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A Mucoralean White Collar-1 Photoreceptor Controls Virulence by Regulating an Intricate Gene-Network during Host Interactions.

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Abstract: Mucolares are an ancient group of fungi encompassing the causal agents for the lethal infection mucormycosis. The high lethality rates, the emerging character of this disease, and the broad antifungal resistance of its causal agents are mucormycosis features alarming clinicians and researchers. Thus, the research field around mucormycosis is currently focused on finding specific weaknesses and targets in Mucorales for developing new treatments against mucormycosis. In this work, we tested the role of the *white-collar* genes family in the virulence potential of *Mucor lusitanicus*. In this regard, the study of the role in virulence of the three genes of this family, *mcwc-1a, mcwc-1b*, and *mcwc-1c*, resulted in a marked functional specialization, as only *mcwc-1a* was essential to maintain the virulence potential of *M. lusitanicus*. The traditional role of *wc-1* genes regulating light-dependent responses is a thoroughly studied field, whereas their role in virulence remains uncharacterized. In this work, we investigated the mechanism involving *mcwc-1a* in virulence from an integrated transcriptomic and functional approach during the host-pathogen interaction. Our results revealed *mcwc-1a* as a master regulator controlling an extensive gene-network. Further dissection of this gene-network clustering its components by type of regulation and functional criteria disclosed a multifunctional mechanism depending on diverse pathways. In the absence of phagocytic cells, *mcwc-1a* controlled pathways related to cell motility and cytoskeleton that could be associated with the essential tropism during tissue invasion. After phagocytosis, several oxidative responding pathways dependent on *mcwc-1a* were activated during the germination of *M. lusitanicus* spore inside of phagocytic cells, which is the first stage of the infection. The third relevant group of genes involved in virulence and regulated by *mcwc-1a* belonged to the "unknown function," indicating that new and hidden pathways are involved in virulence. The unknown function category is especially pertinent in the study of mucormycosis, as it is highly enriched in specific fungal genes that represent the most promising targets for developing new antifungal compounds. These results essentially unveil a complex multifunctional mechanism used by *wc-1* genes to regulate the pathogenic potential in Mucorales that could also apply to other fungal pathogens.

Keywords: white collar; mucormycosis; virulence; Mucorales; *Mucor lusitanicus*

1. Introduction

Mucormycosis is a threatening infection caused by Mucorales that is the most unexplored host-pathogen interaction [1]. The recent interest in mucormycosis relies on the three main features of this infection: high mortality rates, its emerging character, and its unusual antifungal resistance. The disease presents different forms, including cutaneous, pulmonary, and rhinocerebral. The most common form, the rhinocerebral, is also the most lethal infection [2]. The prognosis of rhinocerebral infections has a mortality rate between

30% and 70%, which increases up to 90% when the infection disseminates to other tissues [3]. The direct cause of these high mortality rates is the lack of effective antifungal compounds against mucormycosis [4]. Only a few years ago, mucormycosis was considered a rare infection affecting immunocompromised patients. However, the continuously increasing number of diagnosed cases and the abundance of new infections affecting immunocompetent patients are making mucormycosis to be considered an emerging disease [5–7]. The fungi of the order Mucorales are resistant to most of the current antifungal compounds [8,9]. The exception is a lipidic formulation of Amphotericin B, one of the first antifungal compounds found in the middle of the 20th century, which has several secondary effects, preventing its use in many cases [10]. Other more modern antifungal compounds, such as isavuconazole and posaconazole of the triazole family, have partial effectiveness depending on the Mucorales species [11]. Thus, the high mortality rates, the increasing incidence, and the recent antifungal resistances found in Mucorales reveal the necessity of new and more effective treatments [12]. In this context, most of the current research is based on searching and characterizing new targets in the physiology of Mucorales that could serve for the development of new antifungal compounds. Different research lines study the role of virulence factors described in Mucorales pathogenesis, such as the CotH proteins, the three components of the high-affinity iron uptake system, and the regulatory proteins involved in dimorphism [13–17]. In addition, several genome-wide strategies have been designed to find new and specific targets in Mucorales, based on the RNAi mechanism of the fungus [18] and in the host-pathogen transcriptome [19,20]. This work aims to study the role of the white-collar-1 (wc-1) genes in the virulence of Mucorales as master regulators controlling many pathways that could represent new promising targets. The family of wc-1 genes has been recently associated with virulence in both Ascomycetes and Basidiomycetes [21–23]; however, it never has been studied in Mucorales.

The wc-1 genes control most of the physiological responses of fungi to light. Mutations in these genes provoke the lack of light-regulated responses like the production of carotenes, the control of the circadian clock, sporulation, and phototropism [24–26]. The structure of the proteins encoded by these genes possesses two specific domains: PAS domains (Per-Arnt-Sim) and a Zinc-finger DNA binding domain. One of its three PAS domains has specialized in a LOV domain (light, oxygen, or voltage). The LOV domain is a light receptor, and the Zinc-finger domain recognizes the elements responding to light found in the promoters of target genes [27]. Thus, the White Collar-1 proteins are both DNA binding transcription factors and light sensors [28]. The role of the wc-1 genes regulating different responses to light has been intensely studied and dissected in several fungi since they were firstly described in Neurospora crassa [29]. However, their role in fungal virulence is a recent discovery described in fungi such as Fusarium oxysporum, Cryptococcus neoformans, and Botrytis cinerea [21–23]. These studies showed that the role of the wc-1 genes in virulence is the same in different fungi, being necessary to maintain full virulence. In addition, wc-1 genes regulate virulence in both plant and animal-specific fungal pathogens. In F. oxysporum, which infects both plant and animal hosts, lack of wc-1 gene only affected virulence in animal hosts [23]. Moreover, the studies made in C. neoformans informed that the role of wc-1 genes in virulence is independent of light [21]. In these studies, a mutational analysis of the different domains in WC-1 proteins showed that light-sensing impaired mutants were still fully virulent [21].

