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Tesis Doctoral

**Molecular mechanisms regulating plant infection in the rice
blast fungus *Magnaporthe oryzae***

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**La vida trata de momentos. No los esperes,
créalos.**

A mi familia

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LIST OF ABBREVIATIONS

%	Percentage
ATMT	<i>Agrobacterium tumefaciens</i> -mediated transformation
ATP	Adenosine triphosphate
AVR	Avirulence
BAS	Biotrophic-associated secreted
BIC	Biotrophic interaction complex
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
°C	Degree Celsius
cAMP	Cyclic 3',5' adenosine monophosphate
cDNA	Complementary DNA
CFW/CW	Calcofluor White
cm	Centimeter
CM	Complete medium
CR	Congo Red
DBD	DNA-binding domain
dH ₂ O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
dpi	Days post-inoculation
EIHM	Extra-invasive hyphal membrane
ER	Endoplasmic reticulum
EST	Expressed sequence tag
ETI	Effector-triggered immunity
EXP5	Exportin-5
f2dU	5-fluoro-2'-deoxyuridine
g	Grams
g L ⁻¹	Grams per litre
GFP	Green fluorescent protein
GPCR	G- protein-coupled receptor
GTP	Guanosine triphosphate
H ₂ O ₂	Hydrogen peroxide
h	Hour
H1	Histone1
hpi	Hours post-inoculation
HR	Hypersensitive response
HYG	Hygromycin
IH	Invasive hyphae
Kap	Karyopherin
Kb	Kilobase
KO	Knock-out
LRT	Likelihood ratio test
µg	Microgram
µL	Microlitre
µm	Micrometre
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase

MAPKKK	Mitogen-activated protein kinase kinase kinase
mg	Milligram
mL	Millilitre
mm	Millimetre
mM	Millimolar
MM	Minimal media
MM-C	Minimal media without carbon source
MM-N	Minimal media without nitrogen source
mRNA	Messenger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NBS-LRR	Nucleotide binding site - leucine-rich repeat
NES	Nuclearexport signal
ng	Nanogram
NLS	Nuclear localisation signal
ORF	Open reading frame
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PFAM	Protein Family
PKS	Polyketide synthase
PRR	PAMP-recognition receptor
PTI	PAMP-triggered immunity
qRT-PCR	Quantitative reverse-transcription polymerase chain reaction
RFP	Red fluorescent protein
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
ROS	Reactive oxygen species
rpm	Revolutions per minute
SD	Standard deviation
SUR	Sulfonylurea
T-DNA	Transfer DNA
tRNA	Transfer RNA
TPC1	Transcription factor for polarity control1
WT	Wild type

RESUMEN

Magnaporthe oryzae es un hongo ascomiceto filamentoso que produce la enfermedad de la piriculariosis del arroz. Este estudio se ha centrado en la caracterización de dos mutantes de T-DNA de *M. oryzae* afectados en patogénesis, M1422 y M1373. M1422 está afectado en un gen que codifica un factor de transcripción implicado en control de polaridad (Transcription factor for polarity control 1; Tpc1). El mutante M1373 carece de una proteína implicada en el transporte núcleo-citoplásmico, la exportina-5 (Exp5).

Tpc1 es un regulador transcripcional con un dominio de unión a ADN del tipo Zn(II)₂Cys₆ que sólo se encuentra en hongos filamentosos. La caracterización del mutante $\Delta tpc1$ ha permitido la identificación de su posible mecanismo de acción, mediante la regulación de especies reactivas de oxígeno y el control del complejo NADPH oxidasa. Los procesos de oxidación-reducción están afectados significativamente en el mutante $\Delta tpc1$, debido a que el gen *NOXD*, homólogo funcional de la subunidad p22^{phox} de la NADPH oxidasa humana está reprimido. El mutante $\Delta noxD$ en *M. oryzae* no es capaz de infectar ni hojas ni raíces de arroz. Además este mutante muestra defectos en la repolarización de los filamentos de actina durante la formación del apresorio y en el proceso autofágico, el cual es esencial para la penetración de hojas.

Experimentos de inmunoprecipitación han permitido identificar diferentes vías de participación de Exp5 en la virulencia del hongo. Nuestros resultados demuestran que Exp5 interacciona con Rac1, una pequeña GTPasa involucrada en la organización de los filamentos de actina y el crecimiento polarizado, ambos esenciales para la infección fúngica. Exp5 interacciona además con dos proteínas quinasas activadas por mitógenos necesarias para la formación y penetración del apresorio, Pmk1 y Mps1. Para el estudio de la participación de Exp5 en el metabolismo del ARN, se secuenciaron librerías de pequeños ARN de la estirpe silvestre y del mutante $\Delta exp5$. Estos experimentos mostraron que la mayoría de los transcritos pequeños en $\Delta exp5$ se encuentran en menor número que en la estirpe silvestre, con excepción de los que derivan específicamente de elementos transponibles, que se encuentran en mayor número en $\Delta exp5$. Experimentos Northern realizados con sondas para retrotransposones como MAGGY y RETRO5, y tARNs mostraron que el mutante tiene alterados su distribución subcelular, lo que sugiere que Exp5 participa en la translocación de transcritos pequeños derivados de

estos elementos. El análisis de pequeños ARNs ha llevado de forma inesperada a la identificación de un nuevo virus ARN en *M. oryzae*. La proteína RNA polimerasa dependiente de RNA del nuevo virus, muestra similitudes con los micovirus del tipo ourmia y con el género de virus de plantas ourmiavirus. Por esta razón se le ha llamado “Magnaporthe oryzae ourmia-like virus” (MOLV1).

SUMMARY

M. oryzae is an ascomycetous filamentous fungus that causes one of the most devastating diseases of rice, the rice blast disease. This study is focused on the characterization of two T-DNA mutants with reduced virulence, M1422 and M1373. The M1422 mutant is affected in a gene that encodes the transcription factor for polarity control 1 (Tpc1). The M1373 mutant lacks the orthologue protein of the nucleo-cytoplasmic transporter exportin-5 (Exp5).

Tpc1 is a transcriptional regulator that contains a Zn(II)₂Cys₆ binuclear cluster DNA-binding domain, which is found exclusively in the fungal kingdom. The characterization of the $\Delta tpc1$ mutant has allowed the identification of a putative mechanism by which it acts, via the regulated synthesis of reactive oxygen species and control of the NADPH oxidase complex. Oxidation-reduction processes are significantly affected in $\Delta tpc1$, likely due in part to the down-regulation of the fungal homologue of the p22^{phox} NADPH subunit, the *NOXD* gene. *M. oryzae* $\Delta noxD$ mutant is unable to infect rice. Furthermore, $\Delta noxD$ defects in repolarization of the F-actin cytoskeleton during infection-related development show that Tpc1 is required for penetration, and is also required for infection-associated autophagy.

The karyopherin Exp5 in *M. oryzae* is also necessary for full disease symptoms production. Immunoprecipitation assays have identified several pathways that link Exp5 with fungal pathogenicity. Exp5 interacts with Rac1, a small GTPase involved in actin cytoskeleton organization and polarized cell growth, essential processes required for appressorium formation and function. Exp5 also interacts with two mitogen-activated protein (MAP) kinases that are involved in appressorium-mediated penetration and invasive growth, Pmk1 and Mps1. To ascertain the involvement of Exp5 with RNA metabolism sRNA libraries from wild type and $\Delta exp5$ strains have been generated and sequenced. Overall, small transcripts are down-regulated in $\Delta exp5$ except those derived from specific transposon elements. Northern blots using sRNA probes for the retrotransposons MAGGY and RETRO5 and tRNA^{Met} suggest that Exp5 is involved in their translocation.

In addition, the analysis of the sRNA libraries led us to the discovery of a new RNA virus in the rice isolate Guy11. The viral protein sequence shows similarities with *Ourmia-like* mycoviruses, and plant

viruses from *Ourmiavirus* genus. For this reason this novel RNA virus has been named *Magnaporthe oryzae ourmia-like virus* (MOLV1).

CHAPTER 1

GENERAL INTRODUCTION

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Major plant pathogens of the Magnaportheae family
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The Magnaporthaceae Family

The Magnaporthaceae is a small ascomycetous family that comprises 17 genera and nearly a hundred fungal species (Kirk, *et al.*, 2008, Thongkantha, *et al.*, 2009). This family belongs to the class of Sordariomycetes (Cannon & Kirk, 2007). Several fungal species of the Magnaporthaceae family cause devastating diseases on cereals and grasses, such as the causal agent of take-all disease of wheat *Gaeumannomyces graminis*, the rice blast fungus *Magnaporthe oryzae* and *Magnaporthe poae*, which causes the grey leaf spot on turfgrasses.

Gaeumannomyces and *Magnaporthe* species were grouped in the Magnaporthaceae family based on the common morphology of their teleomorphs and similarities in host range (Cannon, 1994). The teleomorph is the sexual form (reproductive stage) of a fungus, while the anamorph is its asexual form. It has been a common practice to name differently the anamorph and teleomorph of a particular fungus. Based on the Article 59 of the International Code of Botanical Nomenclature, a particular fungal species with both reproductive stages, the teleomorph name takes prevalence over the anamorph name (Hawksworth, 2011). However, molecular phylogenetic approaches and whole genome sequences have revolutionised the taxonomy world, and this dual nomenclature rule based on morphological features is going to disappear. The rule “one fungus, one name” was approved at the Melbourne International Botanical Congress in 2011 (Hawksworth, 2011).

The blast fungus has received several names since its isolation and characterization. The first original report describing the fungus *Pyricularia grisea* as the causal agent of grey leaf spot on the grass *Digitaria sanguinalis* appeared in 1880 (Saccardo, 1880). Since then, scientists working on blast disease have been using four different names to refer to rice-infecting blast isolates (*P. grisea*, *P. oryzae*, *M. grisea* and *M. oryzae*). To gain clarification on the taxonomy of *Magnaporthe* species, rice and all the other grass isolates have been included within the *M. grisea* species complex (Couch & Kohn, 2002), which contains two intersterile groups. The lineage *M. grisea* has been kept for fungal isolates associated with the host grass *Digitaria* spp. The second lineage contains rice and related fungal isolates from *Triticum*, *Avena* and *Setaria* and has been renamed as a new species, *M. oryzae*, although no morphological differences exist between isolates of these two groups. Whole genome sequencing approaches have corroborated this lineage (Chiapello, *et al.*, 2015). There is an important debate about the maintenance of the genus

Magnaporthe or *Pyricularia* for the rice blast fungus among community members (<http://www.magnaporthe.blogspot.com.es/>). In this thesis, the name *Magnaporthe oryzae* has been kept for the blast isolates that infect rice, following the taxonomic classification by Couch & Kohn (2002).

Rice Blast Disease: An Important Constraint to Rice Production

Rice (*Oryza sativa* L.) is one of the most important cereal crops and staple diet of more than three billion people. Fungal blast is considered a major threat to rice crops and costs farmers a loss of nearly \$5 billion a year (Skamnioti & Gurr, 2009). Not surprisingly, it accounts for the world's largest fungicide market. The Japanese market alone for blast fungicides is estimated at US\$400 million per year (Skamnioti & Gurr, 2009).

Rice blast is caused by the fungus *Magnaporthe oryzae* (Couch & Kohn, 2002), and this fungal species can also cause diseases in other staple food crops including finger millet, maize and wheat, representing a serious risk for food security globally and a significant challenge in developing countries (FAO, 2009). The damage produced by blast in rice crops oscillates between 10 and 30 % every year. Under disease-conducive conditions, the fungus can destroy the entire crop (Thinlay, *et al.*, 2000). Rice blast is present in all rice-growing areas worldwide, including Western Australia where rice-growing areas were free of this disease until 2012 (You, *et al.*, 2012). It is also present in Spain, the second rice producer in Europe after Italy (MAGRAMA, 2016). *M. oryzae* can also cause serious yield losses in wheat fields. *M. oryzae* wheat blast epidemics were first seen in Brazil

Fig. 1.1 Rice blast disease symptoms. (a) Fungal vesicles and (b) microsclerotia produced on root surfaces. (c) Cross section of a barley root infected with a GFP-tagged *M. oryzae* strain showing heavy colonisation of the vascular system. (d) Necrotic blast lesions of a 15-day-old rice seedling infected with *M. oryzae*. (e) Leaf blast symptoms. (f) Panicle blast in the field. (g) Neck blast symptoms.

(Urashima, *et al.*, 2004), and the disease has expanded progressively within South America. More recently, this fungal pathogen has emerged in Asia, causing severe epidemics in Bangladesh (Islam *et al.*, 2016).

Rice blast is a polycyclic disease since the fungus can undergo multiple infection cycles during a rice-growing season. High humidity or long periods of rain followed by relatively warm temperatures favour spore germination and fungal penetration (Ou, 1985). Wind-dispersed or water-splashed conidia are the main source of inoculum in the field (Ou, 1985). However, *M. oryzae* can overwinter on alternative weed hosts and plant debris for almost 3 years, playing possibly an important role in the epidemiology of the disease (Harmon & Latin, 2005). This fungus can form resting structures on roots and plant debris such as microsclerotia and vesicles, which can germinate even after 4 years of dormancy (Gangopadhyay & Row, 1986, Sesma & Osbourn, 2004) (Fig. 1.1a, b). *M. oryzae* can penetrate rice roots and spread through the vascular system to the aerial parts of the plant to produce blast disease symptoms (Fig. 1.1c, d), although the relevance of the underground infection process under field conditions has not been proven yet (Sesma & Osbourn, 2004, Besi, *et al.*, 2009, Marcel, *et al.*, 2010). Domestic travellers and the transport of infected material (souvenirs made with seeds, weeds or rice straw) probably also contribute to the dissemination of the disease (You, *et al.*, 2012). PCR-based methods have been developed for detection of the fungus, offering a quick method to control the dissemination of infected material (Harmon, *et al.*, 2003).

In the field, rice blast disease symptoms are visible at any growth stage and at any part of the aerial plant tissue: leaf, collar, nodes, panicle neck and panicles (Fig. 1.1e–

Fig. 1.2 Age-dependent rice susceptibility to *M. oryzae*. (A) 3-week-old leaf (left) and 2-week-old leaf (right) of Nipponbare infected with FR13 isolate. (B) Fertilizer greatly increases susceptibility in the Maratelli/IT20 interaction: without fertilizer (left). (C) Susceptibility symptoms in the rice/*M. oryzae* isolates. From left to right: Nipponbare/FR13 isolate, IR64/PH14 isolate, Nipponbare/CL26 isolate and IR64/CL26 isolate. Images taken from (Ribot, *et al.*, 2008).

g). The shape, colour and size of the lesions largely depend on the rice cultivar, the age of the lesion and environmental conditions (Ou, 1985, Ribot, *et al.*, 2008) (Fig. 1.2). On leaves, blast lesions are eyespot shaped with white to grey colour and surrounded by a dark red-brown margin. Lesion size varies but commonly ranges between 1–1.5 cm long and 0.3–0.5 cm wide. The collar rot appears on the junction between the leaf blade and leaf sheath affecting the entire leaf. The neck rot is the most damaging symptom in the field. Typically, a necrotic or rotten neck is visible at the base of the panicle and often affects the entire panicle, which becomes white and partially or completely unfilled. The blast symptoms in the panicle or nodes are brown or black. On roots, blast lesions display brown necrotic areas, and root architecture is well maintained, which suggests less aggressive damage compared to take-all disease symptoms caused by *G. graminis*.

From Genome Sequences into Underlying Mechanisms Regulating Fungal Pathogenicity

Due to the genomic resources available for both the rice host and the fungus, the genetic tractability of *M. oryzae* and the economic relevance of blast disease, the rice–*M. oryzae* interaction has become a leading pathosystem for studying fungal pathogenicity and plant immunity in crops (Dean, *et al.*, 2012). The laboratory strain 70–15 was the first *M. oryzae* rice-infecting strain whose genome sequence was made available to the research community (Dean, *et al.*, 2005). It also represented the first genome publication of a fungal plant pathogen. The genome of *M. oryzae* is approximately 41 Mb in size (eight annotation MG8, Magnaporthe comparative Sequencing Project, Broad Institute of Harvard and MIT; <http://www.broadinstitute.org>). Gene prediction programmes estimate the presence of 12,991 protein-coding genes, which are distributed in seven chromosomes. The Broad Institute has stopped supporting the fungal genome databases and *M. oryzae* genome is currently maintained by the EMBL (Ensembl Fungi web://fungi.ensembl.org/index.html).

Different large-scale gene functional studies have been carried out since the release of *M. oryzae* genome sequence, including large-scale insertional mutagenesis (Betts, *et al.*, 2007, Jeon, *et al.*, 2007, Saitoh, *et al.*, 2012), and gene silencing (Nguyen, *et al.*, 2008) and gene deletion analyses (Lu, *et al.*, 2014, Kong, *et al.*, 2015). Transcriptomic approaches have also revealed global gene expression profiles during nitrogen

starvation (Donofrio, *et al.*, 2006), appressorium development (Oh, *et al.*, 2008, Soanes, *et al.*, 2012) and plant infection (Mosquera, *et al.*, 2009).

The genomes of *M. oryzae* rice-infecting field isolates Y34 and P131 have been sequenced and compared against the genome reference of the laboratory strain 70–15 (Xue, *et al.*, 2012). This genomic comparison has pointed out some relevant features of these field isolates. Y34 and P131 strains contain several 100 unique genes and have undergone unique DNA duplication events and expansions of pathogenicity associated gene families. Thousands of transposon-like elements are present on the field isolates, although their genomic locations are poorly conserved among them. This suggests that transposition events might play an important role in genome variation in the rice blast fungus, which can explain the rapid adaptation of *M. oryzae* isolates to new resistant rice varieties (Zeigler, 1998, Kang, *et al.*, 2001). This has been confirmed by additional whole genome comparative studies which have increased our understanding on the genetic mechanisms that drive host specialization in *Magnaporthe* species (Chiapello, *et al.*, 2015, Yoshida, *et al.*, 2016, Zhong, *et al.*, 2016). Gene gain or loss and chromosomal rearrangements facilitated by transposable elements seem to be the main mechanism that drives host adaptability during *Magnaporthe* species coevolution processes (Yoshida, *et al.*, 2016, Zhong, *et al.*, 2016). Whole genome sequencing studies have also confirmed that there is a strong conservation of genome composition among *M. oryzae* field isolates, and this include specific gene families encoding effectors and secondary metabolites that can regulate host specificity in *M. oryzae* (Chiapello, *et al.*, 2015).

Evolutionary Implications of *M. oryzae* Reproduction

M. oryzae is a haploid and heterothallic ascomycetous fungus. Blast isolates with opposite mating types MAT-1.1 and MAT-1.2 (compatible strains) can conjugate and enter into an heterokaryotic stage where mycelia contain unfused nuclei (Valent, *et al.*, 1991). Subsequently, this heterokaryotic mycelium enters into a sexual cycle by fusing both nuclei. Within 3 weeks, sexual fruiting bodies or perithecia are formed. The perithecium is filled with asci, each of which contains eight ascospores (sexual spores). The dissection of ascospores is used for classical genetic studies to determine the genetic basis of phenotypic traits looking at the segregation of genetic markers (Valent & Chumley, 1991, Valent, *et al.*, 1991, Talbot, 2003). The rice blast fungus reproduces mainly asexually

since the same mating type is found normally among local populations and are female sterile (Zeigler, 1998, Couch, *et al.*, 2005). A few examples of fertile rice isolates have been recovered from the field such as the strain Guy11 (Leung, *et al.*, 1988, Valent, *et al.*, 1991). Transposable elements or mutations in the mating alleles are directly involved in this lack of fertility (Zeigler, 1998).

The presence in *M. oryzae* of repeat-induced point mutation (RIP)-like processes, which only occur in the sexual phase of a fungal life cycle, suggests that sexual reproduction in the rice blast fungus exists or existed in nature (Ikeda, *et al.*, 2002). Under laboratory conditions, it is relatively easy to produce sexual crosses between *M. oryzae* isolates from different grasses. This ability has been used to identify several important gene loci and to generate fertile rice-infecting laboratory strains such as 70–15. The relevance of sexual reproduction in the field, with the advantage of increasing pathogen fitness, has been addressed in the blast fungus (Saleh, *et al.*, 2012). It seems that ancestral populations of *M. oryzae* from South and East of Asia, the geographical location where this fungus emerged, show signatures of sexual reproduction (Zeigler, 1998, Kumar, *et al.*, 1999, Couch, *et al.*, 2005, Saleh, *et al.*, 2012, Saleh, *et al.*, 2014). Molecular evidences such as genotypic richness (i.e. total number of genetic characteristics inside each specie) and linkage disequilibrium (i.e. some genes do not segregate independently) data support this theory (Saleh, *et al.*, 2012, Saleh, *et al.*, 2014). Currently, this is the only region in the world where evidences for sexual reproduction of *M. oryzae* have been found, confirming the loss of sexual reproduction outside its original location of emergence. In terms of evolution, this Asian South-East geographical area may represent an initial point where *M. oryzae* isolates have evolved by adaptive selection against new rice cultivars and different hosts (Saleh, *et al.*, 2012, Saleh, *et al.*, 2014).

The *M. oryzae* Leaf Infection Process

Under high relative humidity conditions, a succession of developmental events initiates the *M. oryzae* aerial infection (Tucker & Talbot, 2001) (Fig. 1.3), which begins when a wind-dispersed or water-splashed conidium lands on the leaf surface. Immediately after landing, a preformed adhesive material is secreted from the conidial tip to attach itself to the highly hydrophobic surface. One hour later a polarized short germ tube develops from the apical cell of the conidium. Within a few

hours, the apex of the germ tube swells, and a specialised dome-shaped penetration structure known as appressorium is formed. The appressorium is heavily melanised and a tremendous turgor pressure is generated within this structure (Howard, *et al.*, 1991, De Jong, *et al.*, 1997). A penetration peg emerges at the base of the appressorium and crosses the plant epidermal cell by combining physical force and secretion of cell wall-degrading enzymes (Skamnioti & Gurr, 2007). Subsequently, *M. oryzae* initiates a new morphogenetic programme to colonise the plant epidermal cells. Five to six days after the initial penetration of the fungus, conidiophores emerged on the leaf surface to initiate the last step of the infection with the reproduction of the fungus.

The rice blast research community has built a large amount of information on each of the steps of the blast disease cycle. Here, a description and latest findings of the *M. oryzae* leaf infection biology follows.

Fig. 1.3 Rice blast disease infection cycle. Right panel: *M. oryzae* leaf cycle modified from Ribot et al 2008 (Ribot, *et al.*, 2008). *M. oryzae* leaf infection cycle starts when a conidium lands on a leaf and attaches to the surface. Shortly after, the conidium produces a small germ tube, which differentiates into a melanised appressorium. A penetration peg formed at the base of the appressorium crosses the plant cell wall initiating fungal invasion. Invasive growth is different compared to fungal growth on surfaces. The invasive hypha moves beyond the first infected cell during a few days. Finally, conidiophores emerge and the fungus initiates sporulation between 6 and 15 days, releasing thousands of conidia to the environment. Left panel: *M. oryzae* root infection cycle potentially begins from infected plant debris or dormant structures present in the soil. These resting structures can germinate and penetrate into the plant roots. Fungal hypha colonises the vascular system of the root spreading systemically. The fungus moves to the upper parts of the plant producing typical blast lesions from which conidia are formed. These spores are dispersed to other plants by wind or water, propagating the disease.

An Extracellular Matrix Mediates Fungal Adhesion and Differentiation

Spores of *M. oryzae* get attached immediately to the highly hydrophobic leaf cuticle by secreting a preformed mucilaginous extracellular matrix (ECM). This adhesive material is passively released from the conidial apex upon hydration, meaning that there are no significant metabolic costs involved in this process (Hamer, *et al.*, 1988, Tucker & Talbot, 2001). This attachment is required for conidial anchoring and recognition of the surface, steps that precede the subsequent infection-related development. *M. oryzae* mutants with altered ECM show reduced virulence (Ahn, *et al.*, 2004). Initial studies identified components of *M. oryzae* adhesive material such as glycoproteins and lipids which help to retain moisture, essential for appressorium-mediated penetration (Hamer, *et al.*, 1988, Howard, 1997). α -Mannosyl and α -glucosyl residues are highly abundant in the ECM based on lectin labelling and protease digestions (Hamer, *et al.*, 1988, Xiao, *et al.*, 1994). Additional components of *M. oryzae* ECM have been identified by immunological techniques using antibodies against animal cell adhesion factors (collagen VI, vitronectin, fibronectin, laminin) and integrin $\alpha 3$ (Bae, *et al.*, 2007, Inoue, *et al.*, 2007). Particularly, collagen as a major component, vitronectin as cementing compound, laminin, fibronectin and integrins are present in *M. oryzae* adhesive material (Bae, *et al.*, 2007, Inoue, *et al.*, 2007). Evidences suggest that ECM components are synthesised at two different stages of the infection cycle. Collagen, vitronectin and integrins seem to be formed earlier than fibronectin and laminin components (Inoue, *et al.*, 2007).

Integrins are transmembrane glycoproteins located at the plasma membrane and act as cell surface receptors modulating the cellular response to environmental stimuli (Shattil, *et al.*, 2010, Kim, *et al.*, 2011). Fibronectin and collagen are extracellular ligands of integrin receptors (Shattil, *et al.*, 2010, Kim, *et al.*, 2011). Externally applied peptides containing Arg-Gly-Asp amino acids and antibodies against fibronectin reduce conidial adhesion and appressorium development indicating that these processes are modulated by integrin-like proteins in *M. oryzae*. These defects are restored by manipulating the cAMP response pathway with exogenously applied chemicals (cAMP, cutin monomers or IBMX, a cAMP phosphodiesterase inhibitor) (Bae, *et al.*, 2007). These results suggest that integrin-like proteins and their cognate extracellular ligands (fibronectin, collagen) activate the cAMP-dependent pathway and

possibly other signalling pathways. This activation regulates the subsequent infection-related morphogenesis (Tucker & Talbot, 2001, Bae, *et al.*, 2007). Integrins are also detected in conidial cell walls (Inoue, *et al.*, 2007), suggesting that these transmembrane receptors may play a role in the recognition of substrates by *M. oryzae* spores at earlier stages, immediately after landing.

Recognition of the Surface Precedes Appressorium Differentiation

Upon hydration, the first germ tube emerges, usually from the apical compartment of the conidium. If the fungus perceives that the surface is not adequate, the germ tube will arrest, blocking any further differentiation. Alternatively, it can develop a second germ tube from the opposite end of the conidium. Two germ tubes are often seen in germinating conidia on artificial substrates (Tucker, *et al.*, 2010). It is very unusual to see spores germinating from the middle compartment. The germ tube appears near the adhesion site of the spore in the apical cell and grows in direct contact with the surface of the plant for a short distance. Then, it swells and starts to change direction. This process known as

“hooking” takes place before the appressorium development, and it is believed to be an important recognition step (Bourett & Howard, 1990) (Fig. 1.4a). During germ tube elongation, other processes such as secretion of plant cell wall-degrading enzymes, mobilisation of the metabolic reserves (trehalose) and synthesis of fungal cell wall occur

Fig. 1.4. *M. oryzae* development on artificial and root surfaces. (a) Scanning electron micrograph of a germinating conidium (Co) forming an appressorium (Ap) on hydrophobic coverslips. (b) A two-septate conidium expressing a GFP-tagged nuclear protein; septa (Se) are indicated. (c) Sickleshaped microconidia (Mi). (d) Conidiophore (Cf)- producing conidia in the stem of a rice seedling. (e) Conidia on roots developing hyphopodia (Hy). (f) Differentiated hyphopodia from fungal hyphae producing infection pegs (Ip) to penetrate rice roots. Scale bar numbers indicate micrometres.

