarity between samples in an RNA-Seq dataset. In addition to identifying clusters of samples, it also allows one to identify the genes which contribute to the clustering. Prior to displaying the heat map, the raw gene counts were normalized using the logCPM method, filtered by selecting the 500 genes with the most variable expression and, finally, transformed using the Z-score method.

Transcriptome sequencing and differential gene expression

Transcriptome differences in response to salt stress were determined by RNA-Seq using next-generation sequencing. Consequently, the RNA of the strains M. oryzae 70–15, ΔMohog1 and ΔMohog1(adapted) was isolated using the RNeasy Plant Mini Kit (Qiagen). Two conditions were analyzed: Prior salt stress (0 min) and salt stress incubation after 25 min. We collected M. oryzae samples for the transcriptome sequencing from two different growing conditions and used three replicates each: At 0 and 25 min.

The RNA isolated was subsequently quantified using the 2100 Bioanalyzer High Sensitivity DNA kit (Agilent Technologies, Santa Clara, CA, USA) and sequenced in a 150 bpb paired-end run on an Illumina HiSeq 2500. Library preparation was performed by IMSB (Mainz, Germany) using 1.5 μg of total RNA for each strain. The raw fastq reads were paired and aligned to a 70–15 strain reference sequence (assembly version MG8, http://fungi.ensembl.org/Magnaporthe_oryzae/Info/Index) using default parameters with Geneious RNA Mapper (version 11.1.5).

The Integrative Genomics Viewer ( [52, 53]) was then used to visualize the transcripts assembled. The DEGs were identified by the DEseq2 [54]. Genes with an adjustment of p-values by Benjamini Hochberg FDR (FDR < 0.05) and fold change values > 2.5 were considered to be differentially expressed.

Considering the processed RNA-sequencing (Seq) data, a total of 684 differentially expressed genes (DEGs) (176 up- and 508 down-regulated) were identified in the WT 70–15 vs. ΔMohog1 + ΔMohog1(adapted) comparison; while 49 DEGs (23 up- and 26 down-regulated) were identified in the WT 70–15 + ΔMohog1(adapted) vs. ΔMohog1 comparison. Meanwhile, a total of 16 DEGs (9 up- and 7 down-regulated) were present in the WT 70–15 (untreated) + ΔMohog1 (untreated) + ΔMohog1(adapted) (untreated) vs. WT 70–15 (25 min 0.5 M KCl-stress) + ΔMohog1 (25 min 0.5 M KCl-stress) + ΔMohog1(adapted) (25 min 0.5 M KCl-stress) comparison.

RNA isolation, cDNA amplification and qRT-PCR analysis

We conducted a qPCR analysis of selected genes previously found to be strong regulated in our RNA-seq results for validation of the datasets. The M. oryzae cultures were grown for 96h in CM at 26°C and 100 rpm. Each of the cultures were separated into two samples, one mixed with 0.5 M KCl and one an untreated control. Samples for RNA isolation were taken after 25 min. The RNA was isolated from the mycelium samples and the results of transcript abundance given relative to quantification in the MoWT untreated control. The RNA of the MoWT and the mutants was isolated.
SNP analysis
Sequencing reads mapped against the 70–15 reference genome (as .bam files) were used to detect structural genomic variants. Both SNVs and short indels were detected for all strains in comparison with the reference sequence of 70–15. We used the Genome Analysis ToolKit pipeline for SNP calling. The alignment (.bam) files were manually visualized by the Integrative Genomics Viewer to identify commonly associated SNPs. We used VarScan2 and Pindel for the detection of small indels [56]. We used Pindel for large indels and structural variations as it is described as working well on variations between 1 and 1000 nt. BreakDancer-predicted variants were filtered based on mapping quality greater than 25 read, read depths greater than 10 and strand level evidence (at least one read from both directions). SnpEff software was employed to analyze and annotate the resulting SNPs with the M. oryzae sequence and the GFF annotation file (MG8, http://fungi.ensembl.org/Magnaporthe_oryzae/Info/Index) in terms of their chromosomal location and biological effects, such as synonymous/non-synonymous SNPSs, UTRs, intergenic or upstream/downstream.