In Mucorales, as stated above, there are no studies relating the wc-1 genes to virulence. However, several studies made in the fungus Mucor lusitanicus showed a detailed dissection of a complex wc-1 genes regulatory system, characterizing all the responses controlled by light and these genes [30–32]. In M. lusitanicus, there are three different orthologous genes compared to the wc-1 gene of N. crassa, which acquired functional specialization concerning the different responses triggered by light [30]. These three genes were denominated mcwc-1a, mcwc-1b, and mcwc-1c. First, mutagenesis analysis of these three genes showed that mutants in mcwc-1c were defective in the carotene production, whereas mcwc-1a mutants lacked the phototropism response [30]. A second study found

that mcwc-1b is involved in regulating carotene production and vegetative growth, but in a more complex pathway, including the negative regulator gene crgA. The CrgA protein is a ubiquitin ligase that represses carotenogenesis by mono- and di-ubiquitylation of Mcwc-1b, which activates carotenogenesis when it is non-ubiquitylated. On the contrary, the non-ubiquitylated form of Mcwc-1b suppresses the asexual sporulation of M. lusitanicus [31–34].

Here, we explored the role of the wc-1 genes in the virulence of Mucorales. Using a murine model for the interactions, we dissected the role of the three mcwc-1 genes of M. lusitanicus, finding that indeed virulence is also dependent on this family of genes. As expected from the functional specialization of the three mcwc-1 genes in the responses triggered by light, virulence is specifically controlled by only one of the three genes, mcwc-1a. After these results, we analyzed the pathways regulated by wc-1 genes and related to virulence, which has not been explored in any other fungi. Our group previously developed a system to study host-pathogen interactions using fungal spores phagocyted by macrophages [35]. In this system, we investigated the transcriptional response dependent on mcwc-1a during phagocytosis, finding an intricate gene network involved in the process. The analysis of this gene network could help to understand the mechanism behind the regulation of virulence by the wc-1 gene family.

2. Materials and Methods

2.1. *Fungal strains and cell cultures*

M. lusitanicus strain CBS277.49 served as the parental strain of all the following strains. Previously generated deletion mutant strains in *white collar-1* genes –*mcwc-1a*Δ (MU242), *mcwc-1b*Δ (MU244), and *mcwc-1c*Δ (MU247) – derived from the leucine and uracil auxotrophic strain MU402. R7B was used as the wild-type control strain for RNA-seq analyses and virulence assays [36] because it harbors the same genetic background and leucine auxotrophy as the *wc-1* mutants, except for the aforementioned deletions. *M. lusitanicus* strain NRRL3631 is an independent natural isolate that was used as an avirulent mock control in the virulence assays [37].

Fungal colonies were grown in a solid, rich YPG medium with pH adjusted at 4.5 and an incubation temperature of 26ºC for optimal growth and sporulation (REF book). Spores were harvested and then filtered using Falcon® 70 μm cell strainers to remove unintentionally harvested mycelium pieces before confronting macrophages or animal models.

2.2. *Virulence assays*

Male OF-1 mice weighing 30g (Charles River, Barcelona, Spain) [14,19,37] were used as animal models for virulence assays. The mice were immunosuppressed by intraperitoneal administration of cyclophosphamide (200 mg/kg of body weight), 2 days prior to infection and then once every 5 days. Ten-mouse groups were challenged intravenously by retroorbital injection of 1×10^6 spores [38]. During this procedure, the mice were anesthetized using isoflurane via inhalation and monitored until they recovered from the anesthesia. Mice were housed under established conditions with free food and autoclaved water. The animal welfare was checked twice a day for 20 days, and those mice following the criteria for discomfort were euthanized by CO_2 inhalation. Survival rates during the time were plotted in a Kaplan-Meier curve (Graph Pad Prism), and differences were considered statistically significant with a p-value ≤0.05 in a Mantel-Cox test.

2.3. *Host-pathogen in vitro assays, RNA purification, and sequencing*

Spores from strains *mcwc-1a*Δ were confronted with a mouse macrophage cell-line (J774A.1 or ATCC TIB-67) in a 1.5:1 ratio as described in previous studies to perform host-pathogen interaction assays [19]. Briefly, the confrontation occurred in L15 medium supplemented with 20% fetal bovine serum (both from Capricorn Scientific) at a temperature of 37ºC for 5 hours to ensure all spores were phagocytosed. As a non-confrontation control

or saprotrophic conditions, the same concentration of spores was cultured in L15 medium supplemented with fetal bovine serum. Samples were prepared in quadruplicate.

Duplicates of each sample were pooled together, and RNA was extracted using the standard procedure of the RNeasy plant minikit (Qiagen, Hilden, Germany), resulting in two replicated RNA extractions per sample that were sent for sequencing to BaseClear (Leiden, The Netherlands). There, mRNA was enriched by poly(A) purification capture, and cDNA libraries were prepared using TruSeq RNA library preparation kits. The cDNA library was sequenced using an Illumina HiSeq 2500 system, producing two replicated raw datasets of first-stranded (or reverse), 50-bp, single-end reads.