(Tucker & Talbot, 2001). Concomitantly with the germination process, *M. oryzae* secretes additional compounds that contribute to the adhesion of the germ tube and perception of plant physical signals. Among them, hydrophobins have been shown to play a significant role at the early stages of fungal infection (Talbot, *et al.*, 1996, Kim, *et al.*, 2005, Linder, *et al.*, 2005). These specialised proteins are secreted at the interface between the hyphae and a hydrophobic surface and are involved in fungal development and environmental sensing (Linder, *et al.*, 2005). In *M. oryzae*, two hydrophobins play a role during infection, Mpg1 and Mhp1. Mpg1 has been widely characterised in the rice blast fungus (Talbot, *et al.*, 1993, Beckerman & Ebbole, 1996, Lau & Hamer, 1996, Talbot, *et al.*, 1996, Kershaw, *et al.*, 1998, Soanes, *et al.*, 2012). Mpg1 is a class I hydrophobin highly-expressed during conidiogenesis, appressorium development and carbon and nitrogen starvation. The $\Delta mp g1$ mutants show defects in conidiation and appressoria development, and consequently are less virulent. Mhp1 is a class II hydrophobin and mutants lacking this hydrophobin show pleiotropic effects. Similarly to Mpg1, the hydrophobin Mhp1 is required for fungal morphogenesis, including appressorium development and invasive growth (Kim, *et al.*, 2005).

Cutinases and other methyl esterases are relevant enzymes secreted by fungal plant pathogens during early stages of infection (Kolattukudy, 1985). Sixteen methyl esterase-encoding genes are present in *M. oryzae* genome (Dean, *et al.*, 2005), and some of them can be components of *M. oryzae* adhesive material. It is difficult to define their roles in *M. oryzae* infection biology since they may have redundant functions. Two cutinases have been characterised in the rice blast fungus. The *CUT1* gene is dispensable for *M. oryzae* plant infection (Sweigard, *et al.*, 1992). Among all the *M. oryzae* methyl esterases, *CUT2* was selected for further analysis because it is highly induced at 12 h after inoculation on barley leaves (Skamnioti & Gurr, 2007). In *M. oryzae*, Cut2 acts as a surface sensor activating the cAMP/PKA and DAG/PKC signalling cascades which regulate appressorium-mediated penetration. The $\Delta cut2$ mutants show that Cut2 is required for appressorium differentiation and full disease symptoms production, but have no defects in adhesion, indicating a specific role for a cutinase in signalling and fungal development (Skamnioti & Gurr, 2007).

Orchestrated Cellular Processes Govern Early Stages of Plant Infection

Two important stages take place during the process of appressorium differentiation in *M. oryzae*. During the recognition phase, the apex of the germ tube begins to hook, and vesicles located in the apical area move towards the surface of the plant (Bourett & Howard, 1990, Tucker & Talbot, 2001). Then, the tip of the germ tube swells and the appressorium is formed (Fig. 1.4a). This differentiation process is highly orchestrated and is activated in response to starvation stress and physical cues such as hardness and hydrophobicity (Dean, 1997, Talbot, *et al.*, 1997, Tucker & Talbot, 2001). Several interconnected cellular processes regulate these early stages of infection: cell cycle progression followed by cytokinesis and appressorium differentiation (Saunders, *et al.*, 2010, Saunders, *et al.*, 2010), programmed cell death (Veneault-Fourrey, *et al.*, 2006) and mobilisation of metabolic resources to generate high concentrations of glycerol for turgor-mediated penetration (Howard, *et al.*, 1991, Thines, *et al.*, 2000).

During germ tube elongation, the nucleus of the germinating cell moves towards the middle of the germ tube. Subsequently, the nucleus undergoes mitosis and one of the daughter nuclei moves towards appressoria, whereas the second nucleus returns to the conidium (Veneault-Fourrey, *et al.*, 2006). Concomitantly, storage products are transported towards the appressorium during this first nuclear division, and a septum is formed separating the appressorium from the germ tube (Saunders, *et al.*, 2010). Then, appressorial melanisation begins. Melanin is deposited in the space between cell wall and plasma membrane to maintain appressorium integrity. High levels of glycerol derived from the degradation of stored lipids and glycogen within the appressorium build the osmotic force required for penetration of the cuticle (Thines, *et al.*, 2000). Finally, the conidium and germ tube collapse using an autophagic mechanism which is vital for pathogenicity (Veneault-Fourrey, *et al.*, 2006, Kershaw & Talbot, 2009). In *M. oryzae* has been described 22 autophagy genes (*ATG* genes). *ATG8* has been shown necessary for functional appresoria development (Veneault-Fourrey, *et al.*, 2006, Kershaw & Talbot, 2009). The retromer complex is a part of the endosomal machinery formed by five proteins (Koumandou, *et al.*, 2011). Vps35 is the retromer's protein that interacts with the cargoes for their

transport from the endosome to the proper organelle (Seaman, 2012, Burd & Cullen, 2014). Vps35 interacts with cleaved variant of Atg8 preventing its degradation and promoting its delivering to proper compartments (Zheng, *et al.*, 2015). Defective Vps35 mutants showed the same phenotype that $\Delta atg8$ mutant, including a failure to manage appressoria full turgor pressure impairing host penetration. Therefore retromer has a role in conidial autophagic cell death and fungal pathogenicity (Zheng, *et al.*, 2015).

Autophagy has an inverse relationship with target of rapamycin (TOR) pathway. The TOR signalling cascade is an intracellular regulatory network used by eukaryotic cells to regulate growth according to nutrient availability. Glutamine accumulation in $\Delta sd4$ transcription factor mutant induced TOR pathway, inhibiting autophagy route and impairing appressoria formation. Therefore, activation of TOR pathway leads to inhibition of appressorium development and renders the fungus unable to cause rice blast disease (Marroquin-Guzman and Wilson, 2015). Regulation of TOR pathway is upstream Pmk1 (pathogenicity MAP kinase1) and downstream of CpkA (catalytic subunit of protein kinase A) pathways.

Appressorium morphogenesis, autophagic cell death and mobilisation of carbohydrate and lipid reserves to the appressorium are processes regulated by the mitogen-activated protein kinase Pmk1 pathway (Xu & Hamer, 1996, Thines, *et al.*, 2000, Veneault-Fourrey, *et al.*, 2006). In eukaryotes, MAPKs are involved in the activation of cellular processes in response to environmental cues that help to adapt the cell (Zhao, *et al.*, 2007). In *Saccharomyces cerevisiae*, five MAPK pathways exist and have been characterised in detail (Zhao, *et al.*, 2007). In *M. oryzae*, the MAPK Pmk1 has been identified as the homologue of *S. cerevisiae* FUS3/KSS1 MAPK cascades, which regulate mating and filamentous growth (Xu & Hamer, 1996). The $\Delta pmk1$ mutants are unable to produce appressorium and are non-pathogenic. However, they can recognise hydrophobic surfaces and react to exogenously applied cAMP. In *M. oryzae*, Pmk1 is also required for invasive hyphae growth (Xu & Hamer, 1996). Homologues of Pmk1 are required for pathogenicity in all fungal plant pathogens (biotrophs or necrotrophs) of monocot and dicot plants studied to date, indicating that this MAPK pathway is widely conserved (Zhao, *et al.*, 2007). This pathway is under extensive analysis, and genes acting upstream and downstream of Pmk1 have been identified like MAPK kinases Mst7 and Mst11 (Zhao, *et al.*, 2005). Mst11 has a

Sterile Alpha Motif (SAM) N-terminal domain that activates Pmk1 pathway by phosphorylating Mst7 (Zhou et al. 2014 and Qi et al., 2015). The Mst11 RA-domain is also required for the interaction with Ras2, which activates both the cAMP-PKA and Pmk1 pathways (Zhou, *et al.*, 2014). The link between Pmk1 and cAMP pathways assures that the germ tube formation is arrested when appressoria development starts. Other components of the Pmk1 pathway have been found and include the PAK kinase Chm1 (Li, *et al.*, 2004); the Rho-GTPase MgRac1 (Chen, *et al.*, 2008); the scaffold protein Mst50 that interacts with Ras1, Ras2, Ccd42 and the G β subunit Mgb1 (Park, *et al.*, 2006); two novel Pmk1-interacting proteins Pic1 and Pic5 (Zhang, *et al.*, 2011); and the membrane receptors MoMsb2, Cbp1 and MoSho1 (Liu, *et al.*, 2011) (Fig. 1.5). MoMsb2 together with Cbp1 recognize extracellular signals that lead to Ras2 activation. In addition, several transcription factors regulated by Pmk1 have been described such as Mst12 (Park, *et al.*, 2002), Mig1 (Mehrabi, *et al.*, 2008), MoSLF (Li, *et al.*, 2011) and MoMcm1 (Zhou, *et al.*, 2011).

Two other MAPK signalling pathways have been described in *M. oryzae* (Fig. 1.5). The Mps1-dependent MAPK pathway is implicated in appressoria penetration and cell wall integrity (Xu, *et al.*, 1998), whereas the MAPK Osm1 is involved in the cellular response to osmotic stresses and is not required for plant infection (Dixon, *et al.*, 1999).

Signalling and Cytoskeletal Dynamics Regulate Fungal Plant Penetration

At the base of the appressorium, a pore ring is formed and the fungus initiates the turgor-driven penetration into plant tissues by developing a specialised hypha or penetration peg (Talbot, 2003). The penetration peg enables the fungus to cross the plant cell wall and extend to the epidermal lumen of the plant. This structure is enriched in actin filaments and lacks organelles in its cytoplasm (Bourett & Howard, 1992). Particularly two important signalling pathways regulate this step, the Mps1 MAPK cascade (Xu, *et al.*, 1998) and the cAMP response pathway (Xu, *et al.*, 1997) (Fig. 1.5).

The cAMP cascade is required for surface recognition and penetration peg emergence but not appressorium differentiation (Xu, *et al.*, 1997). The generation of glycerol and high turgor pressure within the appressorium requires a rapid degradation of lipid and glycogen reserves which is under the control of the cAMP-activated PKA pathway (Thines,

et al., 2000). Several key components of this signalling pathway have been studied such as the adenylate cyclase Mac1 (Choi & Dean, 1997), CpkA (Xu, *et al.*, 1997), the phosphodiesterases PdeL and PdeH (Zhang, *et al.*, 2011) and the Mac1-interacting protein Cap1 (Zhou, *et al.*, 2012), which regulates the crosstalk between Pmk1- and cAMP-dependent pathways through its interaction with Ras2 (Zhou *et al.* 2014 and Qi *et al.*, 2015) (Fig. 1.5). Additional genetic determinants have been found to play a role in *M. oryzae* penetration including the tetraspanin PLS1 (Clergeot, *et al.*, 2001, Lambou, *et al.*, 2008), the Pmk1-regulated genes *GAS1* and *GAS2* encoding unknown proteins conserved in filamentous fungi (Xue, *et al.*, 2012), the aminophospholipid translocase *PDE1* (Balhadere & Talbot, 2001) and the isocitrate lyase gene *ICL1* of the glyoxylate cycle (Wang, *et al.*, 2003).

Fig. 1.5. Schematic representation of the different signaling pathways involved in *M. oryzae* infection. The cAMP-PKA cascade (in blue), the mitogen-activated protein kinase (MAPK) cascades (in green) and the calcium-calcineurin pathway (in orange). Surface and environmental signals trigger the signaling cascades that allow landing conidia to germinate, form the appressorium and infect plant tissues. Upon recognition the germ tube develops the appressorium, whose development and function are regulated by different signaling cascades. Pmk1 and Cmd1 are also required for invasive growth. See text for further details.

An actin network organised at the base of the appressorium forces the emergence of a penetration peg (Bourett & Howard, 1992). In *M. oryzae*, this process is regulated by septins (Dagdas, *et al.*, 2012). Septins are highly conserved GTPases present in fungi and animals that participate in cytoskeletal-dependent cellular processes such as cytokinesis, polarity and secretion (Gladfelter, 2006, Mostowy & Cossart, 2012). Septins also act as diffusion barriers. *M. oryzae* genome contain five septin genes, four of which are core septins present in budding yeast. *M. oryzae* septins form a dynamic septin ring that contributes to the formation of a toroidal filamentous actin network surrounding the appressorial pore, where the penetration peg differentiates (Dagdas, *et al.*, 2012, Ryder, *et al.*, 2013). The correct assembly of septin-mediated actin network ring depends on the presence of reactive oxygen species (ROS) generated by NADPH oxydases. In *M. oryzae* has been demonstrated that NADPH oxydases Nox1 and Nox2 are necessary for appresoria maturation, polarized growth and septin-mediated plant penetration (Egan, *et al.*, 2007, Ryder, *et al.*, 2013, Martin-Urdiroz, *et al.*, 2015).

The exocyst, the Spitzenkörper and the polarisome are three different structures involved in hyphal tip growth in filamentous fungi (Steinberg, 2007, Read, 2011, Sudbery, 2011, Riquelme, 2013). The exocyst is a fungal structure that regulates exocytosis and endocytosis processes (Jose, *et al.*, 2015). The exocyst is formed by eight proteins required for targeting of vesicles derived from the Spitzenkörper to the plasma membrane. In *M. oryzae*, the exocyst assembly is mediated by septins and the F-actin network and localises at the tip of the germ tube while the appressorium develops, changing its localization in the mature appressorium to the appressorium pore (Giraldo, *et al.*, 2013, Gupta, *et al.*, 2015). The *M. oryzae* exocyst is necessary for correct appresoria repolarization and effective rice cell invasion (Gupta, *et al.*, 2015).

Insights into *M. oryzae* Invasive Growth

Within the host cell, the fungus develops several types of biotrophic invasive hyphae (IH) (Kankanala, *et al.*, 2007), which contain distinct morphological features compared to the filamentous hyphae produced on the leaf surface or in vitro. Soon after the *M. oryzae* penetration peg has crossed the epidermal cell wall, it differentiates into a short and thin filamentous hypha known as primary IH. This primary IH precedes the formation of a thicker intracellular pseudohypha called bulbous IH. The bulbous IH grows within the cytoplasm and moves beyond the first

invaded cell by crossing with constricted infection pegs at regions of the plasma membrane where plasmodesmata aggregate, also known as pit fields (Bell & Oparka, 2011). Thereafter, the bulbous IH differentiates into filamentous IH in the new invaded cell and subsequent fungal invasion continues into neighbouring cells (Kankanala, *et al.*, 2007). Importantly, bulbous and filamentous IH are not in direct contact with the plant cell cytoplasm since a plant-derived plasma membrane called extra-invasive hyphal membrane (EIHM) surrounds them. There is no well-established matrix between IH and EIHM, and IH grows in close contact with the EIHM (Kankanala, *et al.*, 2007). An additional morphological feature of *M. oryzae* invasive growth is the formation of biotrophic interfacial complexes (BICs) (Khang, *et al.*, 2010, Giraldo, *et al.*, 2013). When *M. oryzae* penetrates the first cell, a BIC is formed at the tip of the first bulbous IH, and is left behind, remaining as a discrete structure while the bulbous IH continues growing. New BICs are observed at the tip of each IH growing inside the plant cell. Five to six days after the initial fungal infection, conidiophores start to emerge in the leaf surface, and spores are produced massively during 2 weeks.

Effectors are secreted fungal proteins that play a very important role in *M. oryzae* virulence during biotrophic phase of infection (Valent & Khang, 2010, Mentlak, *et al.*, 2012, Giraldo & Valent, 2013, de Guillen, *et al.*, 2015). Effectors and other compounds are secreted by two different pathways depending on their host localisation. In the case of Slp1 (Mentlak, *et al.*, 2012) and Bas4 (Mosquera, *et al.*, 2009, Chen, *et al.*, 2014) both are apoplastic effectors secreted into the space between IH and EIHM membranes using the ER- and Golgi-dependent secretory pathway. The effectors secreted into the cytoplasm of host cells accumulate into BIC structures until they are delivered by exocyst components (Giraldo, *et al.*, 2013). The exocyst proteins Exo70 and Sec5 are necessary for secreting *M. oryzae* effectors into cytoplasm rice cells, while the Sso1 t-SNARE protein is essential for a correct BIC development (Giraldo, *et al.*, 2013). An example of cytoplasmic effector is Pwl2. The function of this effector is still unknown even though it is exclusively present in rice *M. oryzae* isolates and not in other cereals or grasses isolates (Mentlak, *et al.*, 2012). More recently, the Max-effector family (*M. oryzae* Avrs and ToxB) have been identified in *M. oryzae* during biotrophic plant colonization. They are found at low frequencies but they seem particularly important (de Guillen, *et al.*, 2015). MAX-effectors usually are secreted into plant cell and share the same

architecture although do not share sequence similarity. Another group of effectors contain plant-like hormonal features and are produced by fungal pathogens during infection. This is the case of the *CKS1* gene, which is conserved in most pathogenic fungi and is required for cytokinins production. The *M. oryzae* *CKS1* gene is essential for full virulence. Fungal-derived cytokinins are key effectors and are required for dampening host defences and altering sugar and amino acid distribution at the infection site (Chanclud, *et al.*, 2016).

The elucidation of the molecular mechanisms involved in *M. oryzae* invasive growth has been largely overlooked because many of the mutants characterised in this organism are penetration defective. There is an extensive coupling between penetration and invasive growth processes since cell wall degradation and mechanical pressure are also required for fungal growth inside the host cells (Heath, *et al.*, 1992, Xu, *et al.*, 1997). As an example, $\Delta mst12$ fails to penetrate onion epidermal cells and to infect wounded leaves although it differentiates melanised appressoria, indicating that Mst12 is required for both penetration peg formation and invasive growth differentiation (Park, *et al.*, 2002). Very few genes have been found to play specifically a critical role during *M. oryzae* plant invasion. MIG1 is involved in the late stages of *M. oryzae* infection since the $\Delta mig1$ mutants form normal appressoria, penetrate host cells and develop primary IH but fail to infect wounded leaves. Mig1 is one of the two MADS-box transcription factors present in *M. oryzae* and a downstream target of the MAPK Mps1 (Mehrabi, *et al.*, 2008). The MIR1 gene specifically expressed in *M. oryzae* IH encodes a protein of unknown function, which is present only in the *M. oryzae* species complex. Despite the fact that MIR1 expression is exclusively found in IH, $\Delta mir1$ mutants have no defects in appressorial penetration and are fully pathogenic (Li, *et al.*, 2007).

Fungal Metabolism and Plant Infection

M. oryzae has to adapt to the changing nutritional environment during host invasion, and consequently metabolism plays an essential role during *M. oryzae* invasive growth. *M. oryzae* is considered a hemibiotrophic fungus based on its nutritional mode during host invasion. However, genes regulating the switch in life style and acquisition of nutrients during plant infection are largely unknown (Kankanala, *et al.*, 2007, Fernandez & Wilson, 2012). The duration of the biotrophic versus the necrotrophic

phase in *M. oryzae* is also unknown. During early stages of rice colonisation, *M. oryzae* grows and fulfils its nutritional needs from the plant tissue without killing the host cells due to its ability to manipulate rice physiology as many other biotrophs do (Mendgen & Hahn, 2002, Mengiste, 2012). During this biotrophic stage, limited amounts of cell wall-degrading enzymes are produced and toxin production is absent according to a biotrophic life style. By contrast, extensive degradation of plant cell walls is observed at later time points of infection and in heavily invaded tissues, both stages associated with the necrotrophic phase of the fungus (Rodrigues, *et al.*, 2003, Kankanala, *et al.*, 2007). Typically, necrotrophs produce phytotoxic compounds and cell wall-degrading enzymes to kill the cells and cause leakage of nutrients (Mengiste, 2012). Plant cell walls nearby *M. oryzae* hyphae show strong enzymatic digestion, correlating with *M. oryzae* necrotrophic phase (Kankanala, *et al.*, 2007).

Possibly one of the cues that trigger the switch from biotrophic to necrotrophic hyphae in *M. oryzae* is the lack of carbon sources within the host cell. It is known that nutrient starvation also can act as an environmental cue for infection-related differentiation (Talbot, *et al.*, 1997). *M. oryzae* has to limit the acquisition of nutrients during its biotrophic phase to maintain host cell integrity. Consequently, the use of nutrients must be highly regulated during *M. oryzae* biotrophic growth in order to respond appropriately to nutrient availability. Several interconnected pathways regulate *M. oryzae* growth in response to nutrients during plant invasion. These include the target of rapamycin (TOR) signalling cascade, carbon catabolite repression (CCR), nitrogen metabolite repression (NMR) and the integration of carbon and nitrogen metabolism by trehalose-6-phosphate synthase 1 (Tps1) (Fernandez, *et al.*, 2014).

The Target of Rapamycin (TOR) signalling cascade regulates cell growth according to nutrient availability. This signaling pathway regulates negatively appressorium formation (Marroquin-Guzman & Wilson, 2015). The RNA-binding protein Rbp35 is an upstream regulator of the TOR signalling pathway (Franceschetti, *et al.*, 2011). Rbp35 is a component of the polyadenylation machinery, and it is required for alternative 3' end processing of pre-mRNAs. One of the Rbp35 targets is 14-3-3 pre-mRNA. 14-3-3 is a scaffold protein that regulates TOR cascade and this could explain the defects exhibited by $\Delta rbp35$ in TOR signalling and plant infection (Rodríguez-Romero, *et al.*, 2015).

NMR is a highly regulated process in which preferred nitrogen sources, such as ammonia, glutamine and glutamate, are used preferentially. Ammonia is the preferred nitrogen source for *M. oryzae*. The NMR in *M. oryzae* occurs through the transcriptional activator Nut1, the *M. oryzae* AreA/Nit2 orthologue (Froeliger & Carpenter, 1996). The expression of a large number of genes encoding enzymes that are involved in the utilisation of various secondary nitrogen sources —nitrate, purines or amino acids— is subject to nitrogen metabolic repression and is positively regulated by Nut1. The $\Delta nut1$ mutant can grow on ammonia, which does not require an active Nut1, but $\Delta nut1$ is unable to grow on certain alternative nitrogen sources such as nitrate. *M. oryzae* mutants in genes involved in nitrate assimilation and whose expression is regulated by Nut1 such as *NIA1* and *NIR1* are fully pathogenic on rice leaves (Lau & Hamer, 1996, Wilson, *et al.*, 2010). This suggests that NMR is not involved in *M. oryzae* leaf colonisation and consequently the fungus can assimilate preferred sources of nitrogen (ammonia, glutamine or glutamate) from aerial host tissues. However, genes involved in response to nitrogen availability are important for infection. Two *M. oryzae* nitrogen-regulatory genes of unknown identity, *NPR1* and *NPR2*, are required for growth on a wide range of secondary nitrogen sources, including nitrate, and do not develop lesions on barley (Lau & Hamer, 1996). Therefore, nitrate is not required for *M. oryzae* leaf infection, but secondary nitrogen sources assimilated via Npr1 or Npr2 are necessary for development of full disease symptoms. Additionally, several studies have shown that nitrogen-limiting conditions result in the expression of genes required for fungal pathogenicity such as the genes encoding the hydrophobin Mpg1 and the vacuolar subtilisin-like protease Spm1 (Donofrio, *et al.*, 2006, Saitoh, *et al.*, 2009, Soanes, *et al.*, 2012). We require further studies to understand the molecular mechanisms underlying NMR and their involvement in nitrogen assimilation during *M. oryzae* plant infection.

CCR is a genetic mechanism that ensures the preferential use of glucose over other, less-preferred carbon sources, and it is also present in *M. oryzae* (Fernandez & Wilson, 2012). *M. oryzae* has the ability to use a wide range of mono- and disaccharides as sole carbon source but has a strong preference for glucose (Tanzer, *et al.*, 2003, Wilson, *et al.*, 2007, Fernandez & Wilson, 2012). In *A. nidulans*, CCR is mediated at DNA level by the global transcriptional repressor CreA. A putative orthologue

of CreA (MGG_ 11201) is present in *M. oryzae*, and its role in fungal pathogenicity has yet to be elucidated.

An interesting interconnection of NMR and CCR is mediated by the sugar sensor trehalose-6-phosphate synthase (Tps1) and trehalose-6-phosphate (Fernandez & Wilson, 2012). Tps1 is one of the three mediators of CCR identified in *M. oryzae*. The other two mediators are the Nmr1/2/3 inhibitor proteins and Mdt1, a multidrug and toxin extrusion (MATE)–family pump. Tps1 is a metabolic enzyme that synthesises trehalose-6-phosphate (T6P, a trehalose intermediate) from UDP-glucose and glucose-6-phosphate (G6P). Tps1 has two roles, as a biosynthetic enzyme and as signalling component of G6P. The sensing of G6P by Tps1 results in activation of the activity of the enzyme glucose-6-phosphate dehydrogenase (G6PDH), which converts NADP to NADPH using G6P in the pentose phosphate pathway. Therefore, Tps1 controls intracellular levels of NADPH (depending on the concentration of G6P) and subsequent activation of NADPH-dependent signalling cascades that interconnect carbon and nitrogen metabolism. When NADPH levels increase in a Tps1-dependent manner, three NADP-dependent inhibitor proteins (Nmr1 to Nmr3) are inactivated. As a result of inactivation of Nmr proteins, at least three GATA transcription factors become active, one of which is the white collar-2 homologue involved in light sensing (Pas1). The other GATA factor is essential for appressorium formation (Asd4), and the third GATA factor is Nut1 (Wilson, *et al.*, 2010). The modulation of GATA factor activity in the NADPH-dependent signalling pathway results in Tps1-dependent expression of at least three known virulence factors: the melanin enzyme Alb1, the seven transmembrane receptor Pth11 and the hydrophobin Mpg1 (Wilson, *et al.*, 2007). Accordingly, $\Delta tps1$ mutants are non-pathogenic. Tps1 regulation of Nut1 results in similar but not identical growth phenotype of $\Delta tps1$ and $\Delta nut1$ strains on a wide range of nitrogen sources. An additional regulator of the CCR signal transduction pathway in *M. oryzae* has been identified during a forward suppressor screening in $\Delta nut1$ background (Fernandez & Wilson, 2012). Mdt1 is a member of the MATE protein family required for sporulation and plant infection but not appressorium differentiation. Mdt1 regulates carbon metabolism via extrusion of citrate during infection and growth, which contributes to *M. oryzae* in planta nutrient adaptation.

In summary, NADPH signalling, CCR, NMR and TOR are mechanisms by which *M. oryzae* can sense and adapt its metabolic status to nutrient availability during in planta growth. Future research will

determine the interplay among these regulatory pathways that play a pivotal role in the establishment of plant disease.

The Dark Phase of Blast: *M. oryzae* Root Infection Biology

Similar to other members of the family like *G. graminis* and *M. poae*, *M. oryzae* infects roots under laboratory conditions and undertakes a set of developmental programmes typical of root-infecting pathogens (Sesma & Osbourn, 2004, Tucker, *et al.*, 2010). Several key differences have been found between the mode of penetration of leaves and roots. In contrast to the melanised appressoria observed on leaves, *M. oryzae* produces hyphal swellings to penetrate roots, resembling the simple hyphopodium seen in root-infecting fungi of the *G. graminis*–*Phialophora* complex (Fig. 1.4e). *M. oryzae* hyphopodia are not melanised and *M. oryzae* melanin-deficient mutants are able to produce hyphopodia and penetrate roots (Sesma & Osbourn, 2004). The PKA regulates the high turgor pressure within appressoria generated by the degradation of lipid and glycogen reserves (Thines, *et al.*, 2000). The *M. oryzae* $\Delta cpka$ mutant produces fully functional hyphopodia, indicating that root penetration is not dependent on CPKA (Sesma & Osbourn, 2004). Consequently, *M. oryzae* penetrates the epidermal root cells through a melanin-independent mechanism and the mechanical entry of the hard leaf surface by osmotic force is not operational during hyphopodia-mediated root penetration. From the host perspective, defence-related gene transcripts of rice showed a different temporal induction pattern during *M. oryzae* infection of leaves or roots (Marcel, *et al.*, 2010), which correlates with the different invasion mechanisms that the rice blast fungus undertakes for colonisation of plant tissues.