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s12864-019-6113-3.

Additional file 1: SVs summary.

Additional file 2: Figure S1. Schematic presentation and verification of the MoWT, the lof mutants and the adapted strains by southern hybridization within the Magnaporthe oryzae genome. Southern blot analysis of gene deletion/disruption mutants in M. oryzae with gene specific probes. Genomic DNA of M. oryzae strain 70–15 and the mutants was isolated and restricted with restriction enzymes. The probes which we used for hybridization with the genomic DNA of the wildtype strain and the corresponding mutant strains were always identical.

Additional file 3: Figure S2. Investigation of the “adaptation-frequency” in Magnaporthe oryzae mutants with inactivated components of the HOG signaling cascade.

Additional file 4: Figure S3. Mycelium dry weight of the Magnaporthe oryzae wildtype strain; mutants with inactivated components of the HOG signaling cascade and the “adapted” strains after growth in liquid culture upon sorbitol-stress. The fungal colonies were grown in 250 ml complete medium inclusive 1.5 M sorbitol for 6 d at 26 °C and 120 rpm. Error bars represent the standard deviation of three biological replicates of each strain.

Additional file 5: Figure S4. Pathogenicity assay of the MoWT, the lof mutants and the “adapted” strains. The plant infection assays were carried out as described in experimental procedures. The error bars represent the standard deviation of three experiments with three replicates each.

Additional file 6: Figure S5. VENN diagram of putative structural variations in promotor [A] and in coding sequences [CDS] [B] within the genome of ΔMohog1, ΔMohog1(adapted) and ΔMopbs2(adapted). Numbers in the intersection regions represent overlapping SNPs among the strains. Numbers in parentheses show the corresponding relative percentage of genes harbouring the SNPs.

Additional file 7: Figure S6. qPCR results of selected genes. qRT-PCR analysis of putative genes in MoWT, the “lof” mutants ΔMohog1 and ΔMohog1(adapted). The M. oryzae cultures were grown for 96 h in CM at 26 °C and 100 rpm. Each of the cultures was separated into two samples, one mixed with 0.5 M KCl and one untreated control further grown in CM at 26 °C and 100 rpm. Samples were taken after 25 min. The RNA was isolated from the mycelium samples and the results of transcript abundance given relative to quantification in the MoWT untreated control. Three biological replicates were used of each.

Abbreviations
CM: Complete medium; DEGs: Differentially expressed genes; ERK: Extracellular signal-regulated kinase; GO: Gene ontology; HK: two-component hybrid histidine kinase; HOG: High osmolarity glycerol; HPAEC-PAD: High-performance anion-exchange chromatography with pulsed amperometric detection; lof: “Loss of function”; MAPK: Mitogen-activated protein kinase; MAPKK: MAPK kinase; MAPKKK: MAPK kinase kinase; MM: Minimal medium; PCs: Principal components; SNPs: Single-nucleotide polymorphisms; SNVs: Single nucleotide variations; UTRs: untranslated regions; WT: 70–15; Magnaporthe oryzae wildtype strain

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Authors’ contributions
SB: acquisition, analysis and interpretation of the data; conception and design; writing. LA: analysis of the data; conception, design and design of analytic methods. CC: analysis of the data; conception and design of analytic methods. AT: analysis and interpretation of the sequencing data; conception and design of bioinformatics data. KA: analysis and interpretation of the sequencing data. SJ: acquisition, analysis and interpretation of the data; conception and design; writing. All authors have read and approved the manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article or deposited at the NCBI GenBank SRA database under accession number PRJNA559166 and Bioproject number 559166.

Ethics approval and consent to participate
Not applicable

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.
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