2.3. *RNA-sequencing analysis for gene expression*

The quality of the raw reads from *M. lusitanicus* wild-type and *mcwc-1a*Δ single- and co-cultures with mouse macrophages was assessed with FASTQC v0.11.8 before and after removing adapter and contaminant sequences using Trim Galore! v.0.6.2 (available at http://www.bioinformatics.babraham.ac.uk/projects/). Reads with low Phred quality score (Q ≤32) and/or total length (length ≤20 nt) were filtered out, as well as adapter sequences with an overlap longer than ≥4 bases in the 3'-end of the read. These high-quality and adapter-free reads were mapped to the *M. lusitanicus* f. *lusitanicus* v2.0 genome (herein Mucci2 [39], available at https://mycocosm.jgi.doe.gov/Mucci2/Mucci2.info.html) using STAR v.2.7.1a [40]. Read counts were calculated by HTSeq v.0.9.1 [41] excluding multi-mapping reads and used to perform a differential gene expression analysis with the *limma* package v.3.38.3 [42], using robust settings for empirical Bayes statistics and the trimmed mean of M values (TMM) method for sample normalization. Genes were considered differentially expressed (DEGs) when meeting the following criteria used in previous studies [19]: False Discovery rate (FDR) ≤0.05, log2 fold change ($\log_2$ FC) ≥1.0, and average count per million reads (CPM) in log2 units ≥0.0). Average gene expression values (as $\log_2$ CPM) and $\log_2$ FC ratios were correlated among samples with scatter plots, highlighting up-and downregulated genes and three housekeeping genes whose expression is equal among samples: translation elongation factor EF-1 (Mucci2 ID: 156959), RNA polymerase III transcription factor IIIC subunit TFIIIC (Mucci2 ID: 106349), and vacuolar-type H^+-ATPase V-ATPase (Mucci2 ID: 154376).

DEGs were clustered with Canberra distance and Ward's clustering method to plot a heatmap using the pheatmap package v1.0.12 (available at https://cran.r-project.org/web/packages/pheatmap/). Eukaryotic Orthologous Groups (KOG) and Gene Ontology (GO) terms were identified with EggNOG-mapper v2.0 [43,44]. This classification was used to conduct a KOG class enrichment analysis using KOGMWU package v1.2 [45]; the difference between the mean rank of all genes within the KOG class and the mean rank of all other genes (or delta-rank) was calculated with a Mann-Whitney U-test to estimate up- or downregulation of any KOG class. Then, DEGs were compared to the total amount of genes in each KOG class, considering a significative enrichment in that particular KOG class if p-value ≤0.05 in a one-sided Fisher's exact test. GO term enrichment was also analyzed using FungiDB (REF), plotting the enriched and non-redundant GO terms with REVIGO (REF).

3. Results

3.1. *The gene mcwc-1a is essential to maintain the virulence potential in M. lusitanicus.*

The virulence potential of a pathogenic organism is a complex process relying on multiple mechanisms and gene pathways [46,47]. The *white collar-1* genes control different and diverse pathways and physiological processes, and among them, virulence has been recently identified as a WC-dependent feature in pathogenic fungi. We postulate that the family of the three *wc-1* genes of *M. lusitanicus* might also be involved in the virulent potential of this fungus. Thus, the *mcwc-1a*Δ, *mcwc-1b*Δ, and *mcwc-1c*Δ mutants were tested in a survival assay using an immunosuppressed mouse model of infection, previously developed as a valid host model to study mucormycosis [48]. Mice were also infected with

two wild-type control strains: the virulent R7B strain and the avirulent NRRL3631 strain, both previously validated as control strains [35]. The results of these survival assays are presented in Figure 1. The lack of the gene *mcwc-1a* resulted in a significant reduction of the virulence potential in *M. lusitanicus* (Mantel-Cox test, p-value = 0.006). In contrast, lacking any of the other two *wc-1* genes, *mcwc-1b* or *mcwc-1c,* did not alter the virulence potential of *M. lusitanicus* because the corresponding mutants showed a similar pathogenic behavior as the virulent wild-type R7B strain. These results indicated that in the *M. lusitanicus wc-1* gene family, only the gene *mcwc-1a* has a specialized role in virulence. More interesting, these results showed that the role of WCs proteins in virulence is also conserved in early-diverging fungi, specifically in Mucorales.

3.2. A high-throughput transcriptomic analysis reveals the gene-network controlled by mcwc-1a.

After identifying the essential role of the gene *mcwc-1a* in the virulence of Mucorales and its consequent functional conservation throughout the fungal kingdom, we explored the transcriptional response controlled by *mcwc-1a* both during regular growth and macrophage phagocytosis, trying to identify the genes required for the fungal response during the first stage of the host-pathogen interaction. We conducted a deep-sequencing approach (RNA-seq) using mRNA from spores of the wild-type R7B strain and the mutant *mcwc-1aΔ* grown in two different conditions (both in the dark). The first condition mimicked a saprotrophic environment, using the rich medium L15. The second condition was a co-culture of spores and the J774A.1 cell-line of mouse macrophages (1.5:1 spore–macrophage ratio) for 5 hours, ensuring the phagocytosis of all the spores. This second condition is a novel study model in Mucorales to investigate the initial step of fungal infections, in which the pathogen must overcome the innate immune response to continuing the infection and tissue invasion [49]. The scatter plots of gene expression values in the two conditions described above (Figure 2, A and B) revealed that the *mcwc-1aΔ* mutant had highly significant different profiles in the two growth conditions. The analysis showed that a reduced proportion of the genes controlled by *mcwc-1a* are involved in response to phagocytosis, the first step of the fungal infection (Table 1). We selected differentially expressed genes (DEGs) as those surpassing a corrected p-value of less than or equal to 0.05 (False Discovery Rate [FDR] ≤ 0.05) and an absolute $\log_2$ fold change (FC) greater than or equal to 1 ($\log_2$ FC ≥ |1.0|). Thus, a total of 3456 genes (Table S1) were directly or indirectly regulated by *mcwc-1aΔ* (1957 upregulated and 1499 downregulated) during saprotrophic conditions. In contrast, after phagocytosis, only 478 genes showed differential expression controlled by this gene (172 upregulated and 306 downregulated) (Table 1). This analysis revealed the master regulation character of the gene *mcwc-1a* due to the high number of regulated genes. More importantly, the *in vitro* model for the macrophage-spore interaction showed a reduced list of regulated genes that will facilitate the identification of those involved in virulence. Among the genes involved in the response during the macrophage-spore interaction, the 306 downregulated genes could include the primary targets of *mcwc-1a*, with the highest chances of being involved in the loss of virulence.