Pre-invasive hyphae (pre-IH) are other type of fungal structures observed on root surfaces that can mediate direct penetration of epidermal root cells (Tucker, *et al.*, 2010). *M. oryzae* pre-IH are developed from hyphopodia or germ tubes and directly penetrate roots. The pre-IH can be followed by differential labelling with concanavalin A and wheat germ agglutinin, which indicates that significant cell wall changes accompanied to this morphogenetic programme. Artificial surfaces such as hydrophilic polystyrene (PHIL-PS) can induce hyphopodia-like structures and pre-IH. The mutant $\Delta pmk1$ is non-pathogenic on roots (Dufresne & Osbourn, 2001), and this mutant is unable to develop pre-IH on roots and PHIL-PS. Consequently, this fungal differentiation is regulated by the MAPK Pmk1 cascade. Other structures typical of root-infecting fungi seen during *M.*

oryzae root colonisation include microsclerotia and resting structures such as vesicles and swollen cells (Gangopadhyay & Row, 1986, Lee, *et al.*, 2000, Sesma & Osbourn, 2004).

Several lines of evidence have led to the hypothesis that the hyphopodium is an intermediate step before appressorium penetration. The screening of a *M. oryzae* insertional library of 2,885 T-DNA transformants on hydrophilic polystyrene (PHIL-PS) surfaces has identified 20 transformants that show reduced virulence or are non-pathogenic on leaves and/or roots (Tucker, *et al.*, 2010). Further analysis of these mutants has revealed that appressorium, hyphopodium and pre-IH formation are highly coupled developmental processes, and very few mutants show an organ-specific involvement for infection (Tucker, *et al.*, 2010). This indicates that a significant set of common genes are necessary for fungal infection on both plant organs. Out of the 20 mutants, M1373 showed a clear root-specific infection-deficient phenotype. This mutant lacks the *M. oryzae* orthologue of exportin-5/Msn5p (Exp5; see chapter 3). It is possible that the primitive hyphopodia evolved by acquisition of melanin and generation of high turgor pressure into a more sophisticated penetration structure, the appressorium (Tucker, *et al.*, 2010).

Differences have been found in the role played by nitrogen-related genes during *M. oryzae* leaf and root colonisation. The assimilation of nitrogen by *M. oryzae* from underground plant tissues is regulated by the global nitrogen regulator Nut1 (Froeliger & Carpenter, 1996). The $\Delta nut1$ mutant is non-pathogenic on roots but infects leaves as well as the wild-type strain (Dufresne & Osbourn, 2001). Consequently, *M. oryzae* absorbs nitrogen from less preferred sources in root tissues, and therefore, the NMR plays a crucial role during root infection. The $\Delta npr1$ and $\Delta npr2$ mutants are non-pathogenic on leaves and show opposite phenotypes on roots (Table I.1), representing an additional evidence of the different roles that nitrogen-related genes play during *M. oryzae* colonisation of leaves and roots.

Table I.1 Organ-specific and general pathogenicity genes regulate *M. oryzae* plant colonisation.^a APP, appressoria; ^b Wild type lesion severity +++; no lesions: -; ^c HY, hyphopodia; ND: Not determined; rd: reduced.

<i>M.oryzae</i> strains	Targeted gene function	APP ^a	Leaf symptom ^b	HY ^c	Root symptom	References
Wild type		yes	+++	yes	+++	
Root specific						
<i>Δmgfow1</i>	Mitochondrial respiration	yes	+++	+	+	(Sesma & Osbourn, 2004)
<i>Δexp5</i>	karyopherin	yes	++	ND	-	(Tucker, et al., 2010)
<i>Δnut1</i>	nitrogen global regulator	yes	+++	+	-	(Froeliger & Carpenter, 1996, Dufresne & Osbourn, 2001)
General						
<i>Δmagb</i>	Gα subunit	yes	-	ND	-	(Fang & Dean, 2000)
<i>Δabc1</i>	ABC transporter	yes	-	ND	-	(Urban, et al., 1999, Dufresne & Osbourn, 2001)
<i>Δmgapt2</i>	P-type ATPase	no	-	ND	-	(Gilbert, et al., 2006)
<i>Δnpr2</i>	nitrogen metabolism	yes	-	ND	-	(Lau & Hamer, 1996, Dufresne & Osbourn, 2001)
<i>Δapf1</i>	app differentiation	no	-	ND	-	(Silué, et al., 1998) (Sesma, unpublished)
<i>Δpmk1</i>	MAP kinase (MAPK)	no	-	no	-	(Xu & Hamer, 1996, Dufresne & Osbourn, 2001)
<i>Δmps1</i>	MAP kinase (MAPK)	no	-	no	-	(Xu, et al., 1998) (Sesma, unpublished)

(Continue next page)

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<i>M.oryzae</i> strains	Targeted gene function	APP ^a	Leaf symptom ^b	HY ^c	Root symptom	References
Leaf specific						
<i>alb1, buf1</i>	melanin synthesis	no	-	yes	+++	(Chumley & Valent, 1990, Dufresne & Osbourn, 2001, Sesma & Osbourn, 2004)
<i>ΔcpkA</i>	cAMP signalling	no	-	yes	++	(Xu, et al., 1997, Sesma & Osbourn, 2004)
<i>Δigd1</i>	invasive growth	yes	+	yes	+	(Balhadere, et al., 1999, Dufresne & Osbourn, 2001)
<i>Δmet1</i>	methionine biosynthesis	yes	+	yes	+	(Balhadere, et al., 1999, Dufresne & Osbourn, 2001)
<i>Δgde1</i>	Glycerophosphodiesterase	yes	++	yes	+++	(Balhadere, et al., 1999, Dufresne & Osbourn, 2001)
<i>Δmpg1</i>	hydrophobin	rd	+	yes	+++	(Talbot, et al., 1993) (Sesma, unpublished)
<i>Δnpr1</i>	nitrogen metabolism	yes	-	ND	+++	(Lau & Hamer, 1996, Dufresne & Osbourn, 2001)
<i>Δpth11</i>	seven transmembrane receptor	rd	+	yes	+++	(DeZwaan, et al., 1999) (Sesma, unpublished)

REFERENCES

- Ahn N, Kim S, Choi W, Im KH & Lee YH (2004) Extracellular Matrix Protein Gene, EMP1, Is Required for Appressorium Formation and Pathogenicity of the Rice Blast Fungus, *Magnaporthe grisea*. *Molecules and Cells* 17: 166-173.
- Bae CY, Kim S, Choi WB & Lee YH (2007) Involvement of extracellular matrix and integrin-like proteins on conidial adhesion and appressorium differentiation in *Magnaporthe oryzae*. *J Microbiol Biotechnol* 17: 1198-1203.
- Balhadere PV & Talbot NJ (2001) *PDE1* Encodes a P-Type ATPase Involved in Appressorium-Mediated Plant Infection by the Rice Blast Fungus *Magnaporthe grisea*. *Plant Cell* 13: 1987-2004.
- Beckerman JL & Ebbole DJ (1996) MPG1, a Gene Encoding a Fungal Hydrophobin of *Magnaporthe grisea*, Is Involved in Surface Recognition. *Molecular Plant-Microbe Interactions* 9: 450-456.
- Bell K & Oparka K (2011) Imaging plasmodesmata. *Protoplasma* 248: 9-25.
- Besi M, Tucker SL & Sesma A (2009) *Magnaporthe* and its relatives. ed.^eds.), p.^pp. John Wiley & Sons, Ltd: Chichester.
- Betts MF, Tucker SL, Galadima N, *et al.* (2007) Development of a high throughput transformation system for insertional mutagenesis in *Magnaporthe oryzae*. *Fungal Genetics and Biology* 44: 1035-1049.
- Bourett TM & Howard RJ (1990) *In vitro* development of penetration structures in the rice blast fungus *Magnaporthe grisea*. *Canadian Journal of Botany* 68: 329-342.
- Bourett TM & Howard RJ (1992) Actin in Penetration Pegs of the Fungal Rice Blast Pathogen, *Magnaporthe grisea*. *Protoplasma* 168: 20-26.
- Burd C & Cullen PJ (2014) Retromer: A Master Conductor of Endosome Sorting. *Cold Spring Harbor Perspectives in Biology* 6.
- Cannon PF (1994) The newly recognized family *Magnaporthaceae* and its interrelationships. *Systema Ascomycetum* 13: 25-42.
- Cannon PF & Kirk PM (2007) *Fungal Families of the World*. CABI Publishing.
- Clergeot PH, Gourgues M, Cots J, *et al.* (2001) *PLS1*, a gene encoding a tetraspanin-like protein, is required for penetration of rice leaf by the fungal pathogen *Magnaporthe grisea*. *Proceedings of the National Academy of Sciences, USA* 98: 6963-6968.
- Couch BC & Kohn LM (2002) A multilocus gene genealogy concordant with host preference indicates segregation of a new species, *Magnaporthe oryzae*, from *M. grisea*. *Mycologia* 94: 683-693.
- Couch BC, Fudal I, Lebrun MH, *et al.* (2005) Origins of host-specific populations of the blast pathogen *Magnaporthe oryzae* in crop domestication with subsequent expansion of pandemic clones on rice and weeds of rice. *Genetics* 170: 613-630.
- Chanclud E, Kisiala A, Emery NR, *et al.* (2016) Cytokinin Production by the Rice Blast Fungus Is a Pivotal Requirement for Full Virulence. *PLoS Pathogen* 12: e1005457.
- Chen J, Zheng W, Zheng S, *et al.* (2008) Rac1 Is Required for Pathogenicity and Chm1-Dependent Conidiogenesis in Rice Fungal Pathogen *Magnaporthe grisea*. *PLoS Pathogens* 4: e1000202.

- Chen X-L, Shi T, Yang J, et al. (2014)** N-Glycosylation of Effector Proteins by an α -1,3-Mannosyltransferase Is Required for the Rice Blast Fungus to Evade Host Innate Immunity. *The Plant Cell Online*.
- Chiapello H, Mallet L, Guerin C, et al. (2015)** Deciphering Genome Content and Evolutionary Relationships of Isolates from the Fungus *Magnaporthe oryzae* Attacking Different Host Plants. *Genome Biology Evolution* 7: 2896-2912.
- Choi W & Dean RA (1997)** The Adenylate Cyclase Gene MAC1 of *Magnaporthe grisea* Controls Appressorium Formation and Other Aspects of Growth and Development. *Plant Cell* 9: 1973-1983.
- Dagdas YF, Yoshino K, Dagdas G, Ryder LS, Bielska E, Steinberg G & Talbot NJ (2012)** Septin-Mediated Plant Cell Invasion by the Rice Blast Fungus, *Magnaporthe oryzae*. *Science* 336: 1590-1595.
- de Guillen K, Ortiz-Vallejo D, Gracy J, Fournier E, Kroj T & Padilla A (2015)** Structure Analysis Uncovers a Highly Diverse but Structurally Conserved Effector Family in Phytopathogenic Fungi. *PLoS Pathog* 11: e1005228.
- De Jong JC, McCormack BJ, Smirnoff N & Talbot NJ (1997)** Glycerol generates turgor in rice blast. *Nature* 389: 244-245.
- Dean R, Van Kan JAL, Pretorius ZA, et al. (2012)** The Top 10 fungal pathogens in molecular plant pathology. *Molecular Plant Pathology* 13: 414-430.
- Dean RA (1997)** Signal pathways and appressorium morphogenesis. *Annual Review of Phytopathology* 35: 211-234.
- Dean RA, Talbot NJ, Ebbole DJ, et al. (2005)** The genome sequence of the rice blast fungus *Magnaporthe grisea*. *Nature* 434: 980-986.
- Dixon KP, Xu JR, Smirnoff N & Talbot NJ (1999)** Independent Signaling Pathways Regulate Cellular Turgor during Hyperosmotic Stress and Appressorium-Mediated Plant Infection by *Magnaporthe grisea*. *Plant Cell* 11: 2045-2058.
- Donofrio NM, Oh Y, Lundy R, et al. (2006)** Global gene expression during nitrogen starvation in the rice blast fungus, *Magnaporthe grisea*. *Fungal Genetics and Biology* 43: 605-617.
- Dufresne M & Osbourn AE (2001)** Definition of tissue-specific and general requirements for plant infection in a phytopathogenic fungus. *Molecular Plant-Microbe Interactions* 14: 300-307.
- Egan MJ, Wang ZY, Jones MA, Smirnoff N & Talbot NJ (2007)** Generation of reactive oxygen species by fungal NADPH oxidases is required for rice blast disease. *Proceedings of the National Academy of Sciences, USA* 104: 11772-11777.
- FAO (2009)** The State of Food Insecurity. *World Economic crises - impacts and lessons learned* Food and Agriculture Organization of the United Nations.
- Fernandez J & Wilson RA (2012)** Why No Feeding Frenzy? Mechanisms of Nutrient Acquisition and Utilization During Infection by the Rice Blast Fungus *Magnaporthe oryzae*. *Molecular Plant-Microbe Interactions* 25: 1286-1293.
- Fernandez J, Marroquin-Guzman M & Wilson RA (2014)** Mechanisms of nutrient acquisition and utilization during fungal infections of leaves. *Annual Review of Phytopathology* 52: 155-174.

- Franceschetti M, Bueno E, Wilson RA, Tucker SL, Gómez-Mena C, Calder G & Sesma A (2011)** Fungal Virulence and Development Is Regulated by Alternative Pre-mRNA 3' End Processing in *Magnaporthe oryzae*. *PLoS Pathogens* 7: e1002441.
- Froeliger EH & Carpenter BE (1996)** *NUT1*, a major nitrogen regulatory gene in *Magnaporthe grisea*, is dispensable for pathogenicity. *Mol. Gen. Genet.* 251: 647-656.
- Gangopadhyay S & Row KVK (1986)** Perennation of *Pyricularia oryzae* briosi *et cav.* in sclerotial state. *International Journal of Tropical Plant Diseases* 4: 187-192.
- Giraldo MC & Valent B (2013)** Filamentous plant pathogen effectors in action. *Nat Rev Microbiol* 11: 800-814.
- Giraldo MC, Dagdas YF, Gupta YK, et al. (2013)** Two distinct secretion systems facilitate tissue invasion by the rice blast fungus *Magnaporthe oryzae*. *Nature Communications* 4: 1996.
- Gladfelter AS (2006)** Control of filamentous fungal cell shape by septins and formins. *Nature Reviews Microbiology* 4: 223-229.
- Gupta YK, Dagdas YF, Martinez-Rocha A-L, et al. (2015)** Septin-Dependent Assembly of the Exocyst Is Essential for Plant Infection by *Magnaporthe oryzae*. *The Plant Cell*.
- Hamer JE, Howard RJ, Chumley F & Valent B (1988)** A Mechanism for Surface Attachment in Spores of a Plant Pathogenic Fungus. *Science* 239: 288-290.
- Harmon PF & Latin R (2005)** Winter survival of the perennial ryegrass pathogen *Magnaporthe oryzae* in north central Indiana. *Plant Disease* 89: 412-418.
- Harmon PF, Dunkle LD & Latin R (2003)** A rapid PCR-based method for the detection of *Magnaporthe oryzae* from infected perennial ryegrass. *Plant Disease* 87: 1072-1076.
- Hawksworth DL (2011)** A new dawn for the naming of fungi: impacts of decisions made in Melbourne in July 2011 on the future publication and regulation of fungal names. *MycKeys* 1: 7-20.
- Heath MC, Valent B, Howard RJ & Chumley FG (1992)** Ultrastructural interactions of one strain of *Magnaporthe grisea* with goosegrass and weeping lovegrass. *Canadian Journal of Botany* 70: 779-787.
- Howard R, Ferrari M, Roach D & Money N (1991)** Penetration of Hard Substrates by a Fungus Employing Enormous Turgor Pressures. *Proceedings of the National Academy of Sciences, USA* 88: 11281-11284.
- Howard RJ (1997)** Breaching the outer barrier - cuticle and cell wall penetration. *The Mycota V. Plant Relationships, Part A*, (GC Carroll PT, ed.^eds.), p.^pp. 43-60. Springer-Verlag, Berlin/Heidelberg.
- Ikeda Ki, Nakayashiki H, Kataoka T, Tamba H, Hashimoto Y, Tosa Y & Mayama S (2002)** Repeat-induced point mutation (RIP) in *Magnaporthe grisea*: implications for its sexual cycle in the natural field context. *Molecular Microbiology* 45: 1355-1364.
- Inoue K, Suzuki T, Ikeda K, et al. (2007)** Extracellular matrix of *Magnaporthe oryzae* may have a role in host adhesion during fungal penetration and is digested by matrix metalloproteinases. *Journal of General Plant Pathology* 73: 388-398.

- Jeon J, Park SY, Chi MH, et al. (2007)** Genome-wide functional analysis of pathogenicity genes in the rice blast fungus. *Nature Genetics* 39: 561-565.
- Jose M, Tollis S, Nair D, et al. (2015)** A quantitative imaging-based screen reveals the exocyst as a network hub connecting endocytosis and exocytosis. *Molecular Biology of the Cell* 26: 2519-2534.
- Kang S, Lebrun MH, Farrall L & Valent B (2001)** Gain of Virulence Caused by Insertion of a Pot3 Transposon in a *Magnaporthe grisea* Avirulence Gene. *Molecular Plant-Microbe Interactions* 14: 671-674.
- Kankanala P, Czymmek K & Valent B (2007)** Roles for Rice Membrane Dynamics and Plasmodesmata during Biotrophic Invasion by the Blast Fungus. *Plant Cell* 19: 706-724.
- Kershaw MJ & Talbot NJ (2009)** Genome-wide functional analysis reveals that infection-associated fungal autophagy is necessary for rice blast disease. *Proceedings of the National Academy of Sciences of the United States of America* 106: 15967-15972.
- Kershaw MJ, Wakley G & Talbot NJ (1998)** Complementation of the Mpg1 mutant phenotype in *Magnaporthe grisea* reveals functional relationships between fungal hydrophobins. *EMBO Journal* 17: 3838-3849.
- Khang CH, Berruyer R, Giraldo MC, et al. (2010)** Translocation of *Magnaporthe oryzae* Effectors into Rice Cells and Their Subsequent Cell-to-Cell Movement. *Plant Cell* 22: 1388-1403.
- Kim C, Ye F & Ginsberg MH (2011)** Regulation of Integrin Activation. *Annual Review of Cell and Developmental Biology*, Vol 27 27: 321-345.
- Kim S, Ahn IP, Rho HS & Lee YH (2005)** MHP1, a *Magnaporthe grisea* hydrophobin gene, is required for fungal development and plant colonization. *Molecular Microbiology* 57: 1224-1237.
- Kirk PM, Cannon PF, Minter DW & Stalpers JA (2008)** *Ainsworth and Bisby's Dictionary of the Fungi*. CABI Publishing.
- Kolattukudy PE (1985)** Enzymatic Penetration of the Plant Cuticle by Fungal Pathogens. *Annual Review of Phytopathology* 23: 223-250.
- Kong S, Park SY & Lee YH (2015)** Systematic characterization of the bZIP transcription factor gene family in the rice blast fungus, *Magnaporthe oryzae*. *Environ Microbiol* 17: 1425-1443.
- Koumandou VL, Klute MJ, Herman EK, Nunez-Miguel R, Dacks JB & Field MC (2011)** Evolutionary reconstruction of the retromer complex and its function in *Trypanosoma brucei*. *Journal of Cell Science* 124: 1496-1509.
- Kumar J, Nelson RJ & Zeigler RS (1999)** Population structure and dynamics of *Magnaporthe grisea* in the Indian Himalayas. *Genetics* 152: 971-984.
- Lambou K, Malagnac F, Barbisan C, Tharreau D, Lebrun MH & Silar P (2008)** The Crucial Role of the Pls1 Tetraspanin during Ascospore Germination in *Podospora anserina* Provides an Example of the Convergent Evolution of Morphogenetic Processes in Fungal Plant Pathogens and Saprobes. *Eukaryotic Cell* 7: 1809-1818.
- Lau G & Hamer JE (1996)** Regulatory Genes Controlling MPG1 Expression and Pathogenicity in the Rice Blast Fungus *Magnaporthe grisea*. *Plant Cell* 8: 771-781.
- Lee FN, Jackson MA & Walker NR (2000)** Characteristics of *Pyricularia grisea* "microsclerotia" produced in shaken culture. *B.R. Wells RICE Research*

- Studies* 1999, Vol. 476 (Norman RJ & Beyrouthy CA, ed.^eds.), p.^pp. 475-479. University of Arkansas.
- Leung H, Borromeo E, Bernardo M & Notteghem JL (1988)** Genetic analysis of virulence in the rice blast fungus *Magnaporthe grisea*. *Phytopathology* 78: 1227-1233.
- Li GT, Zhou XY, Kong LG, et al. (2011)** MoSfl1 Is Important for Virulence and Heat Tolerance in *Magnaporthe oryzae*. *Plos One* 6: e19951.
- Li L, Xue CY, Bruno K, Nishimura M & Xu JR (2004)** Two PAK kinase genes, CHM1 and MST20, have distinct functions in *Magnaporthe grisea*. *Molecular Plant-Microbe Interactions* 17: 547-556.
- Li L, Ding SL, Sharon A, Orbach M & Xu JR (2007)** Mir1 is highly upregulated and localized to nuclei during infectious hyphal growth in the rice blast fungus. *Molecular Plant-Microbe Interactions* 20: 448-458.
- Linder MB, Szilvay GR, Nakari-Setälä T & Penttilä ME (2005)** Hydrophobins: the protein-amphiphiles of filamentous fungi. *FEMS Microbiology Reviews* 29: 877-896.
- Liu WD, Zhou XY, Li GT, et al. (2011)** Multiple Plant Surface Signals are Sensed by Different Mechanisms in the Rice Blast Fungus for Appressorium Formation. *Plos Pathogens* 7: e1001261.
- Lu J, Cao H, Zhang L, Huang P & Lin F (2014)** Systematic analysis of Zn2Cys6 transcription factors required for development and pathogenicity by high-throughput gene knockout in the rice blast fungus. *PLoS Pathog* 10: e1004432.
- MAGRAMA (2016)** Ministerio de Agricultura, Alimentación y Medio Ambiente gobierno de España. .
- Marcel S, Sawers R, Oakeley E, Angliker H & Paszkowski U (2010)** Tissue-Adapted Invasion Strategies of the Rice Blast Fungus *Magnaporthe oryzae*. *Plant Cell* 22: 3177-3187.
- Marroquin-Guzman M & Wilson RA (2015)** GATA-Dependent Glutaminolysis Drives Appressorium Formation in *Magnaporthe oryzae* by Suppressing TOR Inhibition of cAMP/PKA Signaling. *PLoS Pathogens* 11: e1004851.
- Martin-Urdiroz M, Osés-Ruiz M, Ryder LS & Talbot NJ (2015)** Investigating the biology of plant infection by the rice blast fungus *Magnaporthe oryzae*. *Fungal Genetics and Biology*.
- Mehrabi R, Ding S & Xu JR (2008)** MADS-box transcription factor Mig1 is required for infectious growth in *Magnaporthe grisea*. *Eukaryotic Cell* 7: 791-799.
- Mendgen K & Hahn M (2002)** Plant infection and the establishment of fungal biotrophy. *Trends in Plant Science* 7: 352-356.
- Mengiste T (2012)** Plant Immunity to Necrotrophs. *Annual Review of Phytopathology, Vol 50* 50: 267-294.
- Mentlak TA, Kombrink A, Shinya T, et al. (2012)** Effector-Mediated Suppression of Chitin-Triggered Immunity by *Magnaporthe oryzae* Is Necessary for Rice Blast Disease. *Plant Cell* 24: 322-335.
- Mosquera G, Giraldo MC, Khang CH, Coughlan S & Valent B (2009)** Interaction Transcriptome Analysis Identifies *Magnaporthe oryzae* BAS1-4 as Biotrophy-Associated Secreted Proteins in Rice Blast Disease. *Plant Cell* 21: 1273-1290.

- Mostowy S & Cossart P (2012)** Septins: the fourth component of the cytoskeleton. *Nature Reviews Molecular Cell Biology* 13: 183-194.
- Nguyen QB, Kadotani N, Kasahara S, Tosa Y, Mayama S & Nakayashiki H (2008)** Systematic functional analysis of calcium-signalling proteins in the genome of the rice-blast fungus, *Magnaporthe oryzae*, using a high-throughput RNA-silencing system. *Molecular Microbiology* 68: 1348-1365.
- Oh Y, Donofrio N, Pan HQ, et al. (2008)** Transcriptome analysis reveals new insight into appressorium formation and function in the rice blast fungus *Magnaporthe oryzae*. *Genome Biology* 9: R85.
- Ou SH (1985)** *Rice Diseases*. Commonwealth Mycological Institute, Kew, Surrey, UK.
- Park G, Xue C, Zheng L, Lam S & Xu JR (2002)** MST12 Regulates Infectious Growth but not Appressorium Formation in the Rice Blast Fungus *Magnaporthe grisea*. *Molecular Plant-Microbe Interactions* 15: 183-192.
- Park G, Xue C, Zhao X, Kim Y, Orbach M & Xu J-R (2006)** Multiple Upstream Signals Converge on the Adaptor Protein Mst50 in *Magnaporthe grisea*. *Plant Cell* 18: 2822-2835.
- Read ND (2011)** Exocytosis and growth do not occur only at hyphal tips. *Molecular Microbiology* 81: 4-7.
- Ribot C, Hirsch J, Batzergue S, Tharreau D, Notteghem JL, Lebrun MH & Morel JB (2008)** Susceptibility of rice to the blast fungus, *Magnaporthe grisea*. *Journal of Plant Physiology* 165: 114-124.
- Riquelme M (2013)** Tip Growth in Filamentous Fungi: A Road Trip to the Apex. *Annual Review of Microbiology* 67: 587-609.
- Rodrigues FA, Benhamou N, Datnoff LE, Jones JB & Belanger RR (2003)** Ultrastructural and Cytochemical Aspects of Silicon-Mediated Rice Blast Resistance. *Phytopathology* 93: 535-546.
- Rodríguez-Romero J, Franceschetti M, Bueno E & Sesma A (2015)** Multilayer Regulatory Mechanisms Control Cleavage Factor I Proteins in Filamentous Fungi. *Nucleic Acids Research* 43: 179-195.
- Ryder LS, Dagdas YF, Mentlak TA, et al. (2013)** NADPH oxidases regulate septin-mediated cytoskeletal remodeling during plant infection by the rice blast fungus. *Proc Natl Acad Sci U S A* 110: 3179-3184.
- Saccardo PA (1880)** Fungorum extra-europaeorum Pugillus. *Michelia* 2: 136-149.
- Saitoh H, Fujisawa S, Ito A, et al. (2009)** SPM1 encoding a vacuole-localized protease is required for infection-related autophagy of the rice blast fungus *Magnaporthe oryzae*. *Fems Microbiology Letters* 300: 115-121.
- Saitoh H, Fujisawa S, Mitsuoka C, et al. (2012)** Large-Scale Gene Disruption in *Magnaporthe oryzae* Identifies MC69, a Secreted Protein Required for Infection by Monocot and Dicot Fungal Pathogens. *PLoS Pathogens* 8: e1002711.
- Saleh D, Milazzo J, Adreit H, Fournier E & Tharreau DC (2014)** South-East Asia is the center of origin, diversity and dispersion of the rice blast fungus, *Magnaporthe oryzae*. *New Phytologist* 201: 1440-1456.
- Saleh D, Xu P, Shen Y, et al. (2012)** Sex at the origin: an Asian population of the rice blast fungus *Magnaporthe oryzae* reproduces sexually. *Molecular Ecology* 21: 1330-1344.