3.3. Cellular processes regulated by mcwc-1a.

Once the genes regulated by *mcwc-1aΔ* was identified, we performed an enrichment analysis of Eukaryotic Orthologous Groups (KOG), exploring the putative cellular processes controlled by this gene both under saprotrophic conditions (rich medium) and macrophage phagocytosis (Figure 3). This analysis was intended to unveil possible cellular processes that could be involved in virulence and controlled by a transcription factor traditionally related to processes triggered by light. Under saprotrophic conditions without macrophages, several cellular processes showed a significant enrichment. Thus, processes related to the cytoskeleton and cell motility, metabolism and transport of coenzymes, and most importantly, signal transduction are significantly downregulated, probably due to the lack of Mcwc-1a activity. Furthermore, the metabolism of macromolecules (amino ac-

ids, carbohydrates, lipids), energy (oxidation-reduction), and cell wall and membrane biogenesis are altered (most of them showing upregulation). Under conditions with macrophages, the lower number of genes identified probably preclude the detection of cellular processes showing enrichment. Nevertheless, two cellular processes outstood with statistical significance. The first group of genes is classified in the unknown function category, an especially relevant feature of Mucorales, as this group is unusually large compared with other fungi, and most of its components are specific to Mucorales [50]. This feature makes those genes especially relevant to find specific targets for developing new antifungal compounds with no secondary effects in human hosts. The second category contains genes involved in metabolism and inorganic ion transport, which may protect from oxidative damage. This feature could be linked to light responses and its protection to oxidative damage, linking known responses regulated by wc-*1* genes with their influence on virulence.

3.4. In-depth analysis of genes regulated by mcwc-1a during the interaction with the host.

To further delimitate the function of those genes regulated during the interaction with macrophages, we analyzed the distribution of genes after the different regulation types exerted by *mcwc-1a* (Figure 4). After grouping genes under similar regulatory categories, i.e., sharing similar relative expression values, the study was followed by a GO term enrichment analysis (Figure 5).   Only the genes differentially expressed after phagocytosis are shown here, and we have classified them into 4 clusters (Tables S1, S2, S3, and S4). The first one (Figure 4, purple-colored cluster) showed a group of 39 genes that could be directly related to pathogenic processes. It comprises those that are induced in the wild-type virulent strain with macrophages but are downregulated in the *mcwc-1a* mutant with macrophages. Those genes are most likely primary targets of Mcwc-1a and could be directly involved in the virulent response to macrophages. The analysis of GO terms (Figure 5) showed enrichment in antioxidant activity, acting on a group of sulfur donors (which would be related to redox reactions and protection against oxidative damage). Besides, we also found enrichment for signaling receptor activity and molecular transducer activity, which is in congruence with the transcriptional factor activity of WC1a.

The second cluster is the most numerous, counting 267 genes (Figure 4, green-colored cluster). In the wild-type strain, these genes are repressed during the interaction with macrophages and highly downregulated in the *mcwc-1a* mutant, suggesting that they are also primary targets of Mcwc-1a. This could mean that a basal level of expression in these genes might be necessary for fungal development and growth, and the loss of activity could affect virulence and the interaction with macrophages. In the GO term analysis, they are related to iron ion transmembrane transport, which is essential for virulence in Mucorales [51]. In addition, other cellular processes were represented, such as GTPases signaling, phosphotransferase activity, cholesterol delta-isomerase activity, and protein-dimerization activity (Figure 5).

The third cluster contains 118 genes induced in the wild-type strain when phagocytosed but shows further induction in the *mcwc-1a* mutant in the same conditions (Figure 4, yellow-colored cluster). They could be possible secondary targets of Mcwc-1a, and their expression could be contained/repressed by the primary targets of Mcwc-1a. Overexpression of these genes and their corresponding cellular functions could misspend energy during the interaction, affecting the virulence potential. This cluster is enriched in genes involved in several categories of metabolism, such as acetyl-coA, carboxylase activity, and transporter activity (Figure 5). These results could explain the upregulation of macronutrient metabolism observed in the *mcwc-1a* mutant, described in the previous analysis (Figure 3).

The fourth cluster contains 54 genes showing analogous expression to the third cluster, similarly induced in the wild-type and mutant strains in saprophytic conditions, but in this case, it is observed further increase in the mutant during phagocytosis (Figure 4,

red color cluster). Similarly, these genes seem to be secondary targets regulated by primary genes activated by *mcwc-1a*. In this case, the metabolism of porphyrins (porphobilinogen synthase) outstands in the GO term analysis (Figure 5). Porphyrins are required for the heme groups, which are necessary for redox reactions; therefore, playing an essential role in oxidative damage protection [52].

3.5. Figures, Tables and Schemes

Table 1. Differentially expressed genes in the *mcwc*-1aΔ mutant strain compared to the wild-type strain.