- Saunders DG, Dagdas YF & Talbot NJ (2010)** Spatial uncoupling of mitosis and cytokinesis during appressorium-mediated plant infection by the rice blast fungus *Magnaporthe oryzae*. *Plant Cell* 22: 2417-2428.
- Saunders DGO, Aves SJ & Talbot NJ (2010)** Cell Cycle-Mediated Regulation of Plant Infection by the Rice Blast Fungus. *Plant Cell* 22: 497-507.
- Seaman MNJ (2012)** The retromer complex – endosomal protein recycling and beyond. *Journal of Cell Science* 125: 4693-4702.
- Sesma A & Osbourn AE (2004)** The rice leaf blast pathogen undergoes developmental processes typical of root-infecting fungi. *Nature* 431: 582-586.
- Shattil SJ, Kim C & Ginsberg MH (2010)** The final steps of integrin activation: the end game. *Nature Reviews Molecular Cell Biology* 11: 288-300.
- Skamnioti P & Gurr SJ (2007)** *Magnaporthe grisea* cutinase2 mediates appressorium differentiation and host penetration and is required for full virulence. *Plant Cell* 19: 2674-2689.
- Skamnioti P & Gurr SJ (2009)** Against the grain: safeguarding rice from rice blast disease. *Trends in Biotechnology* 27: 141-150.
- Soanes DM, Chakrabarti A, Paszkiewicz KH, Dawe AL & Talbot NJ (2012)** Genome-wide transcriptional profiling of appressorium development by the rice blast fungus *Magnaporthe oryzae*. *PLoS Pathog* 8: e1002514.
- Steinberg G (2007)** Hyphal Growth: a Tale of Motors, Lipids, and the Spitzenkörper. *Eukaryotic Cell* 6: 351-360.
- Sudbery P (2011)** Fluorescent proteins illuminate the structure and function of the hyphal tip apparatus. *Fungal Genetics and Biology* 48: 849-857.
- Sweigard JA, Chumley FG & Valent B (1992)** Disruption of a *Magnaporthe grisea* Cutinase Gene. *Molecular & General Genetics* 232: 183-190.
- Talbot NJ (2003)** On the Trail of a Cereal Killer: Exploring the Biology of *Magnaporthe grisea*. *Annual Review of Microbiology* 57: 177-202.
- Talbot NJ, Salch YP, Ma M & Hamer JE (1993)** Karyotypic Variation within Clonal Lineages of the Rice Blast Fungus, *Magnaporthe grisea*. *Applied and Environmental Microbiology* 59: 585.
- Talbot NJ, McCafferty HRK, Ma M, Moore K & Hamer JE (1997)** Nitrogen starvation of the rice blast fungus *Magnaporthe grisea* may act as an environmental cue for disease symptom expression. *Physiological and Molecular Plant Pathology* 50: 179-198.
- Talbot NJ, Kershaw MJ, Wakley GE, De Vries OMH, Wessels JGH & Hamer JE (1996)** MPG1 Encodes a Fungal Hydrophobin Involved in Surface Interactions during Infection-Related Development of *Magnaporthe grisea*. *Plant Cell* 8: 985-999.
- Tanzer MM, Arst HN, Skalchunes AR, Coffin M, Darveau BA, Heiniger RW & Shuster JR (2003)** Global nutritional profiling for mutant and chemical mode-of-action analysis in filamentous fungi. *Funct Integr Genomics* 3: 160-170.
- Thines E, Weber RWS & Talbot NJ (2000)** MAP Kinase and Protein Kinase A-Dependent Mobilization of Triacylglycerol and Glycogen during Appressorium Turgor Generation by *Magnaporthe grisea*. *Plant Cell* 12: 1703-1718.
- Thinlay X, Finckh MR, Bordeos AC & Zeigler RS (2000)** Effects and possible causes of an unprecedented rice blast epidemic on the traditional farming system of Bhutan. *Agriculture, Ecosystems & Environment* 78: 237-248.

- Thongkantha S, Jeewon R, Vijaykrishna D, Lumyong S, McKenzie EHC & Hyde KD (2009)** Molecular phylogeny of Magnaporthaceae (Sordariomycetes) with a new species *Ophioceras Chiangdaoense* from *Dracaena loureiroi* in Thailand. *Fungal Diversity* 34: 157-173.
- Tucker SL & Talbot NJ (2001)** Surface Attachment and Pre-Penetration Stage Development by Plant Pathogenic Fungi. *Annual Review of Phytopathology* 39: 385-418.
- Tucker SL, Besi MI, Galhano R, et al. (2010)** Common Genetic Pathways Regulate Organ-Specific Infection-Related Development in the Rice Blast Fungus. *Plant Cell* 22: 953-972.
- Urashima AS, Lavorent NA, Goulart ACP & Mehta YR (2004)** Resistance Spectra of Wheat Cultivars and Virulence Diversity of *Magnaporthe grisea* Isolates in Brazil. *Fitopatologia Brasileira* 29: 511-518.
- Valent B & Chumley FG (1991)** Molecular Genetic Analysis of the Rice Blast Fungus, *Magnaporthe grisea*. *Annual Review of Phytopathology* 29: 443-467.
- Valent B & Khang CH (2010)** Recent advances in rice blast effector research. *Current Opinion in Plant Biology* 13: 1-8.
- Valent B, Farrall L & Chumley F (1991)** *Magnaporthe grisea* genes for pathogenicity and virulence identified through a series of backcrosses. *Genetics* 127: 87-101.
- Veneault-Fourrey C, Barooah M, Egan M, Wakley G & Talbot NJ (2006)** Autophagic Fungal Cell Death Is Necessary for Infection by the Rice Blast Fungus. *Science* 312: 580-583.
- Wang ZY, Thornton CR, Kershaw MJ, Debaio L & Talbot NJ (2003)** The glyoxylate cycle is required for temporal regulation of virulence by the plant pathogenic fungus *Magnaporthe grisea*. *Molecular Microbiology* 47: 1601-1612.
- Wilson RA, Gibson RP, Quispe CF, Littlechild JA & Talbot NJ (2010)** An NADPH-dependent genetic switch regulates plant infection by the rice blast fungus. *Proceedings of the National Academy of Sciences of the United States of America* 107: 21902-21907.
- Wilson RA, Jenkinson JM, Gibson RP, Littlechild JA, Wang ZY & Talbot NJ (2007)** Tps1 regulates the pentose phosphate pathway, nitrogen metabolism and fungal virulence. *EMBO Journal* 26: 3673-3685.
- Xiao JZ, Ohshima A, Kamakura T & Ishiyama T (1994)** Extracellular Glycoprotein(s) Associated with Cellular Differentiation in *Magnaporthe grisea*. *Molecular Plant-Microbe Interactions* 7: 639.
- Xu JR & Hamer JE (1996)** MAP kinase and cAMP signalling regulate infection structure formation and pathogenic growth in the rice blast fungus *Magnaporthe grisea*. *Genes and Development* 10: 2696-2706.
- Xu JR, Staiger CJ & Hamer JE (1998)** Inactivation of the mitogen-activated protein kinase Mps1 from the rice blast fungus prevents penetration of host cells but allows activation of plant defense responses. *Proceedings of the National Academy of Sciences, USA* 95: 12713-12718.
- Xu JR, Urban M, Sweigard JA & Hamer JE (1997)** The CPKA Gene of *Magnaporthe grisea* Is Essential for Appressorial Penetration. *Molecular Plant-Microbe Interactions* 10: 187-194.

- Xue MF, Yang J, Li ZG, et al. (2012)** Comparative Analysis of the Genomes of Two Field Isolates of the Rice Blast Fungus *Magnaporthe oryzae*. *PLoS Genetics* 8: e1002869.
- Yoshida K, Saunders DG, Mitsuoka C, et al. (2016)** Host specialization of the blast fungus *Magnaporthe oryzae* is associated with dynamic gain and loss of genes linked to transposable elements. *BMC Genomics* 17: 370.
- You MP, Lanoiselet V, Wang CP, Shivas RG, Li YP & Barbetti MJ (2012)** First Report of Rice Blast (*Magnaporthe oryzae*) on Rice (*Oryza sativa*) in Western Australia. *Plant Disease* 96: 1228-1228.
- Zeigler RS (1998)** Recombination in *Magnaporthe grisea*. *Annual Review of Phytopathology* 36: 249-276.
- Zhang HF, Xue CY, Kong LG, Li GT & Xu JR (2011)** A Pmk1-Interacting Gene Is Involved in Appressorium Differentiation and Plant Infection in *Magnaporthe oryzae*. *Eukaryotic Cell* 10: 1062-1070.
- Zhang HF, Liu KY, Zhang X, et al. (2011)** Two Phosphodiesterase Genes, PDEL and PDEH, Regulate Development and Pathogenicity by Modulating Intracellular Cyclic AMP Levels in *Magnaporthe oryzae*. *Plos One* 6: e17241.
- Zhao X, Kim Y, Park G & Xu J-R (2005)** A Mitogen-Activated Protein Kinase Cascade Regulating Infection-Related Morphogenesis in *Magnaporthe grisea*. *Plant Cell* 17: 1317-1329.
- Zhao XH, Mehrabi R & Xu JR (2007)** Mitogen-activated protein kinase pathways and fungal pathogenesis. *Eukaryotic Cell* 6: 1701-1714.
- Zheng W, Zhou J, He Y, et al. (2015)** Retromer Is Essential for Autophagy-Dependent Plant Infection by the Rice Blast Fungus. *PLoS Genet* 11: e1005704.
- Zhong Z, Norvinyeku J, Chen M, et al. (2016)** Directional Selection from Host Plants Is a Major Force Driving Host Specificity in *Magnaporthe* Species. *Scientific Reports* 6: 25591.
- Zhou X, Zhao X, Xue C, Dai Y & Xu J-R (2014)** Bypassing Both Surface Attachment and Surface Recognition Requirements for Appressorium Formation by Overactive Ras Signaling in *Magnaporthe oryzae*. *Molecular Plant-Microbe Interactions* 27: 996-1004.
- Zhou X, Liu W, Wang C, Xu Q, Wang Y, Ding S & Xu JR (2011)** A MADS-box transcription factor MoMcm1 is required for male fertility, microconidium production and virulence in *Magnaporthe oryzae*. *Molecular Microbiology* 80: 33-53.
- Zhou XY, Zhang HF, Li GT, Shaw B & Xu JR (2012)** The Cyclase-Associated Protein Cap1 Is Important for Proper Regulation of Infection-Related Morphogenesis in *Magnaporthe oryzae*. *PLoS Pathogens* 8: e1002911.

OBJECTIVES

The main objective of this PhD thesis is the characterization of novel genes associated with *M. oryzae* infection processes. It has been achieved through the characterization of two novel pathogenicity genes:

- *TPC1* (Transcription factor for Polarity Control 1, Chapter 2), and
- *EXP5* (Exportin-5, Chapter 3).

The research study carried out with Exp5 also allowed the identification of a single stranded RNA virus in the rice isolate Guy11 (Chapter 4).

CHAPTER 2

Tpc1 is an essential Zn(II)₂Cys₆ transcriptional regulator required for polarized growth and virulence in the rice blast fungus

Results presented in this chapter are included within a manuscript currently under review in PLoS Pathogens:

Tpc1 is an essential Zn(II)₂Cys₆ transcriptional regulator required for polarized growth and virulence in the rice blast fungus

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ABSTRACT

The establishment of polarity is a critical process in pathogenic fungi, mediating infection-related morphogenesis and host tissue invasion. Here, we report the identification of *TPC1* (Transcription factor for Polarity Control 1), which regulates invasive polarized growth in the rice blast fungus *Magnaporthe oryzae*. *TPC1* encodes a putative transcription factor of the fungal Zn(II)₂Cys₆ family, exclusive to filamentous fungi. Tpc1-deficient mutants showed severe defects in conidiogenesis, infection-associated autophagy, glycogen metabolism and plant tissue colonisation. By tracking actin-binding proteins, septin-5 and autophagosome components, we show that Tpc1 regulates cytoskeletal dynamics and infection-associated autophagy during appressorium-mediated plant penetration. Importantly, we found that Tpc1 regulates *NOXD*, the p22^{phox} subunit of the fungal NADPH oxidase complex. Tpc1 possibly controls the spatial and temporal regulation of cortical F-actin through the regulation of the NADPH oxidase complex during appressorium repolarisation. Here, we provide evidence that Tpc1 is a core developmental regulator in filamentous fungi, linking the regulated synthesis of reactive oxygen species with polarity control during host invasion.

INTRODUCTION

Rice blast disease is one of the most serious diseases of cultivated rice worldwide and is caused by the filamentous, ascomycete fungus *Magnaporthe oryzae* (Wilson & Talbot, 2009, Valent & Khang, 2010). The disease is initiated when a conidium lands on the rice leaf surface. Then, it germinates to produce a single germ tube that differentiates at its tip to develop a specialised infection structure called an appressorium (Tucker & Talbot, 2001). During the initial stages of appressorium formation, a septum defines the developing appressorium from the rest of the germ tube following a single mitotic division in the germ tube (Veneault-Fourrey, *et al.*, 2006). When the appressorium matures, the three conidial cells and germ tube collapse due to infection-associated autophagy and are no longer viable after 24h (Veneault-Fourrey, *et al.*, 2006). Subsequently, a penetration peg emerges from the base of the appressorium and ruptures the leaf cuticle. A toroidal filamentous actin network forms at the base of the appressorium pore, which is scaffolded by septin GTPases (Dagdas, *et al.*, 2012). The assembly of septin GTPases is regulated by the Nox2 NADPH oxidase complex, which is required for re-modelling of the F-actin cytoskeleton and for assembling the exocyst (Ryder, *et al.*, 2013, Gupta, *et al.*, 2015, Ryder & Talbot, 2015). A proper acting ring formation is necessary for peg emergence and re-establishment of polarized growth at the point of plant penetration. After penetration, the fungal peg grows as a narrow, short primary invasive hypha (Park, *et al.*, 2004), before differentiating into bulbous invasive hyphae during colonisation of the first invaded host cell (Kankanala, *et al.*, 2007). Disease symptoms appear between 72h and 96h after initial infection and coalesce into large spreading necrotic lesions from which the fungus sporulates. *M. oryzae* has also the capacity to penetrate roots by means of hyphopodia and can colonize root tissue and spread systemically throughout the plant (Sesma & Osbourn, 2004, Marcel, *et al.*, 2010).

In this study, we report the identification of a novel Zn(II)₂Cys₆ transcriptional regulator involved in the early stages of plant infection by *M. oryzae*. The Zn(II)₂Cys₆ binuclear cluster domain (IPR001138, PF00172) is exclusively found in the fungal kingdom (MacPherson, *et al.*, 2006, Campbell, *et al.*, 2008). The six cysteine residues bind two zinc atoms, which coordinate folding of the domain involved in DNA-binding. Most Zn(II)₂Cys₆ proteins have been studied in *Saccharomyces cerevisiae* and *Aspergillus* species (Todd & Andrianopoulos, 1997, MacPherson, *et*

al., 2006, Yin & Keller, 2011). Typically, the Zn(II)₂Cys₆ proteins are pathway-specific activators under the control of major regulators (Todd & Andrianopoulos, 1997, Berger, *et al.*, 2006, Yin & Keller, 2011, Downes, *et al.*, 2014). The regulator of galactose catabolism in yeast, Gal4p (Traven, *et al.*, 2006), and the regulators of acetate assimilation FacB (Todd, *et al.*, 1998) and the aflatoxin cluster AfIR (Fernandes, *et al.*, 1998) in *A. nidulans*, are among the best studied examples. By contrast, there are very few Zn(II)₂Cys₆ transcriptional regulators studied in the rice blast fungus (Annex Table A2.1), and fewer still with a clear biological function defined. Out of the 175 members of the Zn(II)₂Cys₆ binuclear cluster family present in *M. oryzae* (Annex Table A2.2), only eight of them (MoCod1, MoCod2, Pig1, Tra1, Tdg3, Xlr1, Far1 and Far2) have been examined in any detail (Tsuji, *et al.*, 2000, Battaglia, *et al.*, 2013, Breth, *et al.*, 2013, Chung, *et al.*, 2013, bin Yusof, *et al.*, 2014) (Annex Table A2.1). A high-throughput gene knockout approach of 104 Zn(II)₂Cys₆ proteins in *M. oryzae* revealed large variation in their biological functions, and found seven additional Zn(II)₂Cys₆ proteins to be required for plant infection, including Gpf1 and Cln2 (Lu, *et al.*, 2014). However, despite this information, the mechanistic insights into how the Zn(II)₂Cys₆ proteins govern *M. oryzae* cellular processes are largely unknown.

Here, we characterize a novel mutant of *M. oryzae* that shows defects in pathogenicity and vegetative growth following its selection from a *M. oryzae* T-DNA insertional library. The T-DNA insertion is located within a gene (MGG_01285) encoding a Zn(II)₂Cys₆ binuclear cluster protein, which we name *TPC1*. We reveal the involvement of this transcriptional regulator in polarized growth, cell patterning and virulence in *M. oryzae*. Among the genes regulated by Tpc1 we found *NOXD*, an important component of the fungal NADPH complex. We provide mechanistic insights into the role of Tpc1, a key regulator of polarity in *M. oryzae* that controls growth, autophagy and septin-mediated reorientation of the F-actin cytoskeleton to facilitate plant colonisation.

RESULTS

M. oryzae *TPC1* mutants show defects in development and pathogenicity

In order to identify novel infection-related genes we screened a total of 300 T-DNA transformants for their ability to infect rice roots using a *M. oryzae* insertional library (Tucker, *et al.*, 2010). The M1422 mutant developed very restricted disease lesions on roots and was selected for further characterization (Fig. 2.1A). On leaves, M1422 produced only a small number of resistant-type lesions (Fig. 2.1B). Colonies of M1422 were also compact and reduced in size, when compared with the wild-type (Fig. 2.1C).

Fig. 2.1. M1422 mutant shows severe defects in pathogenicity, vegetative growth and increase tolerance to nitrogen depletion. (A) M1422 shows reduced disease symptoms on rice roots. Photographs were taken 15 days after inoculation. Wild-type (WT) strain Guy11 presents maximum lesion severity (score 3) in 95% of the infected roots compared to the M1422-infected seedlings that show mild (score 1-2) or null (score 0) necrotic symptoms on roots. Lesions were scored on a scale 0-3, based on colour intensity and extension of the necrotic lesion (Tucker, *et al.*, 2010). **(B)** M1422 is strongly impaired in its ability to infect rice and barley leaves. **(C)** M1422 displays impaired ability to grow on complete medium (CM) and minimal medium (MM) depleted of carbon (MM-C) and nitrogen (MM-N). Relative to strain growth on MM, WT is more affected than M1422 on MM-N. Colony edge is indicated with a white arrow.

The insertion site of the T-DNA within M1422 was located 0.9 kb downstream of the start codon of locus MGG_01285 in the *M. oryzae* genome (Supplemental Fig. S2.1A). This gene encodes a putative transcription factor that belongs to a Zn(II)₂Cys₆ binuclear cluster family. We named this gene *Transcription factor for Polarity Control 1* (*TPC1*). The predicted coding region of *TPC1* is ~2.6 kb long and encodes 839

amino acids (<http://fungi.ensembl.org/index.html>; MG8). The *TPC1* predicted amino acid sequence includes a putative nuclear localisation signal (NLS), one Zn(II)₂Cys₆ binuclear cluster DNA binding domain, a predicted MAPK docking site, two putative phosphorylation sites and a putative sumoylation site within the NLS (Supplemental Fig. S2.1A).

A single T-DNA insertion in M1422 genome was detected by Southern blot hybridisation using the hygromycin phosphotransferase gene as a probe (Supplemental Fig. S2.1B). We also generated a second mutant in *TPC1* by targeted gene replacement (Supplemental Fig. S2.2A-B). We complemented both M1422 and $\Delta tpc1$ with a C-terminal *TPC1:GFP* gene fusion under control of its native promoter. The complemented mutants recovered normal mycelial growth, colonial morphology and full virulence on rice (Supplemental Figs. S2.1C-D and S2.2C). We conclude that mutants M1422 and $\Delta tpc1$ are impaired in *TPC1* function.

Two striking characteristics of M1422 and $\Delta tpc1$ were their impaired hyphal growth and colony morphology (Fig. 2.1C, and Supplemental Figs. S2.1D and S2.2C). Vegetative growth of Tpc1-lacking strains was severely compromised in both complete (CM) and minimal (MM) medium ($p < 0.01$), and showed compact colonies and non-invasive colony morphology (Fig. 2.1C, Supplemental Figs. S2.1E and S2.2D). In *Neurospora crassa*, a class of mutants with polarity defects also exhibited this type of colony morphology (Seiler & Plamann, 2003). Upon detailed examination of the edge of each colony, it was obvious that the branching pattern of vegetative hyphae of M1422 was different to that of Guy11, with greater branching of hyphae evident (Supplemental Fig. S2.1F). Therefore, the lack of Tpc1 affected plant virulence, vegetative growth and colony morphology.

***M. oryzae* TPC1 is required for conidiogenesis and appressorium development**

We observed that M1422 sporulated poorly compared to wild-type (Fig. 2.2A). In addition, M1422 asexual spores showed defects in septation (numbers of cell per conidium) and conidial morphology (Fig. 2.2B). Wild-type conidia were uniformly pyriform, three-celled spores. By contrast, in M1422, although the majority of conidia were three-celled (80%), a significant percentage of two-celled conidia (17%), single-celled

(2%) and four-celled conidia (1%) were observed. Up to 26% of spores showed abnormal morphology in contrast to wild-type where less than 4% were misshapen ($n > 300$). We also found that appressorium development was affected in M1422 (Fig. 2.2C). On hydrophobic coverslips, wild-type conidia germinated to form one germ tube that emerged from the apical cell and formed an appressorium within 4h-8h (Fig. 2.2C-E). In M1422, 40% of conidia germinated from two cells. This percentage increased to 50%-60% with extended incubation time (4h-8h). Formation of two appressoria was never observed in wild-type conidia. We conclude that M1422 is impaired in the normal spatial patterning of appressorium development.

Fig. 2.2. *M. oryzae* TPC1 is required for conidiogenesis and infection-related development. (A) M1422 mycelia produces less conidia per cm² than mycelia of wild-type (WT) strain on CM. Error bars represent the standard deviation of three independent experiments ($n > 300$). (B) Defects in the number of cells *per* conidium and conidia morphology of M1422. The M1422 mutant produces 1-, 2-, 3- (normal and abnormal morphology) and 4-celled conidia (respectively 1c, 2c, 3c and 4c in the panel) compared to the 3-celled conidia uniformly produced by the WT. Scale bar = 20 μ m. (C) M1422 conidia inoculated onto glass coverslips showed an increased frequency of conidia germination from two cells. Scale bar = 10 μ m. (D) Quantification of M1422 defects in germination rate and development of germ tubes per conidia on coverslips. Two germ tubes germinated frequently from M1422 conidia. (E) Quantification of M1422 defects in the formation of infection-related structures; germ tubes (gt) and appressoria (app). M1422 conidia form at least one appressorium during infection-related development on coverslips (mean from > 300 conidia; three biological repeats).

Polarity is coupled to autophagy and glycogen degradation in *M. oryzae*

The impairment of appressorium-mediated plant infection by *TPC1* mutants suggested that it might play a critical role in penetration peg development (Saunders, *et al.*, 2010). Appressorium function is known to depend on autophagic cell death of conidia, prior to appressorium maturation (Veneault-Fourrey, *et al.*, 2006, Kershaw & Talbot, 2009). Therefore, we investigated whether infection-associated autophagy proceeded normally and if conidia underwent autophagic cell death. A *GFP:MoATG8* construct was introduced into M1422 to determine the spatial and temporal dynamics of autophagy (Fig. 2.3A,B). *MoATG8* encodes an autophagic, ubiquitin-like protein involved in autophagosome function and has been shown to be a reliable marker for autophagy (Veneault-Fourrey, *et al.*, 2006, Kershaw & Talbot, 2009). Compared to wild-type Guy11 (33.5 ± 4.4), *GFP:MoATG8*-labeled autophagosomes accumulated in M1422 conidia in significantly smaller numbers (21.6 ± 5.5 ; $p < 0.01$). In both strains, the number of conidial autophagosomes decreased during germination, appressorium maturation and at the onset of spore cell death (Fig. 2.3B). However, autophagosome numbers increased significantly during appressorium maturation (8h; 16.1 ± 4.9) and dropped considerably after conidial death (24h; 5.0 ± 1.8) in wild-type, whereas autophagosome number remained relatively constant in M1422 during appressorium maturation (8.4 ± 4.1) and even after conidial cell death (7.5 ± 3.3). Appressorium development is also accompanied by rapid degradation of glycogen from conidia during germination and from appressoria during turgor generation (Deng & Naqvi, 2010, Badaruddin, *et al.*, 2013). We therefore determined glycogen levels during appressorium development using potassium iodide (KI). Comparative analysis of KI staining between Guy11 and M1422 showed differences during the onset and later stages of conidial cell death (8h and 24h; Fig. 2.3C, D). In Guy11 glycogen depletion was observed (no staining) within both conidial cells and appressoria during development. In the M1422 mutant, conidial cells were also depleted of glycogen although the appressorium still contained high levels of glycogen (95%) during maturation. When considered together, these observations suggest that autophagy and glycogen metabolism are delayed during appressorium development in the Tpc1-lacking strain M1422.

Fig. 2.3. Infection-associated autophagy and glycogen degradation are impaired in *TPC1* insertional mutant *M1422*. (A) Impaired cellular localisation of autophagosomes in *M1422* during infection-related development. Epifluorescence images of wild-type (WT) Guy11 and *M1422* transformants expressing *GFP:MoATG8*. Scale bar = 10 μ m. (B) Bar charts show mean autophagosome numbers present in conidium, germ tube, and appressorium at the time points indicated for each strain (mean $\pm$ SD; three biological repeats). (C) Glycogen metabolism is delayed in the *M1422* mutant. Conidia from Guy11 and *M1422* inoculated onto glass coverslips were exposed to potassium iodide (KI) solution at 0h, 2h, 4h, 6h, 8h and 24h after inoculation. The KI solution stains glycogen within the conidia, but not simple sugars such as glucose and fructose. Scale bar = 10 μ m. (D) Bar charts showing the relative percentage of stained appressoria with KI solution for each strain at 24h after inoculation (three biological replicates).

Tpc1 is required for re-establishment of polarity during appressorium-mediated plant infection

Following maturation of the appressorium, a penetration peg emerges from the appressorial pore to penetrate the plant cuticle and successfully colonise the plant host. To assess whether repolarization was impaired in the M1422 mutant, a penetration assay was performed on onion epidermis (Fig. 2.4A). After 24h, 91% of wild-type conidia formed an appressorium effectively, penetrated and invaded epidermal cells. The majority of M1422 conidia (60%) germinated and produced an appressorium, but failed to penetrate and invade onion cells. Only 40% of M1422 appressoria formed a penetration peg, but were not able to invade the onion epidermal cells and spread away from the point of penetration.

To examine how formation of the germ tube and penetration peg was compromised, we investigated cellular organization of the F-actin cytoskeleton (Howard & Valent, 1996). We tracked gelsolin:GFP and Sep5:GFP in $\Delta tpc1$ mutant. The use of gelsolin:GFP and Sep5:GFP to follow actin reorganization has helped to understand cytoskeleton dynamics during infection-related development (Ryder, *et al.*, 2013). The disorganisation of the appressorial cytoskeleton and actin ring was evident in $\Delta tpc1$ (Fig. 2.4B). Consequently, *TPC1* is required for the correct penetration peg emergence in *M. oryzae*.

Several protein motifs regulate Tpc1 nuclear localisation and function in *M. oryzae*

The Tpc1:GFP fusion protein co-localised with H1:RFP in nuclei of vegetative hyphae, attached conidia (30 min), and germinated conidia (Fig. 2.5A). Moreover, whenever Tpc1:GFP was observed in nuclei, GFP fluorescence was never observed in the cytoplasm or other organelles within conidia. The results are consistent with *TPC1* encoding a transcription factor that acts within the nucleus during the initial stages of spore germination and appressorium development.

To investigate whether *TPC1* is associated with specific or multiple regulatory networks, *TPC1:GFP* localisation was observed in conidia of different mutant backgrounds (Fig. 2.5B). In the $\Delta pmk1$ MAPK mutant (Xu & Hamer, 1996), Tpc1:GFP was observed within the cytoplasm but not in nuclei. By contrast, strong GFP fluorescence was visualised in the conidial nuclei of autophagy-defective $\Delta atg1$ and $\Delta atg8$ mutants, compared to the fluorescence observed in M1422 complemented

with *TPC1:GFP* or Guy11 expressing *TPC1:GFP*. In $\Delta cpkA$, $\Delta mst12$ and $\Delta mps1$ mutants, *TPC1:GFP* expression was not detected (data not shown). These results suggest that Tpc1 activity is associated with the Pmk1 MAP kinase signalling pathway, which regulates appressorium formation, and the control of autophagy.