Culture conditions	Upregulated genes [1]		Downregulated genes [2]	
	#	Average $\log_2$ FC [3]	#	Average $\log_2$ FC [3]
L15 5h 37 ºC	1957	2.53 ± 1.48	1499	-1.84 ± 0.86
L15 5h 37 ºC + Mφ	172	1.71 ± 1.10	306	-1.62 ± 0.61

[1] FDR ≤ 0.05, $\log_2$ FC ≥ 1.0, average $\log_2$ CPM ≥ 0.0; [2] FDR ≤ 0.05, $\log_2$ FC ≥ -1.0, average $\log_2$ CPM ≥ 0.0; [3] Average of all $\log_2$ fold change values and standard deviation.
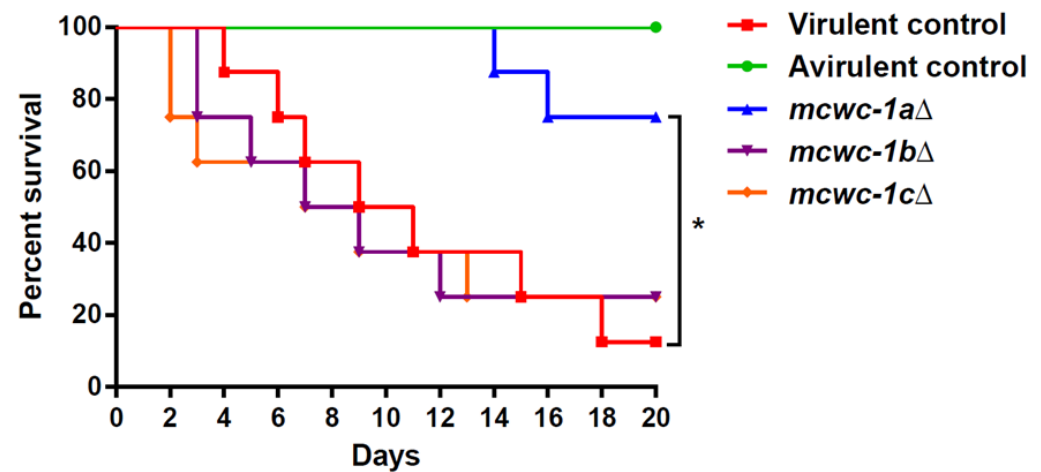
Figure 1. The gene *mcwc-1a* is essential to maintain the virulence potential. Virulence assays in immunosuppressed mice injected with 1×10^6 spores of the mutants in the three *wc-1* genes of *M. lusitanicus*: *mcwc*-1aΔ (blue), *mcwc*-1bΔ (purple), and *mcwc*-1cΔ (orange). In addition, two control strains were also injected: the virulent wild-type strain R7B (red) and the avirulent wild-type strain NRRL3631 (green). The survival rate of the mutant was compared to the virulent control strain and statistically analyzed by a Mantel-Cox test. (*, p-value = 0.006).
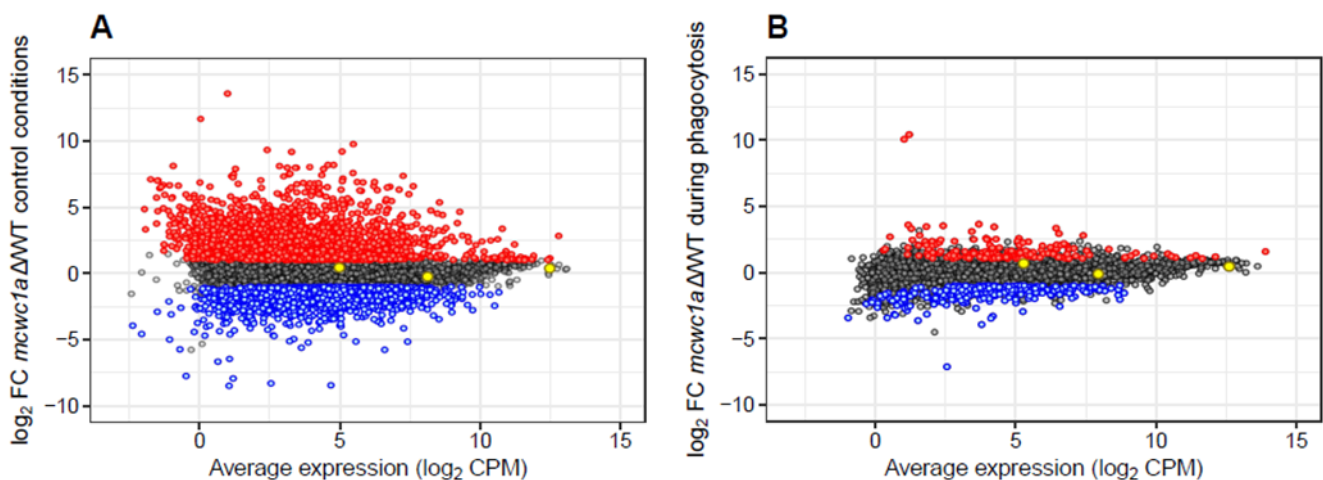