To explore the relevance of specific Tpc1 domains in its expression and function (Fig. 2.5C), site-directed mutagenesis was used to delete entire NLS (*TPC1 Δ NLS:GFP*) or two basic amino acids within the NLS (*TPC1 Δ RR:GFP* and *TPC1 Δ KK:GFP*). Each of these mutations caused mis-localization of Tpc1:GFP in the cytoplasm of conidia (Fig. 2.5D), which impaired its biological activity because each construct failed to complement the growth defect of M1422 (Fig. 2.5E).

Fig. 2.4. *M. oryzae* Tpc1 is required for re-establishment of polarity in appressoria. (A) The M1422 mutant is impaired in appressorium-mediated penetration. After 24 hpi wild-type (WT) appressoria formed on surface of the onion strip have penetrated the underlying epidermal cell and formed invasive hyphae (IH). M1422 shows a defective invasion of onion epidermis. Scale bar = 10 μ m. Bar charts show the relative percentage of appressorial-penetration of onion epidermis for each strain at 24 hpi (three biological replicas). **(B)** Micrographs of F-actin ring organization visualized by expression of gelsolin:GFP and Sep5:GFP in Guy11 and $\Delta tpc1$ strains. The $\Delta tpc1$ mutant produce aberrant septin and actin rings; mis-localization of Sep5:GFP is more severe. The linescan graphs show fluorescence in a transverse section of individual appressoria.

Fig. 2.5. The NLS, sumoylation site and phosphorylation residues are necessary for nuclear localisation and function of *Tpc1*. (A) Nuclear localisation of Tpc1:GFP during infection-related development and vegetative hyphae by confocal microscopy. Conidia and mycelium from a WT strain expressing both histone *H1:RFP* and *TPC1:GFP* protein fusions. (B) Confocal images of conidia harvested from *M. oryzae* $\Delta pmk1$, $\Delta atg1$ and $\Delta atg8$ mutants expressing *TPC1:GFP*. Tpc1 is not visualized in the nuclei of $\Delta pmk1$ mutant. (C) Schematic diagram showing the NLS, the Zn(II)₂Cys₆ cluster domain and phosphorylation residues (Thr168 and Thr349) in the *M. oryzae* Tpc1 predicted amino acid sequence. The sequence within the red box contains the NLS sequence deleted in *TPC1ΔNLS:GFP*. DNA encoding amino acids in red was deleted to obtain *TPC1ΔRR:GFP* and *TPC1ΔKK:GFP* constructs. The lysine residue in blue corresponds to the sumoylation site that was changed to an alanine residue (*TPC1noSUMO:GFP*). The threonine residues 168 and 349 were changed to glycine residues to obtain *TPC1_T168G:GFP* and *TPC1_T349G:GFP* constructs. (D) Images of M1422 strain complemented with Tpc1:GFP mutated variants: *TPC1ΔNLS:GFP* (ΔNLS), *TPC1ΔRR:GFP* (ΔRR), *TPC1ΔKK:GFP* (ΔKK), *TPC1noSUMO:GFP* (noSUMO), *TPC1_T168G:GFP* (T168G) and *TPC1_T349G:GFP* (T349G). Scale bar = 10 μm. (E) Colony images captured at 10 dpi of CM plates inoculated with Guy11, M1422 and M1422 transformants complemented with different *TPC1:GFP* constructs. None of the constructs fully complemented M1422 growth defects.

The Δ KK mutated version, however, partially complemented the mycelial growth phenotype of M1422. Next, we modified putative phosphorylation sites of Tpc1 by expressing *TPC1_T168G:GFP* and *TPC1_T349G:GFP* in M1422. We also modified a putative sumoylation site within the NLS (*TPC1noSUMO:GFP*) and introduced this construct into M1422. In each case, we observed the modified Tpc1:GFP protein fusion in the cytoplasm but not within nuclei. Furthermore, the constructs were unable to complement the reduced growth phenotype of M1422 fully. We conclude that the putative Tpc1 NLS is necessary for nuclear localization and Tpc1 function. Moreover, predicted sumoylation site and phosphorylation residues can regulate Tpc1 function.

Tpc1-like transcriptional regulators are found exclusively in filamentous fungi

We investigated the phylogenetic relationship of Tpc1 to other putative *Magnaporthe* Zn(II)₂Cys₆ proteins and the closest orthologues of Tpc1 in other fungal species (Supplemental Figs. S2.3 and S2.4). We observed that the six cysteine residues of the DNA-binding domain (DBD) in the Zn(II)₂Cys₆ proteins were ordered in a conserved pattern, CX₂CX₆CX₅₋₁₂CX₂CX₆₋₈C (Supplemental Fig. S2.3A-B). In *M. oryzae*, the Zn(II)₂Cys₆ binuclear cluster family is very diverse and composed of 175 members (Annex Table A2.2). The closest orthologues of Tpc1 (MGG_01285) were identified using BLASTP and used to construct a phylogenetic tree (Supplemental Figs. S2.3C and S2.4). Tpc1 clustered in a group with sequences from other Sordariomycetes, such as *Fusarium graminearum*, *N. crassa*, *Chaetomium globosum* and *Podospora anserina*. Based on this tree, Tpc1 is a single copy gene and has not been subject to paralogous duplications. Our phylogenetic analysis reflected the diversification of the Zn(II)₂Cys₆-containing proteins in the fungal kingdom. Interestingly, we did not find putative homologues of Tpc1 in *S. cerevisiae* or *Schizosaccharomyces pombe* using a BLASTP search.

We investigated functional conservation of Tpc1 in the saprotrophic filamentous fungus *N. crassa*, and characterized a *N. crassa* *NcTPC1* deletion mutant (Δ *nctpc1*), obtained from the Fungal Genetic Stock Centre (McCluskey, 2003). Strikingly, the Δ *nctpc1* mutant was severely reduced in vegetative growth compared to an isogenic wild-type strain ($p < 0.01$) (Supplemental Fig. S2.5B), and its vegetative hyphae branched distinctively. In addition, we observed that the Δ *nctpc1* mutant of *N.*

crassa was less severely affected when exposed to increasing osmotic stress, compared with the *N. crassa* wild-type strain ($p < 0.01$) (Supplemental Fig. S2.5C-D). Similar effect was also found in *M. oryzae* Tpc1 (Supplemental Fig. S5E). Consequently, Tpc1 also plays a significant role in growth and development of the fungus and its responses to abiotic stress.

Oxidation-reduction processes are significantly affected in the *Δtpc1* mutant

Tpc1 contains a $\text{Zn(II)}_2\text{Cys}_6$ binuclear cluster DNA binding domain, which is found only in fungal proteins considered bonafide transcription regulators (MacPherson, *et al.*, 2006, Campbell, *et al.*, 2008). We carried out a comparative transcriptome analysis using the wild-type strain and the *TPC1* deletion mutant to identify the biological processes and genes regulated by Tpc1. We identified 215 down-regulated genes and 185 genes to be up-regulated with at least a two-fold change in expression level in the *Δtpc1* mutant (Annex Table A2.3). We classified all the genes that were up- and down-regulated into four functional groups according to potential roles in signalling (13 genes), cell wall biosynthesis or modulation of plant response (secreted proteins; 140 genes), metabolism (127 genes) and other functions (54 genes). Sixty-six genes encoded proteins that lacked any known domain. Remarkably, two gene ontology (GO) terms were found significantly enriched among these differentially expressed genes, the oxidation-reduction process (GO:0055114; 57 genes; $p < 0.001$) and the oxidoreductase activity (GO:0016491; 58 genes; $p < 0.001$).

Within the signalling functional group, two down-regulated genes encoded phosphatidyl ethanolamine-binding proteins (PEBP) that have been shown to regulate protein kinase A (PKA) and mitogen-activated protein kinase (MAPK) pathways (Corbit, *et al.*, 2003, Caesar & Blomberg, 2004). Amongst the up-regulated genes, eight of them encoded transcriptional regulators, which suggested a link between the gene networks controlled by these transcriptional regulators and Tpc1.

The largest group of misregulated genes comprised 140 genes coding for secreted or cell wall-related proteins. Within this group, more than half of the members (74 genes) had no matches in databases. However, twenty-four genes were potentially involved in cell wall remodeling, and encompassed different types of glycosyl hydrolases

(GH10, GH18, GH32, GH43, GH61 and GH81), seventeen proteases and two secreted phospholipases A2. Three Mas3/Gas1 paralogues and several effector proteins such as a Bas2-like, Bas113 and avrPi54 were also found (Xue, *et al.*, 2002, Mosquera, *et al.*, 2009, Giraldo, *et al.*, 2013, Devanna, *et al.*, 2014). The up-down regulation of two CFEM G-protein coupled receptors (Kulkarni, *et al.*, 2005), including *PTH11* (DeZwaan, *et al.*, 1999), suggested an alteration in the ability of the $\Delta tpc1$ mutant to perceive external signals.

The second largest group of genes with altered expression levels encoded proteins related with primary and secondary metabolism (127 genes). We found a significant number of them participating in oxidation-reduction processes (46%) and transport (9%). Alteration in nitrogen and glycerol metabolism was evidenced by the expression changes of four NmrA-like regulatory proteins (Wilson, *et al.*, 2010), enzymes involved in aminoacid biosynthesis, a glycerol kinase and the glycerol dehydrogenase Gcy1, an enzyme also associated with redox regulation in yeast (Costenoble, *et al.*, 2000). Down-regulation of an α -glucosidase supported the glycogen degradation delay of Tpc1-lacking strains.

The last functional group included 54 genes encoding proteins that carry a wide range of biochemical roles. The reduced expression of the autophagy gene *ATG22* and the up-regulation of three small chaperones Hsp20-like suggested the unbalanced signals for survival and cell death existent in $\Delta tpc1$ (Bruey, *et al.*, 2000). Microtubule-dependent vesicle trafficking and cell cycle were also affected in the mutant as inferred from the misregulation of two dynamins, one kinesin light chain, one Marvel protein, two cyclins and the Cdc26 subunit. Genes involved in silencing pathways, spliceosomal snRNP assembly, tRNA processing, RNA-mediated heterochromatin silencing and translational arrest were also misregulated in $\Delta tpc1$, highlighting alterations in other cellular processes that regulate gene expression.

Overall, we found that the gene expression changes due to the lack of Tpc1 correlated with the observed involvement of this protein in glycogen metabolism, autophagy and polar growth. The up-regulation of genes associated with microtubule-dependent transport in $\Delta tpc1$ may be a cellular response to attenuate the polarity-associated defects of the mutant.

Gene-deletion analysis of Tpc1-dependent genes identifies a major pathogenicity gene

The majority of the $\text{Zn(II)}_2\text{Cys}_6$ binuclear cluster proteins are transcriptional activators and only few of them have been shown to act as repressors (Campbell, *et al.*, 2008). To identify novel pathogenicity genes we focused on genes that could play a role in *TPC1*-associated defects. We looked at the 133 down-regulated genes that contain the binuclear cluster recognition motif found with the DREME motif search algorithm (Bailey, 2011) (Annex Table A2.3; Supplemental Fig. S2.6). Motifs enriched in the promoter regions of up-regulated genes were not found. Five genes were selected for gene replacement and included the conidiation-related gene *CON6* (White & Yanofsky, 1993), a glycosyl transferase 18 gene (*GH18*) that lacks the motif but undergoes a 50-fold increased expression *in planta* (Mosquera, *et al.*, 2009), the two signaling-associated *PEBP* genes, and *PRO41/HAM-6*, a gene required for hyphal fusion in *Neurospora crassa* and sexual development in *Sordaria macrospora* (Nowrousian, *et al.*, 2007, Fu, *et al.*, 2011) (Supplemental Fig. S2.7). We confirmed by RT-PCR that all these genes were down-regulated in the Δtpc1 mutant (Supplemental Fig. S2.8A).

Among the six deletion mutants generated, only $\Delta\text{pro41}/\Delta\text{ham-6}$ displayed a severe pathogenicity-deficient phenotype (Supplemental Fig. S2.7). Despite the links found between conidiogenesis and pathogenicity in *M. oryzae* (Shi, *et al.*, 1998, Zhou, *et al.*, 2009, Illana, *et al.*, 2013), the Δcon6 mutant behaved like wild-type *in planta*. Similarly, Δgh18 , Δpebp1 , Δpebp2 , and the double mutant $\Delta\text{pebp1}\Delta\text{pebp2}$ did not show any pathogenicity-associated defects possibly due to redundancy in related gene functions. Consequently, we selected $\Delta\text{pro41}/\Delta\text{ham-6}$ mutants for further characterization.

M. oryzae NOXD/PRO41 is required for superoxide production, sexual development and plant penetration

The open reading frame of *M. oryzae* *PRO41* was initially annotated in the EnsemblFungi database as *HAM-6*, a *N. crassa* gene required for cell fusion (Fu, *et al.*, 2014). However, the orthologue of this protein was first characterized in *S. macrospora* and named Pro41 (Nowrousian, *et al.*, 2007, Nowrousian, *et al.*, 2012). Pro41 is a novel ER membrane protein required for fruiting body maturation in *S. macrospora*. Later, Pro41 was found to be the functional orthologue of the p22^{phox} subunit of the

NADPH oxidase complex in both *Podospora anserina* and *Botrytis cinerea* (Lacaze, *et al.*, 2014, Siegmund, *et al.*, 2015). Therefore, we renamed the Pro41/Ham-6 protein NoxD (Supplemental Fig. S2.8B).

Fig. 2.6. The *M. oryzae* NoxD protein is required for plant penetration and sexual reproduction. (A) Eight-day old wild-type (WT) Guy11 and $\Delta noxD$ strains grown on different media and stress conditions (additional information in Supplemental Fig. S2.7B-C). The $\Delta noxD$ mutant showed accelerated growth rate compared to wild-type (WT) on CM. $\Delta noxD$ growth is affected in MM-N media and shows increase resistance to salts (LiCl, CINA) and CongoRed (C-R) (B) Leaf and root infection assays showing $\Delta noxD$ pathogenicity defects and functional complementation of $Pr_{RP27}:NOXD:mCherry$ construct. (C) Penetration assays using onion epidermal cells. Very few hyphae of $\Delta noxD$ are observed within the onion cell. (D) Fertility assay in oatmeal agar plates pairing Guy11 and $\Delta noxD$ with the tester strain of opposite mating type TH3. No perithecia were observed from the cross of $\Delta noxD \times TH3$.

We looked at the growth of the *M. oryzae* $\Delta noxD$ mutant in different media and stress conditions (Fig. 2.6A and Supplemental Fig. S2.8C). $\Delta noxD$ grew slightly faster than the wild-type on CM and MM, under salt stress (0.2 M LiCl, 0.4 M NaCl) and in Congo Red (CR). However, we did not observe differences in growth under carbon starvation, calcofluor white (CFW) or basic conditions (pH 9.5). Increased resistance to CFW was previously observed for *M. oryzae* $\Delta nox1$ but not for $\Delta nox2$ (Egan, *et al.*, 2007), suggesting NoxD and Nox2 fulfill similar roles during cell wall biogenesis. Growth of *M. oryzae* $\Delta noxD$ and $\Delta nox1\Delta nox2$ mutants in 1mM methyl viologen, 1mM H₂O₂ and 5mM H₂O₂ was similar or improved when compared to wild-type (Fig. S2.8D). Thus, the lack of NoxD did not affect fungal growth under oxidative stressors in contrast to the growth defects displayed by *B. cinerea* NADPH oxidase mutants (Siegmund, *et al.*, 2015).

The infection ability of $\Delta noxD$ was severely affected on leaves and roots (Fig. 2.6B and Supplemental Fig. S2.7), in accordance with the strong penetration defects displayed by $\Delta nox1$ and $\Delta nox2$ (Egan, *et al.*, 2007). The penetration defect displayed by $\Delta noxD$ was confirmed using onion epidermis penetration assays (74% in the wild-type versus 22% in the mutant; Fig. 2.6C). Subsequently, we crossed the $\Delta noxD$ mutant with the rice isolate TH3, a *M. oryzae* strain of opposite mating type (Fig. 2.6D). The inability to produce perithecia indicated that NoxD is required for sexual reproduction in *M. oryzae*.

To define whether superoxide production was impaired in the $\Delta noxD$ mutant, we used nitroblue tetrazolium (NBT), which forms a dark-blue water-insoluble formazan precipitate upon reduction by superoxide radicals (Munkres, 1990, Egan, *et al.*, 2007). In the $\Delta noxD$ mutant, we observed an increase in superoxide production at hyphal tips and a significant reduction in appressoria based on mean pixel intensity measurements ($p < 0.01$) (Fig. 2.7A). This was previously described for $\Delta nox1\Delta nox2$ mutants (Egan, *et al.*, 2007), and supports the existence of alternative routes for cellular ROS generation in *M. oryzae* during hyphal development. A yeast two-hybrid assay was used to identify putative NoxD interactors. We found *M. oryzae* NoxD interacts with the Nox1 NADPH oxidase subunit (Fig. 2.7B) but not with Nox2 or NoxR (data not shown), supporting previous work in *B. cinerea* and *P. anserina* (Lacaze, *et al.*, 2014, Siegmund, *et al.*, 2015).

Fig. 2.7. *M. oryzae* NoxD is an ER- and plasma membrane-localised protein that interacts with Nox1, a component of the fungal NADPH oxidase complex. (A) Superoxide production is significantly affected in the mutants as shown by nitroblue tetrazolium (NBT) staining and quantification of pixel intensities in hyphal tips (mean $\pm$ SD; n=10). Both $\Delta noxD$ and $\Delta nox1\Delta nox2$ produced less ROS in appressoria but higher amounts of ROS in hyphal tips compared to WT. Addition of diphenylene iodonium (DPI) before NBT treatment abolishes the formation of dark precipitates associated with sites of superoxide generation. Values to calculate mean pixel intensity were for white 0 and black for 100. **(B)** A yeast two-hybrid screen reveals that NoxD interacts with Nox1. A yeast colony co-transformed simultaneous with the pGBK-NOX1 (bait Nox1) and pGAD-NOXD (prey NoxD) vectors grows in high stringency media (-His/-Ade/-Leu/-Trp/+X- α -Gal). The interaction of proteins expressed by prey and bait vectors generates a blue-coloured colony due to the activation of α -galactosidase expression in the presence of X- α -Gal. **(C)** Images of NOXD fusion constructs using GFP and mRFP under two different promoters, the NOXD promoter and the strong promoter of ribosomal protein Rp27, respectively. Both NoxD fusions localized in vesicles and plasma membrane. In conidia, fluorescence signal is observed in vesicles and ER-like membranes with NOXD::mRFP. After 8h, fluorescence signal is strongly detected at the plasma membrane of the appressoria. pEGFPKDEL encodes GFP with the ER retention signal KDEL. **(D)** Micrographs of F-actin ring organization visualized by expression of gelsolin::GFP and Sep5::GFP in Guy11 and $\Delta noxD$. This mutant produces aberrant septin and actin rings; mis-localization of Sep5::GFP is more severe. The linescan graphs show fluorescence in a transverse section of individual appressoria.

NoxD is visualized in vesicles and plasma membrane of appressoria and conidia

To localize NoxD we generated C-terminal mRFP and GFP translational fusions under the control of strong or native promoters. Both constructs fully complemented $\Delta noxD$ plant infection defects (Fig. 2.6B and data not shown). *M. oryzae* NoxD was mainly observed in vesicles and the plasma membrane of appressoria and conidia (Fig. 2.7C). ER localization of an overexpressed variant of NoxD, Pr_{RP27}:NOXD:mRFP, was observed in *M. oryzae* spores based on the fluorescence pattern displayed by a GFP that contains the ER retention signal KDEL (pEGFPKDEL) (Nowrousian, *et al.*, 2007). However, the lack of a net-like fluorescence pattern in the $\Delta noxD$ mutant harboring the *NOXD:GFP*, whose expression is driven by the native promoter, suggested that NoxD is found at very low amounts in *M. oryzae* ER.

NoxD is required for septin ring assembly at the appressorial pore

The Nox2-NoxR complex is essential for septin-mediated cytoskeletal reorientation, whereas Nox1 is dispensable although may have important roles to play in maintenance and elongation of the penetration peg (Ryder, *et al.*, 2013). To test if NoxD was also involved in this process, we expressed the actin-binding protein gelsolin:GFP and Sep5:GFP in $\Delta noxD$. In the wild-type, both a septin and gelsolin ring was present at the appressorium pore (Ryder, *et al.*, 2013) (Fig. 2.7D). In the $\Delta noxD$ mutant, however, Sep5:GFP formed a disorganized mass in the infection cell as previously reported for $\Delta nox2$ and $\Delta noxR$ expressing Sep5:GFP (Ryder, *et al.*, 2013). Gelsolin:GFP rings in $\Delta noxD$ also possessed distorted pores. Considering that gelsolin colocalizes with F-actin at the appressorial pore (Ryder, *et al.*, 2013), the altered fluorescence pattern of gelsolin:GFP revealed that the toroidal F-actin ring was disorganized (Fig. 2.7D). Previous reports showed that Sep5:GFP and gelsolin:GFP patterns in the $\Delta nox1$ mutant displayed normal conformation (Ryder, *et al.*, 2013). NoxD and Nox1 therefore appear to play alternative roles in cytoskeletal remodeling in appressoria of *M. oryzae*.

DISCUSSION

To cause disease in rice, *M. oryzae* forms a specialised cell called an appressorium, the development of which involves transitions from

polarised to isotropic cellular growth, followed by rapid turgor-driven polarisation to penetrate the leaf surface. Understanding how these cellular transitions occur is critical to controlling the disease at an early stage, prior to entering the plant. In this study, we have identified a transcription factor, Tpc1 that plays a key role in regulating plant infection, due to its role in polarity control. We have also identified a putative mechanism by which it acts, via the regulated synthesis of reactive oxygen species and control of the NADPH oxidase complex, which regulates septin assembly and F-actin re-modelling at the base of the appressorium. Furthermore, we have found that Tpc1 is required for infection-associated autophagy, which is an essential pre-requisite for appressorium function.

We observed that the *TPC1* mutants formed compact colonies, which resembled the colony morphology shared by a class of mutants with polarity defects in *N. crassa* (Seiler & Plamann, 2003). Conidial germination, and growth and branching of vegetative hyphae, were severely impaired in the two mutants lacking functional Tpc1, supporting defects in establishment of polarity in apically-growing hyphae. The absence of Tpc1 function altered the onset of infection-associated autophagy which occurs during appressorium development (Talbot & Kershaw, 2009). Although M1422 conidia appeared able to undergo conidial cell death, the cellular localization of autophagosomes and glycogen deposits suggested that the process was delayed. Consistent with this observation, Tpc1:GFP was also highly expressed in $\Delta atg1$ and $\Delta atg8$ mutants impaired in autophagy, suggesting that the expression of *TPC1* is de-repressed as a consequence of the inability to carry out autophagy and may therefore be an upstream positive regulator of infection-associated autophagy during appressorium maturation (Fig. 2.8).

Autophagic cell death is linked with appressorium function and penetration in *M. oryzae* (Veneault-Fourrey, *et al.*, 2006), and mutants lacking Tpc1 are also penetration defective. The formation of a penetration peg at the base of the appressorial pore is a cellular process intrinsically linked to polar growth (Macara & Mili, 2008, Riquelme, 2013). The F-actin cytoskeleton plays a crucial role during germ tube repolarisation and penetration peg emergence. We therefore investigated cytoskeletal dynamics during appressorium maturation in the mutant background. The network of F-actin observed with fimbrin:GFP in mature wild-type appressoria (8h-24h) delineated the appressorial pore, which was strikingly absent in M1422. This result together with the mis-

localization of Sep5:GFP and gelsolin:GFP in $\Delta tpc1$ indicated that the F-actin network is disturbed in Tpc1-lacking strains.

Oxidation-reduction processes were also significantly affected in $\Delta tpc1$, likely due in part to the down-regulation of the fungal homologue of the p22^{phox} NADPH subunit, the *NOXD* gene (Lacaze, *et al.*, 2014, Siegmund, *et al.*, 2015). The *M. oryzae* $\Delta noxD$ mutant was unable to infect rice leaves and roots. We established an interaction of NoxD with Nox1, but not with NoxR or Nox2, and confirmed the requirement of NoxD for superoxide generation and sexual reproduction in *M. oryzae*, consistent with NoxD functions in *B. cinerea* and *P. anserina* (Lacaze, *et al.*, 2014, Siegmund, *et al.*, 2015). We also identified $\Delta noxD$ defects in repolarization of the F-actin cytoskeleton during infection-related development, supporting the previous role described for the *M. oryzae* NADPH oxidase complex (Ryder, *et al.*, 2013). Remarkably, the disorganization pattern of gelsolin:GFP and Sep5:GFP in $\Delta noxD$ was similar to that observed in $\Delta nox2$ and $\Delta noxR$ mutants, whereas $\Delta nox1$ formed nearly intact ring shapes (Ryder, *et al.*, 2013). This result suggests that Nox1 and NoxD participate differently in septin-mediated cytoskeleton organization despite their interaction, and strengthens the view of the fungal NADPH oxidase as a dynamic complex (Tudzynski, *et al.*, 2012). It seems likely that a Nox2-dependent process initiates septin ring formation, while Nox1 is necessary for maintenance of this conformation (Fig. 2.8). NoxD may therefore be associated at a relatively early stage in recruiting Nox1 to the appressorium pore, perhaps explaining why its absence results in a more severe phenotype with respect to actin and septin assembly at the pore. The role of NoxD, however, highlights that the Nox1 and Nox2 complexes are both necessary for penetration peg elaboration and extensive polar growth. It is worth noting that the tetraspanin *PLS1* deletion mutants exhibit the phenotype of $\Delta nox2$ in *M. oryzae* and *P. anserina* (Lambou, *et al.*, 2008, Ryder, *et al.*, 2013), pointing out that Pls1 may act as the missing link between Nox2 and NoxD subunits of the fungal NADPH oxidase complex (Fig. 2.8). Interestingly, the *B. cinerea* $\Delta noxD$ mutant showed growth defects in the presence of oxidative stressors in contrast to the wild-type growth exhibited by the *M. oryzae* $\Delta noxD$ mutant, which suggests a diversification of the cellular functions of NoxD in fungi. This result also hints differences in the regulation of ROS-mediated signalling pathways in the fungal kingdom.

Here, we identified a potential mechanism by which the transcription factor Tpc1 regulates appressorium maturation and plant infection. The loss of pathogenicity associated with *M. oryzae* *TPC1* mutants and similar growth defects associated with the *N. crassa* $\Delta nctpc1$ mutant, suggest that Tpc1 plays a key role as a transcriptional regulator in the re-establishment of polarity and the response to numerous signalling pathways, such as the Pmk1 MAP kinase and Atg1 kinase cascades. The role of Tpc1 in appressorium-mediated plant infection appears to be associated with the NADPH oxidase-dependent re-polarisation process of the appressorium, and the associated physiological changes such as autophagy, glycogen mobilisation and asymmetric reorganization of the F-actin cytoskeleton. Future studies will allow further dissection of this role and precise definition of the biological function of Tpc1 in filamentous fungi.

Fig. 2.8. Polar growth and associated processes that take place during appressorium maturation in *M. oryzae* and model of *Tpc1*-mediated plant penetration. (A) Polarity factors regulate the emergence and elongation of the germ tube and the penetration peg required for appressorium development and function. Polarity is concomitant with autophagic cell death and glycogen degradation, cellular processes that also control appressorium function. **(B)** During germination, environmental signals trigger the Pmk1 kinase cascade and the control of autophagy to activate Tpc1 function. Nuclear-localised Tpc1 in turn activates transcription of genes required for polar growth, autophagy, and glycogen degradation. Polarity is associated with cytoskeletal dynamics that is controlled by Nox1 and Nox2 NADPH oxidase complexes. NoxD interacts with Nox1 and maybe with Nox2 indirectly. Interactions of NoxR and Pls1 with Nox1 and/or Nox2 need to be confirmed. To initiate plant infection Nox1/Nox2 NADPH oxidases regulate the formation of the F-actin network at the appressorium pore, which leads to penetration peg emergence and subsequent elongation. Mutant strains used in this study are highlighted in black.