Figure 2. Scatter plots of gene expression values in the *mcwc-1aΔ* mutant at 5 hours during regular growth (A) and macrophage phagocytosis (B) compared to gene expression in the wild-type strain. Each dot represents the expression value of any given gene as CPM (in $\log_2$ units, x-axis) and the fold change (FC) compared to the wild-type expression value (in $\log_2$ units, y-axis). Genes color-coded as red or blue indicate upregulated or downregulated significant genes (FDR ≤ 0.05, average $\log_2$ CPM > 0, $\log_2$ FC ≥ |1|), respectively. Three housekeeping genes –encoding EF-1, TFIIIC, and V-ATPase– are shown colored as yellow to assure proper normalization among samples.
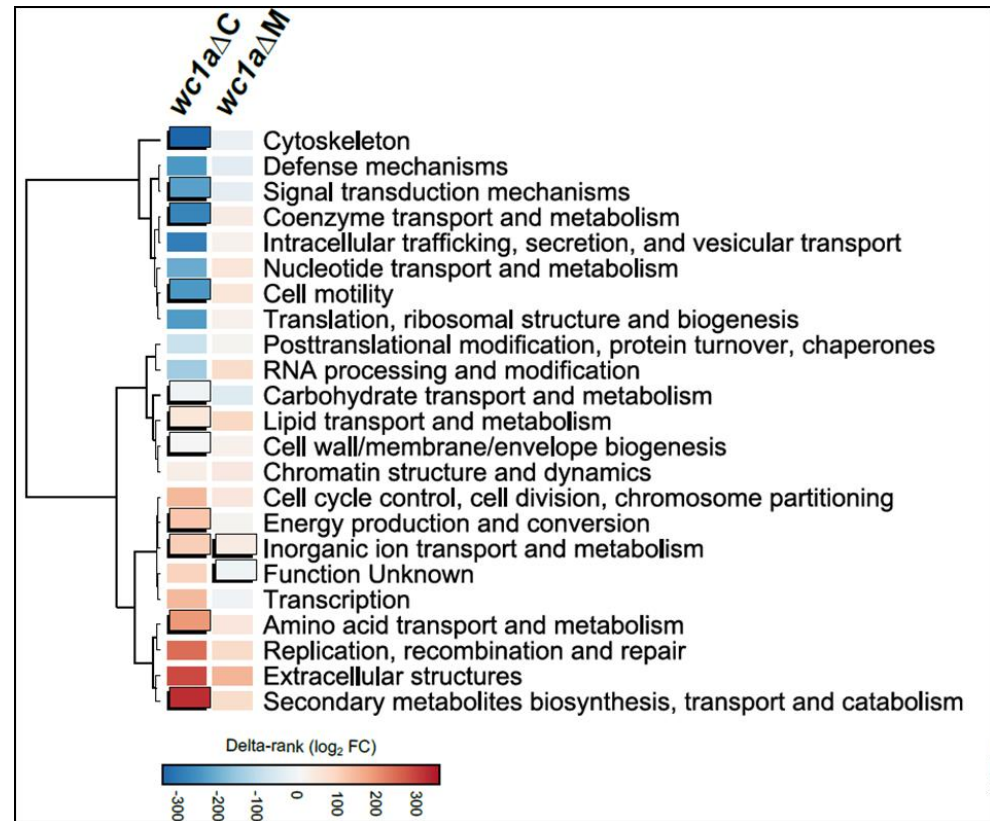
Figure 3. Significant genes during regular growth (wc1aΔc) and macrophage phagocytosis (wc1aΔM) were analyzed for KOG class enrichment. Significant enrichments (Fisher's exact test, P ≤ 0.05) are shown as uplifted rectangles. A measure of up-or downregulation for each KOG class is indicated according to a colored-scale of delta-rank values (the difference between the mean rank differential expression value of all genes in a particular KOG class and the mean rank differential expression value of all other genes). KOG classes are clustered according to the similarity of their delta rank values.
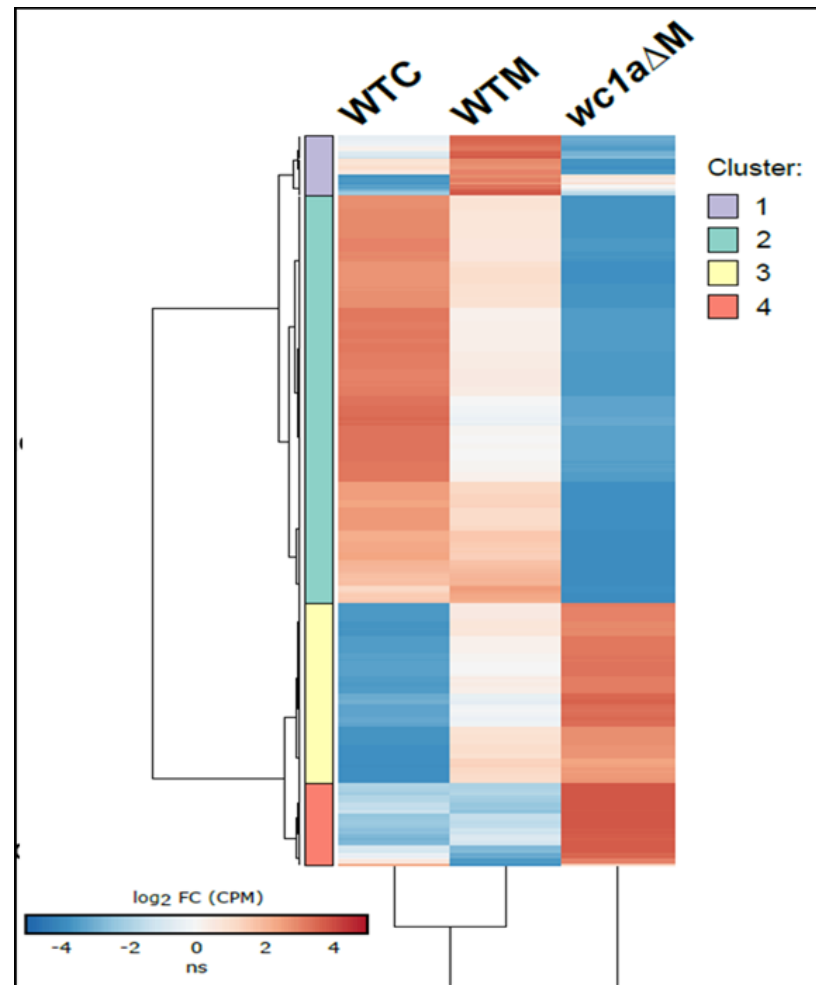