MATERIALS AND METHODS

Strains, media and plant infections

M. oryzae was routinely incubated in a controlled temperature room at 25°C with a 12h light/dark cycle. Media composition of complete medium (CM), minimal medium (MM), minimal medium without carbon (MM-C) or nitrogen (MM-N), and DNA extraction and hybridisation were all as previously described (Talbot, *et al.*, 1993). Growth tests were carried out with 7 mm plugs of mycelium from Guy11 and the M1422 mutant strains as initial inoculum. The wild-type *Neurospora crassa* strain and isogenic deletion mutant NCU05996 were obtained from the Fungal Genetics Stock Centre (FGSC, Kansas City, Missouri, USA).

Vogel's minimal medium was used for cultivation of *N. crassa* strains at 25°C with a 12h light/dark cycle and for stock-keeping at 4°C (<http://www.fgsc.net/Neurospora/NeurosporaProtocolGuide.htm>). Growth tests were carried out on Vogel plates with 5 mm plugs of mycelium from *N. crassa* wild type (wt) and *NcTPC1* KO strains. Plates were incubated at 25°C for 2 days. *M. oryzae* leaf and root infection assays were carried out, as previously described (Tucker, *et al.*, 2010, Franceschetti, *et al.*, 2011).

Conidiation and onion penetration assays

Conidia were harvested after fungal cultures were incubated at 25°C for a period of 10 days. Calculations were then carried out to determine the number of conidia generated *per* cm² of mycelium. Values are the mean $\pm$ SD from >300 conidia of each strain, which were measured using the ImageJ software (Collins, 2007). Photographs were taken using the Zeiss Axioskop 2 microscope camera using differential interference contrast (DIC) microscopy or epifluorescence. Conidia were stained with 5 μ l calcofluor white (CFW) solution (Fluka) and incubated at 25°C for 30 minutes. Cell number *per* conidium was determined by visualizing septa with CFW. Appressorium-mediated penetration of onion epidermal strips was assessed using a procedure based on Chida and Sisler (Chida & Sisler, 1987). A conidial suspension at a concentration of 1×10^5 conidia mL⁻¹ was prepared and dropped onto the adaxial surface of epidermal layers taken from onion. The strips were incubated in a moist chamber at 25°C and penetration events scored 24h to 48h later by recording images with an Olympus IX81 inverted microscope system. Glycogen staining solution contained 60 mg of KI and 10 mg of I₂ per milliliter of distilled water. Glycogen deposits are visible immediately.

Generation of mutant strains by gene replacement

Gene deletion constructs were generated using multisite gateway technology (Invitrogen) as previously described (Franceschetti, *et al.*, 2011, Rodríguez-Romero, *et al.*, 2015). *TPC1*, *CON6*, *GH18*, *PEBP2* and *NOXD* coding sequences were replaced by the hygromycin resistance cassette and *PEBP1* by the sulfonyleurea resistance cassette in the gene replacement constructs. Primers for constructing entry plasmids are described in Annex Table A2.4. Fungal transformants generated by *Agrobacterium*-mediated transformation were selected growing in DCM solid media supplied with 5-fluoro-2'-deoxyuridine (50µM) and 200µg/ml Hygromycin or 150µg/ml Chlorymuronethyl in the case of $\Delta pebp1$. Knockout strains were confirmed by PCR or Southern blotting. Sequence data and gene numbers used in this study were taken from EnsemblFungi (*Magnaporthe oryzae* MG8; <http://fungi.ensembl.org/index.html>).

Generation and cellular localisation of fluorescent proteins

To determine the localisation of Tpc1, live-cell imaging was performed using a *M. oryzae* Guy11 strain containing two constructs, histone H1 tagged with red fluorescent protein (*H1:RFP*) to visualize nuclei (Saunders, *et al.*, 2010), and *TPC1:GFP*. For the construction of a functional *TPC1:GFP* gene fusion, primers were designed in order to amplify the *TPC1* (MGG_01285) promoter region and ORF from genomic DNA of *M. oryzae* Guy11 (Annex Table A2.4). The *TPC1_GFP_F* forward primer was designed approximately 1.3 kb upstream from the *TPC1* start codon to include a substantial component of the promoter sequence. The *TPC1_GFP_R* reverse primer spanned the stop codon and contained a complementary region to the *GFP* sequence. GFP primers were designed to amplify the 1.4 kb sGFP:TrpC construct cloned in pGEMT. Both fragments were joined together by fusion nested PCR. The amplicons were cloned into pGEMT-easy digested with *EcoRI*. The 4.3 kb *TPC1:GFP* fragment was gel purified and cloned into pCB1532 that had previously been digested with *EcoRI*. The pCB1532 vector contains the 2.8 kb *ILVI* gene, which encodes the acetolactate synthase-encoding allele bestowing resistance to sulfonyleurea (Sweigard, *et al.*, 1997). The resulting plasmid pCB1532-*TPC1:GFP* was used to transform protoplasts of M1422 mutant. For all rounds of PCR amplification, Phusion® High-Fidelity DNA polymerase (Finnzymes,

Thermo Fischer Scientific Inc.) was used, following the manufacturers' guidelines for PCR conditions.

The *GFP:MoATG8* (Kershaw & Talbot, 2009) and the *FIM:GFP* (A.L. Martinez-Rocha and N.J. Talbot, unpublished data) constructs were used to transform protoplasts of M1422 mutant. The *GFP:MoATG8* and the *FIM:GFP* protein fusion vectors were generated using the native *M. oryzae* *MoATG8* gene (MGG_01062) and the native *M. oryzae* fimbrin-encoding gene (MGG_04478), respectively. Both fragments were cloned into pCB1532 vector that contains the 2.8 kb *ILVI* gene, which encodes the acetolactate synthase allele conferring sulfonylurea resistance. Transformants showing identical growth and colony morphology to the background strain were selected for further examination using epifluorescence or confocal microscopy. At least three different transformants of each were independently analysed.

The *TPC1:GFP* gene fusion was cloned into pCB1532 vector (SUR^R) and used to transform protoplasts of Guy11 expressing Histone H1 fused with red fluorescent protein (*H1:RFP*) (Saunders, *et al.*, 2010), and also introduced into isogenic $\Delta pmk1$, $\Delta atg1$ and $\Delta atg8$ mutants. Transformants were selected for further examination using confocal microscopy and verified as containing a single copy of the gene fusion construct by Southern blot hybridisation. At least three different transformants of each were used in all experiments.

PCR site-directed mutagenesis of *TPC1:GFP* construct

To create a mutation or deletion of a specific region of *TPC1:GFP*, primers were designed to contain each desired mutation (Annex Table A2.4). A first PCR round was used to amplify two parts of *TPC1:GFP*. To amplify part 1, a reverse primer was used containing the mutation or deletion desired, whereas to amplify *TPC1:GFP* part 2 the forward primer containing the specific mutation or deletion was used. A second PCR round was then used to join both fragments using flanking primers. To obtain *TPC1* Δ NLS:GFP, *TPC1* Δ RR:GFP and *TPC1* Δ KK:GFP, primers were engineered to delete the NLS, or two basic arginine (RR) and lysine (KK) amino acids present within the NLS. The other constructs were made in order to mutate a specific amino acid without changing the reading frame. *TPC1*_T168G:GFP, *TPC1*_T349G:GFP and *TPC1*noSUMO:GFP in which the putative phosphorylation sites (Thr at position 168 and 349) changed to a Gly residue and putative sumoylation site (Lys) changed to an Ala residue, respectively. Fragments were gel

purified and cloned into pGEMT-easy and mutations confirmed by DNA sequencing. The constructs with the mutation or deletion introduced correctly were then cloned into pCB1532 (SUR^R). The resulting plasmids were used to transform protoplasts of the wild-type strain Guy11 and the M1422 mutant background. At least three transformants were selected for further examination using confocal microscopy.

RNA isolation and global gene expression profile using microarrays

Using a modified protocol of LiCl method (Franceschetti, *et al.*, 2011), RNA was extracted from 8-day old fungal mycelia grown on cellophane placed on top of CM agar plates (Supplemental Fig. S2.2D). Two to three additional washes with phenol:chloroform were implemented to avoid RNA degradation from cellophane samples. RNA quality control was carried out with Agilent RNA 6000 Nano kit (ref. 5067-1504). Four biological replicates were independently hybridized for each transcriptomic comparison. Each of these replicates derived from three technical repetitions. Slides were Agilent Magnaporthe II Oligo Microarrays 4x44K (ref. 015060). Background correction and normalization of expression data were performed as previously described (Franceschetti, *et al.*, 2011). Hybridizations and statistical analysis were conducted by the Genomics Facility at the National Biotechnology Centre (Madrid, Spain). The GO term analysis was carried out with gProfiler (Reimand, *et al.*, 2011). Using the 215 down-regulated genes in $\Delta tpc1$ mutant two motifs in regions 1 kb upstream from the start codon were detected with the DREME motif discovery algorithm (Bailey, 2011). CentriMo, a position-specific motif-enrichment analysis method, was also used for a statistical assessment of the positional preference of the motifs (Bailey & Machanick, 2012). Enriched motifs were not found when using the promoter regions of the 185 up-regulated genes. Microarray data are available in the ArrayExpress database (EMBL_EBI) under accession number E-MTAB-4127.

Yeast-two hybrid screen

In-Fusion Cloning based on *in vitro* homologous recombination was performed to generate vectors including NoxD into the pGADT7 prey vector, and Nox1, Nox2 and NoxR into the pGBKT7 bait vector. Genes were amplified from *M. oryzae* cDNA using primers with a 15bp

overhang and restriction site complementary to the target vector (Annex Table A2.4). For NoxD, a 435bp fragment was amplified, for Nox1, a 1662bp fragment was amplified, for Nox2, a 1749bp fragment was amplified, and for NoxR, a 1578bp fragment was amplified. Respective fragments were cloned into pGBKT7 and pGADT7 plasmids linearized by digestion with *EcoRI* and *SmaI*. Yeast two-hybrid assays using pGADT7 or pGBKT7 (Clontech) based constructs were performed according to the manufacturer's instructions (MATCHMAKER Gold Yeast Two-Hybrid System).

Imaging of fluorescent fusion proteins

For the construction of NoxD:GFP, primers were designed to amplify the ORF including 2kb upstream of the start codon, GFP and TrpC terminator with 15bp overhangs complementary to adjacent fragments. Fragments were ligated into pCB1532 (Sweigard, *et al.*, 1997), which carries the sulphonyl urea resistance cassette and had been digested with *Bam*HI and *Hind*III and this construct transformed into of the wild-type strain Guy11 using protoplasts (Ryder, *et al.*, 2013). The NoxD:mRFP construct was generated using multi-site gateway technology (Life Technologies) with the entry mCherry-withSTOP and destination SULPH-R3R4 vectors (Franceschetti, *et al.*, 2011), and PCR fragments amplified from *M. oryzae* genomic DNA using Phusion DNA polymerase (NEB) and primers detailed in Annex Table A2.4. Appressorium development assays were performed on hydrophobic borosilicate glass coverslips (Fisher Scientific), as described previously (Ryder, *et al.*, 2013). For epifluorescence microscopy, conidia were incubated on coverslips and observed at each time point using an IX-81 inverted microscope (Olympus) and a UPlanSApo X100/1.40 oil objective. All microscopic images were analyzed using MetaMorph (Molecular Devices). Confocal imaging was performed with a Leica SP8 microscope.

qPCR and ROS detection

To confirm microarray results, the relative abundance of gene transcripts were analysed by qPCR. One μ g of total RNA was reverse transcribed using PrimeScriptTM RT reagent Kit (Takara). The average threshold cycle (Ct) was normalized against actin transcript and relative quantification of gene expression was calculated by the $2^{\Delta\Delta C_t}$ method (Pfaffl, 2001). Primer efficiency was tested using dilutions of cDNA samples. qPCR reactions

were carried out with 1 μ l of reverse transcribed products and fast-start DNA master SYBR green I kit (Roche Diagnostics) in a final reaction of 20 μ l using the following program: one cycle of 95°C for 4 min and 40 cycles of 94°C for 30 s and 60°C for 30 s. The Ct (threshold cycle) provided a measure for the starting copy numbers of the target genes. Three technical repetitions from three independent biological experiments were used for each gene. For ROS detection in *M. oryzae* fungal structures, NBT staining(Egan, *et al.*, 2007) and quantification method of pixel intensities in hyphal tips(Burgess, *et al.*, 2010) were carried out as previously described.

Phylogenetic analysis of Tpc1

First, 141 *M. oryzae* protein sequences containing a fungal Zn(II)₂Cys₆ binuclear cluster domain (PF00172) were identified from the *Magnaporthe* sequence database at the Broad Institute (<http://www.broadinstitute.org/>) and the Fungal Transcription Factor Database (<http://ftfd.snu.ac.kr/intro.php>). HMM search from HMMER3(Eddy, 2011) was used to screen the genome assembly of *M. oryzae* proteins with the fungal Zn₂Cys₆ profile hidden Markov model pHMM zn_clus_ls.hmm (PF00172.13) from Pfam database (Punta, *et al.*, 2012) (<http://pfam.sanger.ac.uk/>). Subsequently, gene numbers were updated using the MG8 genome version of EnsemblFungi database (<http://fungi.ensembl.org/index.html>). Out of these 141 sequences, only 113 had a full length zinc cluster domain, and extra six closest sequences were included to build Supplemental Fig. S2.4. Additional Zn(II)₂Cys₆ proteins found in Lu et colleagues (Lu, *et al.*, 2014) were included in Annex Table A2.2. Basic Local Alignment Search Tool (BLAST) was used to find orthologous proteins of TPC1/MGG_01285 (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Protein sequences were pre-aligned using HMMalign and the pHMM zn_clus_ls.hmm (Supplemental Fig. S2.3) from Pfam. The Zn(II)₂Cys₆ binuclear cluster domain region was extensively manually aligned in BioEdit (<http://www.mbio.ncsu.edu/BioEdit/BioEdit.html>). Unambiguous aligned positions were used for the subsequent phylogenetic analyses. The maximum likelihood (ML) analyses were performed with the program PhyML version 3.0.1(Guindon & Gascuel, 2003). All trees were visualised using the program Figtree (<http://tree.bio.ed.ac.uk/software/figtree/>).

REFERENCES

- Badaruddin, M., Holcombe, L. J., Wilson, R. A., Wang, Z. Y., Kershaw, M. J. & Talbot, N. J. (2013).** Glycogen Metabolic Genes Are Involved in Trehalose-6-Phosphate Synthase-Mediated Regulation of Pathogenicity by the Rice Blast Fungus *Magnaporthe oryzae*. *Plos Pathogens* **9**.
- Bailey, T. L. (2011).** DREME: motif discovery in transcription factor ChIP-seq data. *Bioinformatics* **27**, 1653-1659.
- Bailey, T. L. & Machanick, P. (2012).** Inferring direct DNA binding from ChIP-seq. *Nucleic Acids Res* **40**, e128.
- Battaglia, E., Klaubauf, S., Vallet, J., Ribot, C., Lebrun, M. H. & de Vries, R. P. (2013).** Xlr1 is involved in the transcriptional control of the pentose catabolic pathway, but not hemi-cellulolytic enzymes in *Magnaporthe oryzae*. *Fungal Genetics and Biology* **57**, 76-84.
- Berger, H., Pachlinger, R., Morozov, I., Goller, S., Narendja, F., Caddick, M. & Strauss, J. (2006).** The GATA factor AreA regulates localization and *in vivo* binding site occupancy of the nitrate activator NirA. *Molecular Microbiology* **59**, 433-446.
- bin Yusof, M. T., Kershaw, M. J., Soanes, D. M. & Talbot, N. J. (2014).** FAR1 and FAR2 regulate the expression of genes associated with lipid metabolism in the rice blast fungus *Magnaporthe oryzae*. *PLoS One* **9**, e99760.
- Breth, B., Odenbach, D., Yemelin, A. & other authors (2013).** The role of the Tra1p transcription factor of *Magnaporthe oryzae* in spore adhesion and pathogenic development. *Fungal Genetics and Biology* **57**, 11-22.
- Bruey, J. M., Ducasse, C., Bonniaud, P. & other authors (2000).** Hsp27 negatively regulates cell death by interacting with cytochrome c. *Nature Cell Biology* **2**, 645-652.
- Burgess, A., Vigneron, S., Brioudes, E., Labbe, J. C., Lorca, T. & Castro, A. (2010).** Loss of human Greatwall results in G2 arrest and multiple mitotic defects due to deregulation of the cyclin B-Cdc2/PP2A balance. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 12564-12569.
- Caesar, R. & Blomberg, A. (2004).** The stress-induced Tfs1p requires NatB-mediated acetylation to inhibit carboxypeptidase Y and to regulate the protein kinase A pathway. *J Biol Chem* **279**, 38532-38543.
- Campbell, R. N., Leverenz, M. K., Ryan, L. A. & Reece, R. J. (2008).** Metabolic control of transcription: paradigms and lessons from *Saccharomyces cerevisiae*. *Biochemical Journal* **414**, 177-187.
- Collins, T. J. (2007).** ImageJ for microscopy. *BioTechniques* **43**, 25-30.
- Corbit, K. C., Trakul, N., Eves, E. M., Diaz, B., Marshall, M. & Rosner, M. R. (2003).** Activation of Raf-1 signaling by protein kinase C through a mechanism involving Raf kinase inhibitory protein. *J Biol Chem* **278**, 13061-13068.
- Costenoble, R., Valadi, H., Gustafsson, L., Niklasson, C. & Franzen, C. J. (2000).** Microaerobic glycerol formation in *Saccharomyces cerevisiae*. *Yeast* **16**, 1483-1495.
- Chida, T. & Sisler, H. D. (1987).** Restoration of Appressorial Penetration Ability by Melanin Precursors in *Pyricularia oryzae* Treated with Antipenetrants and in Melanin-Deficient Mutants. *Journal of Pesticide Science* **12**, 49-55.

- Chung, H., Choi, J., Park, S. Y., Jeon, J. & Lee, Y. H. (2013).** Two conidiation-related Zn(II)2Cys6 transcription factor genes in the rice blast fungus. *Fungal Genet Biol* **61**, 133-141.
- Dagdas, Y. F., Yoshino, K., Dagdas, G., Ryder, L. S., Bielska, E., Steinberg, G. & Talbot, N. J. (2012).** Septin-Mediated Plant Cell Invasion by the Rice Blast Fungus, *Magnaporthe oryzae*. *Science* **336**, 1590-1595.
- Deng, Y. Z. & Naqvi, N. I. (2010).** A vacuolar glucoamylase, Sga1, participates in glycogen autophagy for proper asexual differentiation in *Magnaporthe oryzae*. *Autophagy* **6**.
- Devanna, N. B., Vijayan, J. & Sharma, T. R. (2014).** The Blast Resistance Gene Pi54 of Cloned from *Oryza officinalis* Interacts with Avr-Pi54 through Its Novel Non-LRR Domains. *Plos One* **9**.
- DeZwaan, T. M., Carroll, A. M., Valent, B. & Sweigard, J. A. (1999).** *Magnaporthe grisea* Pth11p Is a Novel Plasma Membrane Protein That Mediates Appressorium Differentiation in Response to Inductive Substrate Cues. *Plant Cell* **11**, 2013-2030.
- Downes, D. J., Davis, M. A., Wong, K. H., Kreutzberger, S. D., Hynes, M. J. & Todd, R. B. (2014).** Dual DNA binding and coactivator functions of *Aspergillus nidulans* TamA, a Zn(II)2Cys6 transcription factor. *Mol Microbiol* **92**, 1198-1211.
- Eddy, S. R. (2011).** Accelerated Profile HMM Searches. *PLoS Computational Biology* **7**.
- Egan, M. J., Wang, Z. Y., Jones, M. A., Smirnoff, N. & Talbot, N. J. (2007).** Generation of reactive oxygen species by fungal NADPH oxidases is required for rice blast disease. *Proceedings of the National Academy of Sciences, USA* **104**, 11772-11777.
- Fernandes, M., Keller, N. P. & Adams, T. H. (1998).** Sequence-specific binding by *Aspergillus nidulans* AfIR, a C6 zinc cluster protein regulating mycotoxin biosynthesis. *Mol Microbiol* **28**, 1355-1365.
- Franceschetti, M., Bueno, E., Wilson, R. A., Tucker, S. L., Gómez-Mena, C., Calder, G. & Sesma, A. (2011).** Fungal Virulence and Development Is Regulated by Alternative Pre-mRNA 3' End Processing in *Magnaporthe oryzae*. *PLoS Pathogens* **7**, e1002441.
- Fu, C., Iyer, P., Herkal, A., Abdullah, J., Stout, A. & Free, S. J. (2011).** Identification and Characterization of Genes Required for Cell-to-Cell Fusion in *Neurospora crassa*. *Eukaryotic Cell* **10**, 1100-1109.
- Fu, C., Ao, J., Dettmann, A., Seiler, S. & Free, S. J. (2014).** Characterization of the *Neurospora crassa* Cell Fusion Proteins, HAM-6, HAM-7, HAM-8, HAM-9, HAM-10, AMPH-1 and WHI-2. *Plos One* **9**, e107773.
- Giraldo, M. C., Dagdas, Y. F., Gupta, Y. K. & other authors (2013).** Two distinct secretion systems facilitate tissue invasion by the rice blast fungus *Magnaporthe oryzae*. *Nature Communications* **4**, 1996.
- Guindon, S. & Gascuel, O. (2003).** A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* **52**, 696-704.
- Gupta, Y. K., Dagdas, Y. F., Martinez-Rocha, A.-L., Kershaw, M. J., Littlejohn, G. R., Ryder, L. S., Sklenar, J., Menke, F. & Talbot, N. J. (2015).** Septin-Dependent Assembly of the Exocyst Is Essential for Plant Infection by *Magnaporthe oryzae*. *The Plant Cell*.

- Howard, R. J. & Valent, B. (1996).** Breaking and Entering: Host Penetration by the Fungal Rice Blast Pathogen *Magnaporthe grisea*. *Annual Review of Microbiology* **50**, 491-512.
- Illana, A., Rodriguez-Romero, J. & Sesma, A. (2013).** Major Plant Pathogens of the Magnaporthaceae family. In *Genomics of Soil- and Plant-associated Fungi*, pp. 45-88. Edited by B. A. Horwitz. Berlin Heidelberg: Springer-Verlag Ltd.
- Kankanala, P., Czymmek, K. & Valent, B. (2007).** Roles for Rice Membrane Dynamics and Plasmodesmata during Biotrophic Invasion by the Blast Fungus. *Plant Cell* **19**, 706-724.
- Kershaw, M. J. & Talbot, N. J. (2009).** Genome-wide functional analysis reveals that infection-associated fungal autophagy is necessary for rice blast disease. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 15967-15972.
- Kim, S., Singh, P., Park, J., Park, S., Friedman, A., Zheng, T., Lee, Y. H. & Lee, K. (2011).** Genetic and molecular characterization of a blue light photoreceptor MGWC-1 in *Magnaporthe oryzae*. *Fungal Genetics and Biology* **48**, 400-407.
- Kulkarni, R. D., Thon, M. R., Pan, H. & Dean, R. A. (2005).** Novel G-protein-coupled receptor-like proteins in the plant pathogenic fungus *Magnaporthe grisea*. *Genome Biology* **6**, R24.
- Lacaze, I., Lalucque, H., Siegmund, U., Silar, P. & Brun, S. (2014).** Identification of NoxD/Pro41 as the homologue of the p22(phox) NADPH oxidase subunit in fungi. *Molecular Microbiology* **95**, 1006-1024.
- Lai, J., Ng, S. K., Liu, F. F., Patkar, R. N., Lu, Y., Chan, J. R., Suresh, A., Naqvi, N. & Jedd, G. (2010).** Marker fusion tagging, a new method for production of chromosomally encoded fusion proteins. *Eukaryot Cell* **9**, 827-830.
- Lambou, K., Malagnac, F., Barbisan, C., Tharreau, D., Lebrun, M. H. & Silar, P. (2008).** The Crucial Role of the Pls1 Tetraspanin during Ascospore Germination in *Podospora anserina* Provides an Example of the Convergent Evolution of Morphogenetic Processes in Fungal Plant Pathogens and Saprobies. *Eukaryotic Cell* **7**, 1809-1818.
- Lu, J., Cao, H., Zhang, L., Huang, P. & Lin, F. (2014).** Systematic analysis of Zn2Cys6 transcription factors required for development and pathogenicity by high-throughput gene knockout in the rice blast fungus. *PLoS Pathog* **10**, e1004432.
- Macara, I. G. & Mili, S. (2008).** Polarity and Differential Inheritance-Universal Attributes of Life? *Cell* **135**, 801-812.
- MacPherson, S., Larochelle, M. & Turcotte, B. (2006).** A fungal family of transcriptional regulators: The zinc cluster proteins. *Microbiology and Molecular Biology Reviews* **70**, 583-604.
- Marcel, S., Sawers, R., Oakeley, E., Angliker, H. & Paszkowski, U. (2010).** Tissue-Adapted Invasion Strategies of the Rice Blast Fungus *Magnaporthe oryzae*. *Plant Cell* **22**, 3177-3187.
- McCluskey, K. (2003).** The fungal genetics stock center: From molds to molecules. *Advances in Applied Microbiology, Vol 52* **52**, 245-262.
- Mosquera, G., Giraldo, M. C., Khang, C. H., Coughlan, S. & Valent, B. (2009).** Interaction Transcriptome Analysis Identifies *Magnaporthe oryzae*

- BAS1-4 as Biotrophy-Associated Secreted Proteins in Rice Blast Disease. *Plant Cell* **21**, 1273-1290.
- Munkres, K. D. (1990).** Histochemical detection of superoxide radicals and hydrogen peroxide by *Age-1* mutants of *Neurospora*. *Fungal Genetics Newsletter* **37**, 24-25.
- Nowrousian, M., Frank, S., Koers, S., Strauch, P., Weitner, T., Ringelberg, C., Dunlap, J. C., Loros, J. J. & Kuck, U. (2007).** The novel ER membrane protein PRO41 is essential for sexual development in the filamentous fungus *Sordaria macrospora*. *Molecular Microbiology* **64**, 923-937.
- Nowrousian, M., Teichert, I., Masloff, S. & Kuck, U. (2012).** Whole-Genome Sequencing of *Sordaria macrospora* Mutants Identifies Developmental Genes. *G3-Genes Genomes Genetics* **2**, 261-270.
- Odenbach, D., Breth, B., Thines, E., Weber, R. W., Anke, H. & Foster, A. J. (2007).** The transcription factor Con7p is a central regulator of infection-related morphogenesis in the rice blast fungus *Magnaporthe grisea*. *Molecular Microbiology* **64**, 293-307.
- Oh, Y., Donofrio, N., Pan, H. Q., Coughlan, S., Brown, D. E., Meng, S. W., Mitchell, T. & Dean, R. A. (2008).** Transcriptome analysis reveals new insight into appressorium formation and function in the rice blast fungus *Magnaporthe oryzae*. *Genome Biology* **9**, R85.
- Park, G., Bruno, K. S., Staiger, C. J., Talbot, N. J. & Xu, J.-R. (2004).** Independent genetic mechanisms mediate turgor generation and penetration peg formation during plant infection in the rice blast fungus. *Molecular Microbiology* **53**, 1695-1707.
- Pfaffl, M. W. (2001).** A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29**, e45.
- Punta, M., Coghill, P. C., Eberhardt, R. Y. & other authors (2012).** The Pfam protein families database. *Nucleic Acids Research* **40**, D290-D301.
- Reimand, J., Arak, T. & Vilo, J. (2011).** g:Profiler--a web server for functional interpretation of gene lists (2011 update). *Nucleic Acids Research* **39**, W307-315.
- Riquelme, M. (2013).** Tip Growth in Filamentous Fungi: A Road Trip to the Apex. *Annual Review of Microbiology* **67**, 587-609.
- Rodríguez-Romero, J., Franceschetti, M., Bueno, E. & Sesma, A. (2015).** Multilayer Regulatory Mechanisms Control Cleavage Factor I Proteins in Filamentous Fungi. *Nucleic Acids Research* **43**, 179-195.
- Ryder, L. S., Dagdas, Y. F., Mentlak, T. A., Kershaw, M. J., Thornton, C. R., Schuster, M., Chen, J., Wang, Z. & Talbot, N. J. (2013).** NADPH oxidases regulate septin-mediated cytoskeletal remodeling during plant infection by the rice blast fungus. *Proc Natl Acad Sci U S A* **110**, 3179-3184.
- Ryder, L. S. & Talbot, N. J. (2015).** Regulation of appressorium development in pathogenic fungi. *Curr Opin Plant Biol* **26**, 8-13.
- Saunders, D. G., Dagdas, Y. F. & Talbot, N. J. (2010).** Spatial uncoupling of mitosis and cytokinesis during appressorium-mediated plant infection by the rice blast fungus *Magnaporthe oryzae*. *Plant Cell* **22**, 2417-2428.
- Seiler, S. & Plamann, M. (2003).** The genetic basis of cellular morphogenesis in the filamentous fungus *Neurospora crassa*. *Mol Biol Cell* **14**, 4352-4364.
- Sesma, A. & Osbourn, A. E. (2004).** The rice leaf blast pathogen undergoes developmental processes typical of root-infecting fungi. *Nature* **431**, 582-586.

- Shi, Z., Christian, D. & Leung, H. (1998).** Interactions Between Spore Morphogenetic Mutations Affect Cell Types, Sporulation, and Pathogenesis in *Magnaporthe grisea*. *Molecular Plant-Microbe Interactions* **11**, 199-207.
- Siegmund, U., Marschall, R. & Tudzynski, P. (2015).** BcNoxD, a putative ER protein, is a new component of the NADPH oxidase complex in *Botrytis cinerea*. *Molecular Microbiology* **95**, 988-1005.
- Swiegaard, J. A., Chumley, F., Carroll, A., Farrall, L. & Valent, B. (1997).** A series of vectors for fungal transformation. *Fungal Genetics Newsletter* **44**, 52-53.
- Szilagyi, M., Kwon, N. J., Dorogi, C., Pocs, I., Yu, J. H. & Emri, T. (2010).** The extracellular beta-1,3-endoglucanase EngA is involved in autolysis of *Aspergillus nidulans*. *Journal of Applied Microbiology* **109**, 1498-1508.
- Talbot, N. J., Ebbole, D. J. & Hamer, J. E. (1993).** Identification and Characterization of *MPG1*, a Gene Involved in Pathogenicity from the Rice Blast Fungus *Magnaporthe grisea*. *Plant Cell* **5**, 1575-1590.
- Talbot, N. J. & Kershaw, M. J. (2009).** The emerging role of autophagy in plant pathogen attack and host defence. *Current Opinion in Plant Biology* **12**, 444-450.
- Todd, R. B. & Andrianopoulos, A. (1997).** Evolution of a fungal regulatory gene family: the Zn(II)₂Cys₆ binuclear cluster DNA binding motif. *Fungal Genet Biol* **21**, 388-405.
- Todd, R. B., Andrianopoulos, A., Davis, M. A. & Hynes, M. J. (1998).** FacB, the *Aspergillus nidulans* activator of acetate utilization genes, binds dissimilar DNA sequences. *EMBO J* **17**, 2042-2054.
- Traven, A., Jelcic, B. & Söpta, M. (2006).** Yeast Gal4: a transcriptional paradigm revisited. *EMBO Rep* **7**, 496-499.
- Tsuji, G., Kenmochi, Y., Takano, Y., Swiegaard, J., Farrall, L., Furusawa, I., Horino, O. & Kubo, Y. (2000).** Novel fungal transcriptional activators, Cmr1p of *Colletotrichum lagenarium* and Pig1p of *Magnaporthe grisea*, contain Cys₂His₂ zinc finger and Zn(II)₂Cys₆ binuclear cluster DNA-binding motifs and regulate transcription of melanin biosynthesis genes in a developmentally specific manner. *Molecular Microbiology* **38**, 940-954.
- Tucker, S. L. & Talbot, N. J. (2001).** Surface Attachment and Pre-Penetration Stage Development by Plant Pathogenic Fungi. *Annual Review of Phytopathology* **39**, 385-418.
- Tucker, S. L., Besi, M. I., Galhano, R., Franceschetti, M., Goetz, S., Lenhart, S., Osbourn, A. & Sesma, A. (2010).** Common Genetic Pathways Regulate Organ-Specific Infection-Related Development in the Rice Blast Fungus. *Plant Cell* **22**, 953-972.
- Tudzynski, P., Heller, J. & Siegmund, U. (2012).** Reactive oxygen species generation in fungal development and pathogenesis. *Curr Opin Microbiol* **15**, 653-659.
- Valent, B. & Khang, C. H. (2010).** Recent advances in rice blast effector research. *Current Opinion in Plant Biology* **13**, 1-8.
- Veneault-Fourrey, C., Barooah, M., Egan, M., Wakley, G. & Talbot, N. J. (2006).** Autophagic Fungal Cell Death Is Necessary for Infection by the Rice Blast Fungus. *Science* **312**, 580-583.

- White, B. T. & Yanofsky, C. (1993).** Structural Characterization and Expression Analysis of the *Neurospora* Conidiation Gene Con-6. *Developmental Biology* **160**, 254-264.
- Wilson, R. A. & Talbot, N. J. (2009).** Under pressure: investigating the biology of plant infection by *Magnaporthe oryzae*. *Nature Reviews Microbiology* **7**, 185-195.
- Wilson, R. A., Gibson, R. P., Quispe, C. F., Littlechild, J. A. & Talbot, N. J. (2010).** An NADPH-dependent genetic switch regulates plant infection by the rice blast fungus. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 21902-21907.
- Xu, J. R. & Hamer, J. E. (1996).** MAP kinase and cAMP signalling regulate infection structure formation and pathogenic growth in the rice blast fungus *Magnaporthe grisea*. *Genes and Development* **10**, 2696-2706.
- Xue, C., Park, G., Choi, W., Zheng, L., Dean, R. A. & Xu, J.-R. (2002).** Two Novel Fungal Virulence Genes Specifically Expressed in Appressoria of the Rice Blast Fungus. *Plant Cell* **14**, 2107-2119.
- Yan, X., Li, Y., Yue, X., Wang, C., Que, Y., Kong, D., Ma, Z., Talbot, N. J. & Wang, Z. (2011).** Two Novel Transcriptional Regulators Are Essential for Infection-related Morphogenesis and Pathogenicity of the Rice Blast Fungus *Magnaporthe oryzae*. *PLoS Pathog* **7**, e1002385.
- Yin, W. & Keller, N. P. (2011).** Transcriptional regulatory elements in fungal secondary metabolism. *J Microbiol* **49**, 329-339.
- Zhou, Z. Z., Li, G. H., Lin, C. H. & He, C. Z. (2009).** Conidiophore Stalk-less1 Encodes a Putative Zinc-Finger Protein Involved in the Early Stage of Conidiation and Mycelial Infection in *Magnaporthe oryzae*. *Molecular Plant-Microbe Interactions* **22**, 402-410.

SUPPLEMENTAL INFORMATION

Figure S2.1

Fig. S2.1. M1422 phenotype defects are restored by re-introduction of TPC1:GFP construct. (A) The T-DNA in M1422 is positioned 0.9 kb after the start codon of the MGG_01285 gene. This gene has been termed *TPC1* (*Transcription factor for Polarity Control-1*). Features of *TPC1*-encoding protein: NLS, nuclear localisation signal (brown box); Zn(II)₂Cys₆ binuclear cluster DNA binding domain (green box); MAPK docking site (blue box); phosphorylation sites Thr168 and Thr349 (indicated by one and two asterisks, respectively). (B) Southern hybridization of total genomic DNA digested with *MfeI* and probed with hygromycin phosphotransferase. The presence of a single hybridising restriction fragment of ~9 kb confirms a single T-DNA insertion event in M1422 genome. (C) Rice cv. CO39 leaves infected with a conidial suspension (10⁵ conidia ml⁻¹) of Guy11, M1422 and M1422 complemented with *TPC1:GFP*. (D) Colonies of the wild-type (WT) strain Guy11, M1422 and M1422 complemented with *TPC1:GFP*. Photographs were taken after incubating on CM at 25°C for 10 days. (E) M1422 also displays impaired ability to grow on minimal medium (MM) depleted of carbon (MM-C) and nitrogen (MMN). (F) Colony edges of WT Guy11 strain show complex branching hyphae compared to mutant M1422.

Figure S2.2

Fig. S2.2. Gene-replacement strategy to generate *TPC1* deletion mutants. (A) Gene replacement construct lacking the entire coding sequence (CDS) of *TPC1* was introduced into wild-type (WT) strain Guy11. (B) Confirmation of $\Delta tpc1$ mutants was carried out by Southern blotting. Total genomic DNA digested with *MluI* and probed with *TPC1* CDS (right panel) gave the expected band size (8.2 kb) in the WT strain and no bands in knockout strains. Similarly, total genomic DNA digested with *SphI* and probed with *TPC1* 5'UTR (left panel) confirmed the presence of a 7.4 kb band in *TPC1* deletion mutants. (C) Introduction of *TPC1::GFP* fusion construct into the $\Delta tpc1$ mutant recovered its severe growth defects. (D) CM plates covered with cellophane were used to grow WT and $\Delta tpc1$. Eight-day old mycelia on CM plates covered with cellophane (upper plates) and sixday grown mycelia after removing the cellophane (lower plate). In contrast to the WT, $\Delta tpc1$ is unable to grow on CM after removing the cellophane, which suggested its inability to cross this plastic barrier.

Figure S2.3

Fig. S2.3. Phylogenetic analysis of *M. oryzae* Tpc1 protein. (A) HMM pattern for Zn(II)2Cys6 binuclear cluster protein family. The fungal Zn(II)2Cys6 cluster protein family is characterised by 6 cysteine residues arranged as the motif CX2CX6CX5-12CX2CX6-8C that bind to two zinc atoms. (B) Homology of the Zn(II)2Cys6 binuclear cluster motif. Alignment constructed using HMMER3 and fungal Zn2Cys6 binuclear cluster domain Hidden Markov Model (PF00172.13) with Zn2Cys6 regions of the following fungal transcription factors: *Magnaporthe oryzae* TPC1 (MGG_01285) and PIG1 (MGG_07215); *Saccharomyces cerevisiae* GAL4 (YPL248C); *Aspergillus nidulans* FacB (ANID_00689); *Neurospora crassa* NIT4 (NCU08294); *Schizosaccharomyces pombe* MOC3 (SPAC821.07c) and *Nectria hematococca* CTF1α (AAA96824.1). Conservative aminoacid substitutions are indicated in gray boxes and identical aminoacids in black boxes. (C) Maximum likelihood tree of *M. oryzae* Tpc1 protein (MGG_01285) with its closest fungal orthologues. Orthologous proteins of MGG_01285 were identified using BLAST and aligned manually in BioEdit program. This unrooted phylogenetic tree was constructed using an alignment of 22 fungal sequences from different fungal species: *Ajellomyces capsulatus* (EH07230.1), *A. clavatus* (XP_001271908.1), *A. fischerianus* (XP_001266982.1), *A. flavus* (EED55362.1), *A. fumigatus* (XP_751792.1), *A. niger* (An04g06640), *A. oryzae* (XP_001820273.1), *A. terreus* (XP_001210781.1), *Botryotinia fuckeliana* (BC1G_06121), *Chaetomium globosum* (CHGG_09110), *Coccidioides immitis* (CIMG_00566), *Fusarium graminearum* (FG08769.1), *N. crassa* (NCU05996), *M. oryzae* (MGG_01285), *Podospora anserine* (XP_001906056.1), *Paracoccidioides brasiliensis* (EEH45457.1), *Penicillium chrysogenum* (Pc22g12400), *P. marneffeii* (XP_002151174.1), *Phaeosphaeria nodorum* (SNOG_06665), *P. stipitatus* (EED24478.1), *Pyrenophora tritici* (XP_001931826.1) and *Sclerotinia sclerotiorum* (SS1G_00170). Fungal species within a pink, green and yellow balloon belong to the Dothideomycetes, Sordariomycetes and Leotiomycetes classes, respectively. The other species belong to Eurotiomycetes (*Ajellomyces*, *Aspergillus*, *Paracoccidioides* and *Penicillium* species) and Euascomycetes (*Coccidioides*) classes. Asterisks in the clades denote high likelihood ratio test (LRT) support values (*LRT > 80%).

Figure S2.4

Fig. S2.4. Maximum likelihood tree of *M. oryzae* Zn(II)₂Cys₆ cluster proteins. Proteins containing Zn(II)₂Cys₆ domain were identified in the *Magnaporthe* database using PFAM pHMM zn_clus_ls.hmm. The unrooted phylogenetic tree was constructed using an alignment of 119 zinc cluster *M. oryzae* sequences. Asterisks in the clades denote high LRT support values (* LRT > 80%).

Figure S2.5

Fig. S2.5. *M. oryzae* Tpc1 has an orthologue in *N. crassa* with similar roles in polarized growth development. (A) Alignment of *M. oryzae* Tpc1 (MGG_01285) with *N. crassa* Tpc1 (NCU05996). Amino acid residues of the nuclear localisation signal (NLS), Zn(II)2Cys6 cluster domain and MAPK docking site are within boxes. The red arrowheads indicate the putative phosphorylation sites (Thr residues), while the black arrowhead indicates the putative sumoylation site (Lys residue) present in the *M. oryzae* Tpc1. **(B)** Vegetative growth and colony morphology of *NcTPC1* deletion mutant. The *NcTPC1* KO mutant colony was severely reduced in vegetative growth and the branching of the vegetative hyphae was different to that observed for the isogenic *N. crassa* wild-type (WT) strain. **(C)** Diameter of WT and *NcTPC1* KO colonies reveals the increased tolerance to NaCl (mean±SD; three biological replica). **(D)** Colony images of Vogel's medium plates with increased concentrations of sodium chloride (NaCl). Colony edge is indicated with a black arrow. **(E)** Colony images of CM plates with increased concentrations of sodium chloride [NaCl] (left). Diameter of WT and M1422 colonies measured at 10 days post-inoculation (dpi) reveals the increased tolerance to NaCl (right graph). Error bars represent the standard deviation of three independent biological replicas.

Figure S2.6

Fig. S2.6. Motif enrichment analysis using the 215 down-regulated genes in *Δtpc1*.

Using 1,000 nucleotides upstream the start codon of each gene, two motifs were detected based on the DREME motif discovery algorithm, and were followed by a statistical assessment of the positional preference of the motifs using CentriMo. **(A)** The logo GGGGAK is similar to the DNA sequence recognized by the yeast Zn2(Cys)6 protein Oaf1 (DREME E value 3.5×10^{-4}), and it is present in 133 genes. **(B)** The logo CCCCTTK is similar to the DNA sequence recognized by the yeast Zn finger C2H2 protein Com2 (DREME E value 2.8×10^{-3}), and it is present in 123 genes, 70 of which contain the GGGGAK motif. Dash lines in the centrimo graphs represent the negative dataset used in the analysis. The negative dataset was obtained using 1,000 nucleotides upstream the start codon of the rest of *M. oryzae* genes (12,591). Motifs enriched in the promoter regions of up-regulated genes were not found.

Figure S2.8

Fig. S2.8. NoxD is not involved in *M. oryzae* response to stress conditions. (A) Comparison of expression levels of five Tpc1-dependent genes. qPCR experiments confirmed microarray expression changes for selected genes. qPCR data were normalized against actin and wild-type (WT) expression of the gene. **(B)** Fungal NoxD proteins were aligned using CLUSTAL Omega (EMBL-EBI server). Bc, *Botrytis cinerea* (EMR81583); Pa, *Podospira anserina* (CDP23073); Sm, *Sordaria macrospora* (CAL68654); Mo, *Magnaporthe oryzae* (MGG_09956.8). TM, transmembrane regions predicted using *M. oryzae* NoxD and TMHMM Server v. 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>). TargetP 1.1 localizes *M. oryzae* NoxD in the secretory pathway although lacks a clear signal peptide prediction. **(C)** Growth tests on CM and MM. The $\Delta noxD$ mutant grows slightly faster on CM while no differential growth of $\Delta noxD$ is observed upon exposure to carbon starvation, calcofluor white and pH 9.5. **(D)** Effect of different oxidative stresses in WT, $\Delta noxD$ and $\Delta nox1\Delta nox2$ strains. Both mutants were more resistant than WT on complete medium (CM) supplemented with 1mM Methyl-V and 1mM H₂O₂ but not on CM with 5 mM H₂O₂. Growth was monitored at 8 dpi. CM is taken as reference for each strain.

CHAPTER 3

Exp5, a *Magnaporthe oryzae* nucleo-cytoplasmic receptor involved in non-coding RNA metabolism and plant pathogenesis

Results presented in this chapter are included within a manuscript currently in preparation:

Exp5, a *Magnaporthe oryzae* nucleo-cytoplasmic receptor involved in non-coding RNA metabolism and plant pathogenesis

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ABSTRACT

Magnaporthe oryzae is an important plant pathogenic ascomycetous fungus that causes the rice blast disease, one of the most damaging fungal diseases of rice. The *M. oryzae* karyopherin Exp5 protein is the orthologue of the human karyopherin exportin-5 and the *Saccharomyces cerevisiae* Msn5. Karyopherins are involved in the translocation of proteins and/or RNAs between the nucleus and the cytoplasm. The *M. oryzae* $\Delta exp5$ mutant is strongly impaired in plant disease symptoms production, suggesting that cargoes translocated by *M. oryzae* Exp5 play an important role during infection. To better understand the role of this karyopherin during plant infection we set out to identify its cargoes. Several t-RNA synthetases, seven subunits of the 26S proteasome and key components of pathogenesis-related signal transduction pathways such the mitogen activated protein kinases Pmk1 and Mps1 were found to immunoprecipitate with Exp5. An important set of mitochondrial proteins including TIM44 and TOM70 also immunoprecipitate with Exp5, which suggest that mitochondrial dysfunction can be contributing to the strong root infection defects exhibited by $\Delta exp5$. Additional deficiencies such as accumulation of sRNAs derived from transposon elements and tRNA^{Met} in the mutant supports the involvement of Exp5 in sRNA metabolism and tRNA transport. Our results suggest that Exp5 is required for the transport of specific classes of proteins, tRNAs and small RNAs, several of which contribute to *M. oryzae* pathogenicity.

INTRODUCTION

Magnaporthe oryzae is one of the most important airborne fungal pathogens of rice worldwide and the causal agent of the devastating rice blast disease. *M. oryzae* is closely related to root-infecting pathogens such as *M. poae*, which infects turfgrasses and *Gaeumannomyces graminis*, which causes the take-all disease on wheat. *M. oryzae* leaf infection process has been studied with exquisite detail, and this fungus can also infect roots under laboratory conditions (Sesma & Osbourn, 2004, Besi, *et al.*, 2009). Different mechanisms and signaling pathways mediate *M. oryzae* infection of leaves or roots although common genes and pathways have also been found necessary for fungal infection on both plant organs such as the mitogen activated kinases Pmk1 and Mps1 (Dufresne & Osbourn, 2001, Sesma & Osbourn, 2004, Besi, *et al.*, 2009, Tucker, *et al.*, 2010).

Hydrophilic polystyrene surfaces (PHIL-PS) can induce the infection-related development observed during *M. oryzae* root colonization (Tucker, *et al.*, 2010). Twenty mutants with altered ability to differentiate pre-invasive hyphae (pre-IH) on PHIL-PS were identified from an insertional library of 2,885 *M. oryzae* T-DNA transformants. Among them, the mutant M1373 presented reduced ability to infect roots and leaves although the defects on roots were remarkably stronger (Tucker, *et al.*, 2010). The T-DNA insertion in M1373 is located in the open reading frame of the gene MGG_09560, which encodes a protein homologue to the yeast Msn5 and the human exportin-5 karyopherins. The deletion mutant $\Delta exp5$ confirmed the *in planta* phenotype of M1373. Colony morphology and growth in complete (CM) and minimal (MM) media of the $\Delta exp5$ mutant were very similar compared to the wild type strain (Tucker, *et al.*, 2010). However, in addition to the altered pre-IH growth on PHIL-PS $\Delta exp5$ showed a reduction in conidia production, and it was unable to produce appressoria on artificial surfaces. A strong reduction in root disease symptoms production was also confirmed in the deletion mutant $\Delta exp5$ (Tucker, *et al.*, 2010). Remarkably, this mutant produced similar number of lesions on leaves compared to the wild type strain, indicating that appressoria development was restored *in planta*. However, the size of these lesions was smaller than those produced by the wild type, which suggested a defect in invasive growth of aerial tissues. The Exp5 protein showed a steady-state nuclear localization under all conditions tested (Tucker, *et al.*, 2010).

In eukaryotic organisms, the nucleus is physically separated from the cell cytoplasm and the transport of proteins and RNAs between these two compartments is essential for living cells. All nuclear proteins such as histones and transcription factors need to be imported into the nucleus from the cytoplasm where they are synthesized. Equally, transfer RNA (tRNA), ribosomal RNA (rRNA) and messenger RNA (mRNA), which are synthesized in the nucleus during transcription, have to be imported into the cytoplasm where translation occurs. Nuclear pore complexes (NPCs) mediate these transport processes. Each NPC is formed of approximately 30 different proteins present in multiple copies called nucleoporins (Nups) (Doucet, *et al.*, 2010). Lower and higher eukaryotes have a conserved NPC structure. The central channel of the NPCs is formed by a class of Nups that contain Phe/Gly amino acid repeats named FG-Nups. The FG-Nups proteins limit the diffusion of macromolecules across the nuclear envelope, recognize the transport factors carrying their cargo and mediate their movement through the NPC channel (Fig. 3.1) (Gorlich & Kutay, 1999, Cook, *et al.*, 2007, Terry, *et al.*, 2007, Chook & Süel, 2011). Movement of small molecules occurs passively through the NPCs. Cargoes larger than 40kDa are translocated actively by transport receptors that recognize signals to import (NLS, nuclear localization signal) or export (NES, nuclear export signal) present in their cargoes. The karyopherin protein family constitutes the largest group of nuclear transport receptors that mediate active nuclear transport of specific cargoes in a Ran-dependent mechanism (Harel & Forbes, 2004). Ran is a small GTPase whose nucleocytoplasmic gradient controls the direction of karyopherins transport. Karyopherins represent one major class of nuclear transport receptors found in all eukaryotic organisms from yeast to humans (Kimura & Imamoto, 2014, Cautain, *et al.*, 2015, Sloan, *et al.*, 2016). Fourteen karyopherins have been identified in *S. cerevisiae* and twenty-two in humans. In *M. oryzae*, thirteen out of the fourteen yeast karyopherins are present (Supplemental Table. S3.1).

A range of cellular processes linked to stress responses, pseudohyphal differentiation, glucose repression, phosphate metabolism, nitrogen metabolism and cell cycle have been assigned to the yeast Msn5. This protein exports from the nucleus several transcription factors including Pho4p (Kaffman, *et al.*, 1998), Mig1p (DeVit & Johnston, 1999), Msn2p-Msn4p (Gorner, *et al.*, 2002), Crz1 (Boustany & Cyert, 2002), Rtg1p-Rtg3p (Komeili, *et al.*, 2000), Swi6p (Queralt & Igual, 2003), and other proteins such as the Cln-Cdc28p inhibitor Far1p

(Blondel, *et al.*, 1999), the MAPK scaffold protein Ste5 (Mahanty, *et al.*, 1999), the chaperone hsp70-Ssa4p (Quan, *et al.*, 2006) and the Ho endonuclease (Bakhrat, *et al.*, 2008).

Fig. 3.1. β -Karyopherins mediate the transport of cargoes between the cytoplasm and the nucleus. (A) Proteinaceous and RNA cargoes containing nuclear export signals (NES) associate with exportins (Exp; β -karyopherins with export functions) and RanGTP in the nucleus (Nuc.). After translocation through the NPC (white oval), hydrolysis of RanGTP to RanGDP and inorganic phosphate (Pi) releases the cargo into the cytoplasm (Cyt.). Then, the exportin is recycled back to the nucleus. **(B)** Cargos containing nuclear localization signals (NLS) bind importins (Imp; β -karyopherins with import functions) at low RanGTP concentrations in the cytoplasm and translocate to the nucleus as a dimeric complex. Association in the nucleus of RanGTP with the importin releases the cargo and the importin-RanGTP complex is exported to cytoplasm. Picture based on (Sloan, *et al.*, 2016).

To our knowledge, the replication protein A (RPA) is the only cargo shown to be imported to the nucleus by Msn5 (Yoshida & Blobel, 2001). Mns5 also binds mature (spliced and aminoacylated) tRNAs and short non-coding dsRNAs in a Ran-GTP dependent mechanism (Shibata, *et al.*, 2006, Murthi, *et al.*, 2010, Phizicky & Hopper, 2010, Wu, *et al.*, 2015). Yeast strains lacking either Los1 or Msn5 alone revealed only moderate defects in the nuclear export of tRNAs, and the double knockout is still viable although it displays a strong synthetic defect in mature tRNA export (Takano, *et al.*, 2005, Huang & Hopper, 2015, Wu, *et al.*, 2015). This suggests that Los1 and Msn5 are not the only tRNA nuclear exporters. Since splicing of tRNA introns occurs in the cytoplasmic side of the mitochondrial membrane in yeast, the accumulation of mature tRNAs in the nucleus under certain conditions led to the discovery of the tRNA retrograde pathway (Hopper, 2013). Although Msn5 covers a minor

role in the primary tRNA export route (i.e. export of tRNAs encoded by intron-containing genes), it is a main player in the re-export of these tRNAs to the cytoplasm together with Mtr10 (Murthi, *et al.*, 2010). Surprisingly, no sequence similarity has been found among the protein cargoes and the nuclear export signal (NES) recognised by Msn5 (Shibata, *et al.*, 2006, Cautain, *et al.*, 2015), which suggest that the NES motif recognised by Msn5 is more likely to be determined by protein structure or post-translational modifications.

In animals and plants, much less is known about exportin-5 protein cargoes. Metazoan exportin-5 is required for 60S ribosome biogenesis, and the 60S export is dependent on at least two exportins, Crm1 and Exp5 (Wild, *et al.*, 2010). Metazoan Exportin-5 also exports dsRNA-binding proteins in a dsRNA-dependent manner (Brownawell & Macara, 2002, Gwizdek, *et al.*, 2004) and tRNAs by forming a ternary complex with tRNA along with the translation factor eEF1A (Bohnsack, *et al.*, 2002, Calado, *et al.*, 2002, Sloan, *et al.*, 2016). Furthermore, human exportin-5 recognizes directly precursor of miRNAs (pre-miRNAs) and is an important component of the miRNA biogenesis machinery. It directly recognizes the 2-nt single-stranded 3' overhang of the ~60-70 pre-miRNA to transport them to the cytoplasm where they are cleaved by Dicer (Bohnsack, *et al.*, 2004, Lund, *et al.*, 2004, Czech & Hannon, 2011, Kimura & Imamoto, 2014). In *A. thaliana*, loss-of-function mutations in HASTY (the plant exportin-5 orthologue) reduce the level of most miRNAs but do not affect the accumulation of tRNAs, and the hemimethylated miRNA duplexes are transported to the cytoplasm either by HASTY or through HASTY-independent mechanisms (Park, *et al.*, 2005), although the exact form of sRNAs exported cargo by HASTY remains to be discovered. These cumulative findings suggest that in most organisms Exp5 orthologues may play major roles in the export of non-coding RNAs. Tef1, the yeast orthologue of the translation elongation factor eEF1A, is also required for the tRNA reexport and its subcellular localization relies on the karyopherins Mtr10 and Msn5 (Murthi, *et al.*, 2010, Sloan, *et al.*, 2016).

In contrast to the yeast $\Delta msn5$ mutant, which presents defects in carbon-sources utilization and higher sensitivity to high concentration of several ions (Alepuz, *et al.*, 1999 1530), the $\Delta exp5$ mutant only shows different colony morphology and growth at pH 9.5 and 0.3 M LiCl (Tucker, *et al.*, 2010). These results hint a functional divergence among the Exp5-like karyopherins in the fungal kingdom. In *M. oryzae* Exp5 is

required for full disease symptoms production on leaves and roots. We therefore focused this study on deciphering the protein and RNA cargoes transported by *M. oryzae* Exp5 in order to increase our knowledge on the mechanisms that regulate infection processes in this fungal pathogen of rice.