Figure 4. The expression values (calculated as the Z-score of CPM) of significant genes during macrophage phagocytosis are clustered by similarity to analyze phagocytosis response in the *mcwc-1a*Δ mutant. Expression values for wild-type strain in control conditions (WTC) and during macrophage phagocytosis (WTM), as well as for the *mcwc-1a*Δ during macrophage phagocytosis (*wc1a*ΔM), are shown. Four major clusters were identified and shown as colored blocks that indicate the full-length of the clusters.
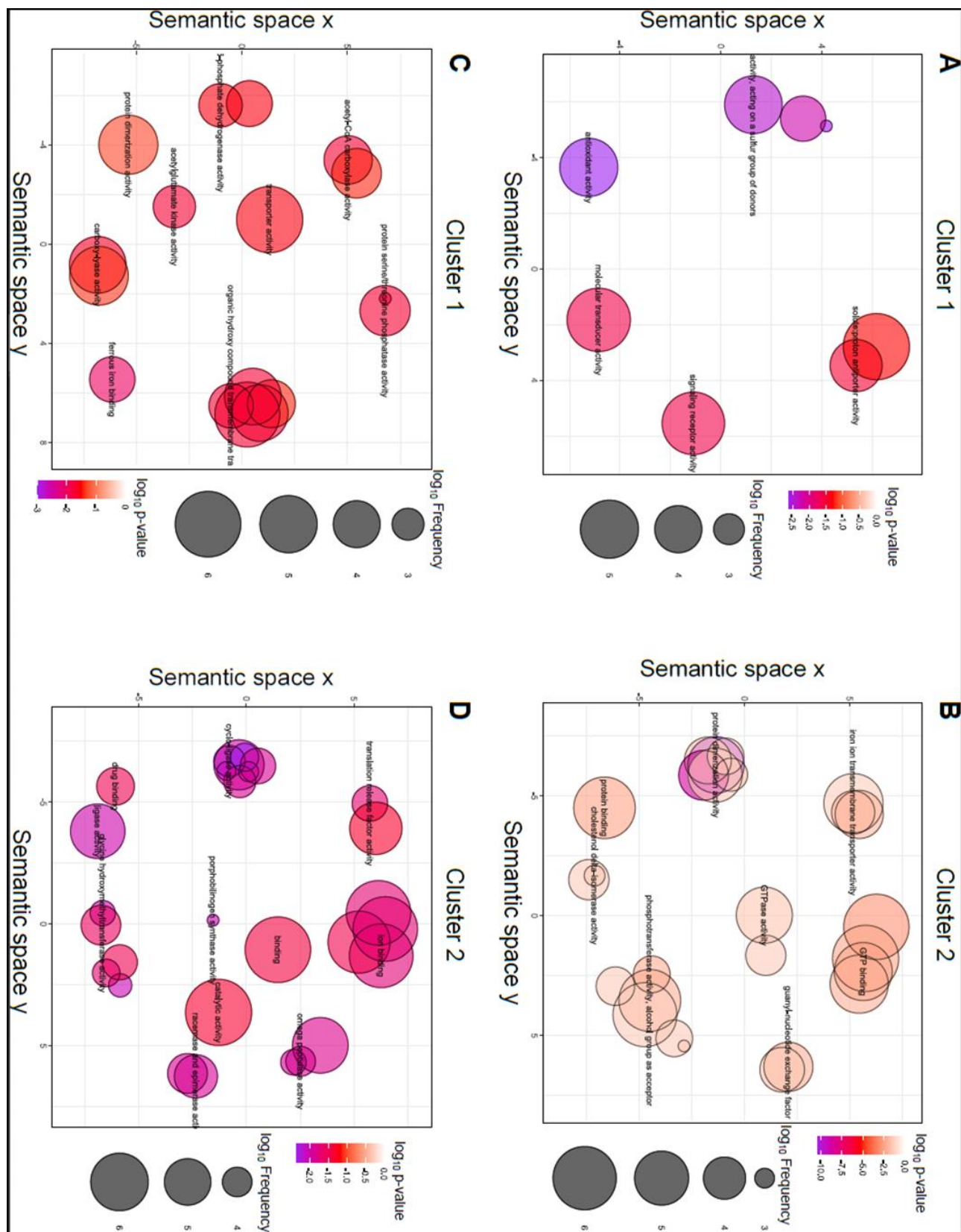
Figure 5. Gene Ontology molecular function terms enrichment in the *mcwc*-1a-dependent response to macrophage phagocytosis. (A-D) Each dot in the scatter plots represents a molecular function GO term, color-coded to indicate the p-value of the enrichment and size-coded to show the frequency of the term in the whole gene set, both in $\log_{10}$ units. The plots show GO terms remaining after redundancy reduction. The axes display a bidimensional space that shows semantic similarity among GO terms, i.e., GO terms close together are involved in similar GO molecular functions. Each plot shows GO molecular function enrichment in the genes comprised in clusters 1 (A), 2 (B), 3 (C), and 4 (D).

4. Discussion

We analyzed the role of the three mc*wc-1* genes during host-interaction and showed that *mcwc-1a* is involved in the virulence potential of *M. lusitanicus*. Previous works demonstrated the role in virulence of *wc-1* genes in different fungi of Basidiomycota and Ascomycota divisions [21–23]. Our results completed the analysis of the *wc-1* genes in the evolutionary tree of pathogenic fungi, showing its highly conserved function from the early-diverging fungi Mucorales to the more recent Basidiomycetes. Besides the high conservation, the essential role of *wc-1* genes in both plant and animal fungal pathogens made evident the fundamental functions of these genes in the transition from saprophytic to pathogenic fungi, independently of the host type. In the specific case of the exceptional fungus *F. oxysporum*, which infects both plants and animals, the *wc-1* gene controls virulence only in animal hosts, suggesting that different genes and pathways assumed the virulent role in plant hosts [23].

The *wc-1* genes are a photoreceptor family that controls most of the physiological changes triggered in the mycelium after the light exposition. In a saprophytic environment, like a soil rich in organic material, the mycelia that reach the surfaces encounter the stressing conditions of sunlight and air exposition. The rapid response controlled by the *wc-1* genes activates the production of secondary metabolites like carotenes as protection for the UV light damage and the production of asexual spores for air spreading over a large area. In this context, it is difficult to relate the pathways and processes controlled by WCs proteins with virulence regulation. However, the functional dissection of the WC structural domains has enlightened the evolution of the WC proteins that resulted in virulence regulation. Two independent studies demonstrated in two different fungal models, *C. neoformans* and *Fusarium asiaticum*, that the photoreception domains are not required in virulence, whereas the DNA binding zinc-finger domains are essential for full virulence [21,53]. These results explain why the *mcwc-1b* gene of *M. lusitanicus* is not involved in virulence because this gene only encodes for the photoreceptor domain and lacks a zinc-finger domain, being involved in the regulation of carotenogenesis [30]. The three *wc-1* genes of *M. lusitanicus, mcwc-1a, mcwc-1b,* and *mcwc-1c,* acquired a specific functional specialization. Thus, *mcwc-1a* regulates phototropism and virulence, whereas *mcwc-1b and mcwc-1c* control carotenogenesis by ubiquitination or light induction, respectively [31,32]. The existence of gene families with different paralogues exhibiting a functional specialization has been described several times in *M. lusitanicus* [51,54–57]. A study in the fungus *F. asiaticum* reported a similar functionalization of the *wc-1* genes, showing that only one of its two WC proteins is involved in virulence [23].

Once the conservation of the essential role of WCs in virulence has been established in Mucorales, the question to resolve is the mechanism used by these proteins to regulate virulence. In *F. oxysporum*, a hypothesis based on the production of secondary metabolites under dark conditions was suggested as a possible explanation for the lack of virulence in the *wc-1* mutant [23]. Other studies showed that the production of gibberellins and bikaverins required a WC-1 protein in a light-independent manner in the fungus *Fusarium fujikuroi*, but without a clear link to virulence [58]. In *M. lusitanicus*, the genes *mcwc-1b* and *mcwc-1c* regulate the production of carotenes (which are the main secondary metabolites) without affecting virulence. However, these results do not discard the regulation of *mcwc-1a* of other unknown secondary metabolites with a putative role in virulence. Our results showing the functional specialization of the three *wc-1* genes in *M. lusitanicus* could suggest another hypothesis for its role in virulence. The gene related to virulence, *mcwc-1a*, also controls phototropism. Tropism is an essential process for growth and sporulation and also could be related to the pathogenic potential because it might control the hyphae movement during tissue and blood vessel invasion for dissemination. Thus, a set of genes involved in tropism and controlled by *mcwc-1a* could link to virulence.

Independently of how the WC-dependent mechanism works regulating virulence, all the studies conclude that finding the WC-dependent genes is the necessary next step to understand this mechanism.   Our results investigating the gene-network controlled by

mcwc-1a showed a substantial number of genes (almost one-third of the total predicted genes) differentially expressed during saprotrophic growth conditions. The general interpretation of these results clearly defines the *wc-1* genes as master regulators controlling the development of *M. lusitanicus*. However, a more specific and reduced set of genes was found differentially expressed during the interaction with macrophages, in a context simulating the first stage of the infection. A detailed analysis clustering all the differentially expressed genes and dissecting their putative functional associations led us to suggest three non-exclusive hypotheses to explain the role of WCs controlling virulence. First, we have found repetitively groups of genes in different clusters involved in the protection against oxidative damage. This process is an essential fungal function required both after the exposition to the light and during the macrophage confrontation, linking the traditional response of WCs with its role in controlling virulence. Thus, our results suggested that *mcwc-1a* might be influencing the oxidative-damage protection pathways in the two scenarios. Secondly, we found an enrichment of differentially expressed genes with unknown functions during the interaction with the macrophages. These results indicated that besides the protection against oxidative stress, *mcwc-1a* must be controlling other cellular processes with essential roles in the survival inside the macrophages. This group of unknown function genes in Mucorales contains a large proportion of unique genes not found in other organisms [50], which makes them the perfect candidates to find targets for new antifungal compounds. Finally, our results showed an important enrichment of genes involved in cell motility and cytoskeleton out of the interaction with macrophages. These results could suggest a role of *mcwc-1a* in the second stage of the infection, during the tissue invasion, angioinvasion, and dissemination of the infection. This stage could be a link to essential processes related to tropism, cell motility, and cytoskeleton-reorganization functions. These could be the first results suggesting a link between the regulation of phototropism and virulence by Mcwc-1a in a light-independent manner.

The virulence potential of fungal pathogens depends on complex mechanisms controlled by diverse pathways and cellular processes. The intricate gene-network controlled by *mcwc-1a* described in this work first enlightens the oxidative stress, tropism, and a group of unknown functions as the main processes that this master regulator might control to regulate virulence potential in fungal pathogens.

strain in the same conditions, previously available at GEO [19] under the following sample accession numbers: GSM3293661 and GSM3293662 (wild-type strain single-cultured); and GSM3293663 and GSM3293664 (wild-type strain co-cultured with mouse macrophages). The Mucci2 [39] genome and annotation files can be accessed at the Joint Genome Institute (JGI) website (http://genome.jgi.doe.gov/) and used under the JGI Data Usage Policy.

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