RESULTS

Lack of Exp5 increases the expression of the karyopherins Kap104, Pse1, Cse1, and Kap123 in *M. oryzae* under complex and carbon-depleted media

Thirteen karyopherin (kap) proteins have been identified in *M. oryzae* based on their yeast counterparts (Supplemental Table S3.1). To understand the relevance of these set proteins and their potential links with Exp5, we started by analysing their expression levels in the wild type and $\Delta exp5$ strains under rich and poor media, i.e. liquid complex medium (CM), carbon starvation (MM-C) and nitrogen starvation (MM-N). All the karyopherin genes showed lower expression levels than the actin gene in these media (Fig. 3.2A, 3.3A and Supplemental Fig. S3.1A). In CM, we classified the karyopherins in four groups based on their expression profiles in the wild type strain (Fig. 3.2A). The first group contained only the karyopherin Kap120, which showed the lowest expression levels. The second group included three kaps (Kap104, Pse1 and Rsl1), and the third group seven kaps (Exp5, Pdr6, Los1, Kap114, Kap123, Cse1 and Crm1). Kap mRNA levels in the second and third groups were two and three orders of magnitudes higher than Kap120, respectively. The fourth group included Nmd5 and Mtr10, which displayed the strongest expression. In yeast, Nmd5 mainly imports into the nucleus the transcription factor IIS (TFIIS) (Albertini, *et al.*, 1998). TFIIS binds RNA polymerase II and stimulates cleavage and elongation of nascent pre-mRNA in the transcription elongation complex (Luse, 1991, Borukhov, *et al.*, 1993). High transcriptional activity is expected in actively growing fungal cells. Consequently, the increased expression of Nmd5 in rich media suggests that Nmd5 transport activity is supporting RNA polymerase II-mediated transcription by increasing TFIIS import into the nucleus. The yeast Mtr10 imports into the nucleus mRNA-binding proteins involved in splicing and/or nuclear export of mRNAs (Senger, *et al.*, 1998), and it is also required for the re-export of mature tRNAs. The mRNA and tRNA re-export pathways are known to be regulated by nutrient availability

since both are required to maintain high translation rates during active fungal growth (Murthi, *et al.*, 2010). Therefore, Nmd5 and Mtr10 expression levels correlated with the expected high translation rates in CM.

To identify the links between *M. oryzae* Exp5 and the rest of the kaps, gene expression ratios between the $\Delta exp5$ mutant and wild type strains were analysed under all conditions tested (Fig. 3.2B, 3.3B, Table 3.1 and Supplemental Fig. S3.1B).

In CM, $\Delta exp5$ showed increased expression in all the karyopherin genes. The biggest variations were observed in *PSE1* (group II), *NMD5* (group IV) and *KAP123*, *LOS1* and *CSE1* (group III) with a 95-, 61-, 31-, 13- and 11-fold change, respectively (Fig. 3.1B). In yeast, Pse1 interacts with the NPC and imports several transcriptional regulators including Pdr1 that is involved in the pleiotropic drug response, the regulator of mating and pseudohyphal/invasive growth Ste12, and Yap1 and Aft1, which are required for oxidative stress tolerance and iron homeostasis (Bardwell, *et al.*, 1998, Mamnun, *et al.*, 2002, Ryo Ueta, 2003, Gulshan, *et al.*, 2012, Ueta, *et al.*, 2012). Nmd5 and Sxm1 are two distinct but related karyopherin proteins in yeast (Supplemental Table S3.1). We found only one orthogue (MGG_02927) for both proteins in *M. oryzae*. In addition to TFIIS, the yeast Nmd5 also imports Crz1, a transcriptional factor that regulates calcium-dependent signaling (Albertini, *et al.*, 1998, Ferrigno, *et al.*, 1998, Polizotto & Cyert, 2001) whereas Kap123 mediates nuclear import of ribosomal proteins and histones H3 and H4. Kap123 can interact with Pse1, Sxm1 and Nmd5 in yeast (Sydorsky, *et al.*, 2003), suggesting already a close link between them. Cse1 is a recycling factor that mediates the nuclear export of the α -karyopherin (Kap α /Srp1) back to the cytoplasm, thereby allowing its participation in multiple rounds of nuclear import through its interaction with Rsl1 (Schroeder AJ, 1999, Yuksel, *et al.*, 2015), which interacts with Nups to carry out the Kap α nuclear import (Lonhienne, *et al.*, 2009, MacKinnon, *et al.*, 2009). Overall, our results suggest that in the absence of Exp5, the fungal cell increase the expression of these five kaps (*PSE1*, *NMD5*, *KAP123*, *LOS1* and *CSE1*) in order to supplant the transport role of Exp5 during active growth. This correlates with the weak phenotype display by the $\Delta exp5$ mutant in CM.

In carbon starved cells, expression of mostly all kaps in the wild type strain was reduced compared to CM except for the *PSE1* (group II), *EXP5* and *RSL1* (group III), and *KAP120* (group I), which showed a 15-,

6.9-, 3.2- and 2.2-fold increase in their expression levels (Fig 3.3A-B and Table 3.1). Possibly Pse1, Exp5, Rsl1 and Kap120 play an important role in the adaptation to carbon starvation, particularly Pse1 and Exp5.

With regard to the behaviour of kap genes in $\Delta exp5$ carbon depleted cells, a two-fold up-regulation in the expression of *KAP104*, *KAP123*, and *CSE1* (group III) and three-fold in *PSE1* (group II) was found while other kap genes from group III such as *LOS1*, *KAP114* and *PDR6* showed a three-fold reduction (Supplemental Fig. S3.3A-B). It is worth noting that many of the altered kaps in the mutant under this condition display important roles controlling RNA polymerase II transcription and cell cycle. The yeast Kap104 mediates nuclear import and re-import of mRNA-binding proteins, and it regulates asymmetric protein synthesis in daughter cells during mitosis (van den Bogaart, *et al.*, 2009). In addition to recycling Kap α , Cse1 is also required for cell division and mitotic chromosome segregation (Xiao, *et al.*, 1993) whereas Kap114 is responsible for the import of specific proteins such as the transcription factors Spt15 and Sua7 and histones H2A and H2B. The Spt15 and Sua7 proteins are involved in the formation of the preinitiation complex at promoter regions and the start site selection of RNA polymerase II, respectively (Hodges, *et al.*, 2005). Similarly, Pdr6 imports the RNA polymerase II TFIIA complex into the nucleus (Titov & Blobel, 1999). The increase expression of Pse1 and Kap123 in $\Delta exp5$ carbon starved cells strengthens the previous link observed between these two kaps and Exp5 in CM.

A similar analysis was also carried out in nitrogen starved cells. Pse1 (group II), Kap120 (group I) and Exp5 (group III) were the only up-regulated kaps in the wild type strain with a 4.9-, 4.0- and 2.3-fold change, respectively (Supplemental Fig. S3.2A-B). In the mutant, the expression of kaps was down-regulated except for Pse1 (group II), Cse1 and Crm1 (group III) and Nmd5 (group IV). Interestingly, both Kap123 (group III) and Nmd5 (group IV) showed the lowest levels of expression in both carbon- and nitrogen-starved cells (Fig. 3.3B and Supplemental Fig. S3.1 A-B).

A

B

Fig. 3.2. Pse1, Nmd5, Kap123, Los1 and Cse1 are overexpressed in $\Delta exp5$ cells grown in complex medium (CM). (A) Expression analysis of *M. oryzae* karyopherins in wild type and $\Delta exp5$ strains. Gene expression is represented by fold change ($2^{-\Delta\Delta Ct}$) normalized against actin. Karyopherins show lower levels of expression than the actin gene (decimal values in Y-axis). (B) Gene expression ratios between wild type and $\Delta exp5$ strains.

A **B**

Fig. 3.3. Kap104, Kap123, Cse1 and Pse1 are overexpressed in carbon-starved $\Delta exp5$ cells. (A) Expression analysis of karyopherins in wild type and $\Delta exp5$ strains in carbon starved cells (MM-C). Gene expression is represented by fold change ($2^{-\Delta\Delta Ct}$) normalized against actin. Karyopherins show lower levels of expression than the actin gene (decimal values in Y-axis). (B) Gene expression ratio between $\Delta exp5$ and Guy11 in MM-C.

		WT		$\Delta exp5$ mutant		
		MM-C	MM-N	CM	MM-C	MM-N
		MM-C/CM ratio	MM-N/CM ratio	$\Delta exp5$ /WT ratio	$\Delta exp5$ /WT ratio	$\Delta exp5$ /WT ratio
I	<i>KAP120</i>	2.2 ± 0.0	4.0 ± 0.0	2.2 ± 0.9	-1.6 ± 1.3	-3.5 ± 2.2
II	<i>KAP104</i>	-2.2 ± 0.9	-3.2 ± 1.4	2.6 ± 0.8	2.4 ± 0.4	-1.6 ± 0.1
	<i>RSL1</i>	3.2 ± 0.0	1.3 ± 0.0	5.6 ± 2.5	-1.3 ± 0.9	-1.7 ± 0.6
	<i>PSE1</i>	15.2 ± 0.0	4.9 ± 0.0	95.3 ± 29.8	1.9 ± 0.5	1.4 ± 0.2
III	<i>EXP5</i>	6.9 ± 0.1	2.3 ± 0.2			
	<i>PDR6</i>	1.4 ± 0.3	1.1 ± 0.4	1.8 ± 0.5	-3.0 ± 1.3	-2.5 ± 0.9
	<i>LOS1</i>	-1.4 ± 4.9	-3.5 ± 1.4	13.3 ± 3.4	-3.5 ± 1.9	-3.9 ± 1.6
	<i>KAP114</i>	-1.2 ± 2.2	-13.9 ± 5.3	4.7 ± 2.1	-3.7 ± 0.7	-1.2 ± 0.3
	<i>KAP123</i>	-32.5 ± 16.1	-124.4 ± 61.7	31.7 ± 15.8	2.9 ± 0.7	-2.6 ± 0.5
	<i>CSE1</i>	-4.9 ± 2.5	-16.7 ± 8.3	11.2 ± 5.5	2.0 ± 0.5	1.5 ± 0.3
	<i>CRM1</i>	-1.4 ± 1.7	-7.5 ± 1.8	5.5 ± 2.7	1.1 ± 0.2	1.2 ± 0.1
IV	<i>NMD5</i>	-30.4 ± 14.2	-196.6 ± 91.7	60.9 ± 30.5	-1.1 ± 0.5	1.5 ± 0.1
	<i>MTR10</i>	-3.2 ± 1.6	-5.8 ± 2.9	5.4 ± 2.7	1.1 ± 0.3	-1.9 ± 0.4

Table. 3.1. Summary of the expression analysis of karyopherins in wild type and $\Delta exp5$ strains in different media. Bold numbers represent fold of change. Standards deviation are also written. Dark red: highly reduced; red: reduced; dark green: highly up-regulated; green: up-regulated.

Proteins involved in mitochondrial protein import, primary metabolism, protein homeostasis and MAPK signaling immunoprecipitate with Exp5

In order to identify proteins that directly interact with Exp5 amino and carboxy fusions using HA-Flag were generated for immunoprecipitation assays. The $\Delta exp5$ mutant strains complemented with *Flag-HA:EXP5* and *EXP5:HA-Flag* constructs were selected based on their expression levels and their ability to restore appressoria formation on coverslips (Fig. 3.4 A-B).

The immunoprecipitation assays were carried out with the C9 strain, which contains the HA-Flag fused to the C-terminus of the protein (Fig. 3.5). After tandem affinity protein purification, wild type and C9 eluates obtained from the last purification step were analysed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS; final HA-eluate in Fig. 3.5B). We found a total of 258 proteins with at least two unique peptides of difference between wild type and C9 strains after validating the results using a False Discovery Rate (FDR) >20 for protein match (Table 3.2). These parameters are usually set when the reference database is not well annotated or the total proteome is too small as it occurs in our case, i.e. a genome of 40 Mb in size and ca. 13,000 proteins.

Fig 3.4. Identification of $\Delta exp5$ strains expressing Exp5:HA-Flag fusion constructs. (A) Eight-day old plates of wild type (WT) Guy11, $\Delta exp5$ and $\Delta exp5$ complemented with *EXP5:HA-Flag* amino and carboxy fusions in CM. Conidia (c) formed appressoria (a) on coverslips. Scale bar: 20 μ m. **(B)** Western blot using total protein extracts of $\Delta exp5$ complemented with amino and carboxy *EXP5:HA-Flag* fusions. Strains expressing amino (RBP35N+) and carboxy (RBP35C+) HA-Flag fusions of Rbp35 were used as positive controls. Carboxy HA-Flag constructs of Exp5 showed higher expression levels than the amino constructs. The C9 strain ($\Delta exp5$ /*EXP5:Flag-HA*) was selected for immunoprecipitation assays (marked with an asterisk).

We classified all the proteins identified in the immunoprecipitation assay into eight functional groups according to their potential roles in nucleo-cytoplasmic transport (7 proteins), general metabolism (95 proteins), mitochondria (33 proteins), RNA metabolism and ribosome biogenesis (32 proteins), endocytosis/secretion/cell wall (13 proteins), cytoskeleton (8 proteins), signaling (7 proteins) and other functions (53 proteins). In addition, we found nine proteins that lacked any known domain.

Fig. 3.5. Control tests carried out during immunoprecipitation assays. Left panel: initial protein extracts oriole stained in a polyacrylamide gel. 20 μ g of protein per well was used. Right panel: Western blot using anti-HA antibody of the different aliquots obtained during each immunoprecipitation step. Red arrow indicates the expected protein band size corresponding to the Exp5:HA-Flag protein.

The validity of the immunoprecipitation assay was supported by the presence of RanGTP and the nucleoporin Ndc1 among Exp5-interacting proteins. We found additional proteins associated with nucleocytoplasmic transport including the group III kaps Cse1, Kap123 and Crm1, and two additional kaps from group II, Rsl1 and Pse1. The interaction of Exp5 with Pse1, Cse1 and Kap123 correlated with their overexpression in the absence of Exp5.

Within the metabolism group several pathogenicity-related proteins were identified including the homocitrate synthase (MGG_01092) (Zhang, *et al.*, 2014), the neutral trehalase Nth1 (MGG_09471) (Foster, *et al.*, 2003), the S-adenosylmethionine synthase (MGG_00383) (Dean, *et al.*, 2005), the trehalose synthase and glucose sensor Tps1 (MGG_03860) (Bell, *et al.*, 1998), the tetrahydroxynaphthalene reductase involved in melanin synthesis (MGG_02252) (Andersson, *et al.*), a glutathione dehydrogenase (MGG_06011) (Lee, *et al.*, 2012), a glycogen synthase (MGG_07289) (Badaruddin, *et al.*, 2013) and a serine hydroxymethyltransferase (MGG_13781) (Cui, *et al.*, 2015). Alterations in their transport can affect the ability to grow inside the plant cell. This correlates with the invasive growth defect observed in aerial parts of the plant in the $\Delta exp5$ mutant.

The next most abundant groups of Exp5-interacting proteins included 33 mitochondria-related proteins and 32 proteins involved in RNA metabolism and ribosome biogenesis (Table 3.2). Two important pathways of protein import into mitochondria are mediated by the TOM and the TIM protein complexes (Harbauer, *et al.*, 2014). Pre-proteins that carry a cleavable presequence are transferred in the mitochondria from TOM, which is a translocase located in the outer membrane into the translocase TIM23 complex of the inner membrane (Harbauer, *et al.*, 2014). This process is driven by the mitochondrial membrane potential. In our experiment, Tom70 and Tim44, both of which are components of the TOM and TIM23 complexes respectively, immunoprecipitated with Exp5. This possibly explained the amount of mitochondrial proteins found in these experiments. With respect to the proteins involved in RNA metabolism and ribosome biogenesis, it is particularly abundant the involvement of proteins in translation elongation. We found four translation elongation factors, 12 t-RNA synthetases, the complex Gcn1/Gcn20 that also regulates translation elongation and 13 ribosomal proteins.

Eleven out of the thirteen proteins within the endocytosis/secretion/cell wall group were predicted to participate in vesicle trafficking and sorting. Two chitin synthases also associated with fungal cell wall biosynthesis were within this group. Due to the relevance of this pathway in fungal growth and the little defects in colony morphology displayed by the $\Delta exp5$ mutant, it is highly probable that several kaps including Exp5 participate in the translocation of proteins associated with these biological processes. With regard to the cytoskeleton group, the presence of actin and tubulin supported the role of karyopherins in microfilament regulation as previously suggested (Mosammaparast & Pemberton, 2004, Sloan, *et al.*, 2016). Among this group was also found the Rac1 protein, which has a significant role in pathogenesis due to its role in regulating actin cytoskeleton and polarized cell growth (Jeon, *et al.*, 2007, Chen, *et al.*, 2008).

Several proteins involved in signal transduction pathways associated with *M. oryzae* infection process were also found to interact with Exp5, including the mitogen-activated protein kinases (MAPKs) Mps1 (MGG_04943.8) and Pmk1 (MGG_09565). Mps1 is essential for appressoria penetration and regulates the cell wall integrity pathway while Pmk1 is required for appressorium development and invasive growth (Fig. 1.5) (Xu & Hamer, 1996, Xu, *et al.*, 1998). Their potential translocation

by Exp5 supports the relevance of this protein in the biology of *M. oryzae* infection. Exp5 also seemed to be involved in protein homeostasis due to their interaction with the cytosolic 26S proteasome (12 subunits were found in the immunoprecipitation experiments), the assembly factor of E3 ubiquitin ligases and 5 different proteases. Two of them, the subtilisin-like proteinase Spm1 and the prenyl protease 1 were previously reported to be involved in pathogenicity (Jeon, *et al.*, 2007, Saitoh, *et al.*, 2009).

In conclusion, these immunoprecipitation experiments allowed us to identify several cellular processes in which Exp5 potentially participates due to its role as nucleo-cytoplasmic transporter. These include translation elongation, mitochondrial protein import, glucose metabolism, endocytosis/exocytosis pathways, MAPK signaling, polarized growth and protein homeostasis.

Nuclear accumulation of sRNAs in the $\Delta exp5$ mutant

To elucidate the involvement of *M. oryzae* Exp5 in sRNA pathways we carried out the sequencing of sRNA libraries derived from wild type and $\Delta exp5$ strains. To achieve this, we used a new approach focused in high definition adapters (HD). These adapters allow a higher number of ligation combinations with sRNAs, reducing the bias produced by standard sRNA-seq adapters (Sorefan, *et al.*, 2012). The experiment was carried out by triplicate. We obtained good quality of reads with less than 1 error per 100 nucleotides (Supplemental Table S3.2). The read length after removal of internal adapters oscillated between 13-26 bp in both strains and their distribution reflected the presence of two sRNA population sizes, the biggest one of 20 ± 3 nt and the smallest of 14 ± 1 nt in length (Supplementary Fig. S3.4).

A total of 23,907 transcripts with more than one read per million were assembled from the sRNA sequencing data (Fig. 3.6A). Most of the transcripts were derived from transposable elements (38.4 %) or from viruses (22.4 %). (19.3 %) had an unknown origin and (7.2 %) came from intergenic regions. (3.9 %) of the transcripts matched to rDNA, and other unclassified ESTs represented (4.9 %) of total transcripts. (3.2 %) of the transcripts were cDNAs, and tRNAs and introns were (0.3 %) each other.

The small transcriptome data differed between $\Delta exp5$ and wild type. The size distribution of the mutant changed with the disappearance of the 14-nt peak (Supplementary Fig. S3.4). Possibly, this 14-nt peak contained fragments derived from tRNAs and rDNAs since the $\Delta exp5$ mutant showed a significant alteration in the sRNA populations derived

from these two groups. Additionally, almost all the transcripts were down-regulated in the mutant, i.e. tRNAs, rDNA, cDNA, viruses, other classified ESTs and unknown transcripts. Only transposable elements and transcripts located in intergenic regions and introns were up-regulated in $\Delta exp5$ (Fig. 3.6B).

In order to elucidate the role of Exp5 in sRNA transport Northern blots were carried out using sRNAs extracted from the nuclear and cytoplasmic fractions of wild type and $\Delta exp5$ strains (Supplemental Fig. S3.5). The small nuclear RNAs U6 and U2 are components of different subunits of the spliceosome, and they were used as specific markers of the nuclear fraction. Presence of their corresponding bands in the nuclear fractions of wild type and mutant strains confirmed the right separation of nuclear and cytoplasmic sRNA pools (Fig. 3.7A). Additional probes identified from the sRNA sequencing data were used to compare their profile patterns in different subcellular compartments.

Two probes were used for the analysis of tRNAs, tRNA^{Met} and tRNA^{Lys(CUU)}. Their corresponding genes are found in nine and eight different genomic loci, respectively and can vary in size. We observed an enrichment of the mature tRNA^{Met} and the ~150 nt precursor band of tRNA^{Lys} in the nuclear fractions of $\Delta exp5$, which suggested that both tRNAs were being retained in the nucleus of the mutant (Fig. 3.7B). The tRNA^{Met} size varies between 72 nt for the mature spliced form and 104 to 113 nt for its precursors. Similarly, the size of the tRNA^{Lys(CUU)} varies between 73 nt for the mature tRNA and 94 nt/101 nt/114 nt if it contains the intron. In our experiments we only detected one band of tRNA^{Met} corresponding to the mature tRNA^{Met} form, which was barely seen in the $\Delta exp5$ cytoplasmic fraction despite of its overexpression in the total RNA fraction (Fig. 3.7B). With regard to the tRNA^{Lys(CUU)} two different bands were observed. A band of approximately 150 nt that matched with the size of one of the tRNA^{Lys(CUU)} precursors was retained in the nuclear fraction of the $\Delta exp5$ mutant and was similarly distributed in the cytoplasm of both strains.

Fig. 3.7. Small RNA libraries were sequenced to compare transcriptional profiles between $\Delta exp5$ and WT strains. (A) Total of transcripts assembled in the wild type strain compared to total unique reads. **(B)** Bar graph indicating the number of contigs differentially expressed in $\Delta exp5$.

This suggested that Exp5 was not the only karyopherin involved in the transport of tRNA^{Lys(CUU)} to the cytoplasm. The second band of 80 nt corresponded to the mature form of tRNA^{Lys(CUU)} and it was exclusively detected in the cytoplasmic fraction of both wild type and $\Delta exp5$. Since no change was observed for this band between the two strains, our results indicated that Exp5 is required for the export of the precursor but not the mature form of tRNA^{Lys(CUU)}.

MAGGY and RETRO5 are two retrotransposons that belong to the gypsy-like transposable elements and contain long terminal repeats (LTR) (Farman, *et al.*, 1996, Farman, *et al.*, 2002). One of the roles of the RNA silencing machinery in a living cell is to silence transposon elements and viruses (Murata, *et al.*, 2007). Since the sRNA derived from these two retrotransposons were found up-regulated in $\Delta exp5$, probes were also designed to confirm these results by Northern blotting. We observed a conserved pattern for both retrotransposons (Fig. 3.7C). An increase of ~50 nt sRNAs of RETRO5 was found in the mutant nucleus compared to the wild type strain. Similarly, nuclear sRNAs ~30-40 nt in size of MAGGY accumulated in $\Delta exp5$. However, both RETRO5 and MAGGY sRNAs were detected at the same level in the cytoplasmic fractions of the wild type and $\Delta exp5$ strains.

Remarkably, the analysis of the sRNA sequencing data allowed us to discover the presence of a single stranded RNA (ssRNA) virus in the *M. oryzae* wild type isolate used in this study named as Guy11. The Guy11 strain is a rice-infecting isolate recovered from an infected rice field of the French Guiana (Leung, *et al.*, 1988, Valent & Chumley, 1991). This new mycovirus was named MOLV1 (Magnaporthe oryzae ourmia-like virus 1; see chapter 4). We used a probe of MOLV1 to confirm the presence of sRNAs derived from this virus in *M. oryzae* (Fig. 3.7D). It is worth noting that Exp5 was not required for silencing the RNA levels of MOLV1 (Fig. 3.7D).

Overall, we observed differences in the sRNA patterns between the nuclear and cytoplasmic fractions of wild type and $\Delta exp5$ strains, which supported the idea that Exp5 is involved in the export of sRNAs to the cytoplasm. With respect to tRNA transport, our data suggest that Exp5 is involved in the primary export of tRNA^{Lys(CUU)} and the re-export of tRNA^{Met} in *M. oryzae*.

Fig. 3.8. Nuclear accumulation of sRNAs in the $\Delta exp5$ mutant. Detection of sRNAs by Northern blotting using probes against **A)** small nuclear RNAs (snRNAs) U6 and U2; **B)** tRNA^{Met} and tRNA^{Lys(CUU)}; **C)** RETRO5 and MAGGY retrotransposons; and **D)** MOLV1 virus. Probes were designed anti-sense to our sRNA targets except for those used against retrotransposons (RETRO5 and MAGGY). Retrotransposon probes were designed to identify their anti-sense small RNAs, which were up-regulated in the mutant. Numbers represent band intensities, which were quantified using ImageJ program as follows. Ratio 1 was given to pixel intensities of the wild type (WT) under each condition and represents the ratio between Northern blot and EtBr band intensities. Ratios in the mutant were obtained in the same way and each ratio was normalized against the corresponding WT ratio value. Green and red numbers indicate accumulation or reduction of sRNAs in $\Delta exp5$ compared to WT.

DISCUSSION

Karyopherins belong to a conserved protein family that represents the largest group of nuclear transport receptors found across organisms from yeast to humans (Harel & Forbes, 2004). Karyopherins interact with their cargoes, with specific proteins of the NPC and RanGTP (Mosammaparast & Pemberton, 2004). Msn5 is the only yeast karyopherin identified to date with roles in both import and export of different RNAs and proteins between the cytoplasm and the nucleus (Sloan, *et al.*, 2016). Exp5, the *M. oryzae* orthologue of Msn5 is one of the thirteen karyopherins present in *M. oryzae*. Although Exp5 was reported to be involved in plant virulence (Tucker, *et al.*, 2010), the mechanisms by which it regulates *M. oryzae* pathogenicity have not been elucidated yet.

To characterise the role played by Exp5 in the rice blast fungus, we started by analyzing the expression levels of all kaps under different nutritional conditions and looking at their potential links with Exp5. Expression of the *EXP5* gene is significant in CM and MM-C, suggesting that Exp5 is involved in fungal adaptation to these two nutritional conditions. In the $\Delta exp5$ mutant, all the kaps increase their expression in CM, particularly Pse1 (group II), Kap123 (group III), and Nmd5 (group IV). In addition to the involvement in ribosomal proteins export, Pse1 and Kap123 can also transport transcription factors and histones, respectively. Pse1 also interacts with Nups. The yeast Nmd5 translocates MAPK-dependent transcription factors to the cytoplasm for its activation by phosphorylation as in the case of Ste12 (Errede & Ammerer, 1989), which activates genes involved in mating or pseudohyphal/invasive growth pathways (Roberts & Fink, 1994, Bardwell, *et al.*, 1998). The yeast MAPK Fus3/Kss1 involved in this pathway is well conserved in fungi and controls the infection process of all fungal pathogenic species tested (Turrà, *et al.*, 2014). Overexpression of Nmd5 in the $\Delta exp5$ background can be due to nuclear transport defects in Pmk1, the *M. oryzae* orthologue of the yeast Fus3/Kss1 proteins and one of the two MAPKs that immunoprecipitate with Exp5. During *M. oryzae* vegetative growth Pmk1 is located in the cytoplasm while Pmk1 localises in the nucleus during appressoria development (Bruno *et al.*, 2004). Exp5 is possibly involved in the translocation of Pmk1 during this infection-related development. The inability of $\Delta exp5$ to form appressoria on coverslips supported this idea. Further experiments are needed to confirm the Exp5 links with this relevant pathogenesis-related signaling pathway.

The low expression levels of all the kaps under carbon and nitrogen starvation is an indication of the low metabolic activity carried out by the fungal cell. Under these conditions, a small increase in karyopherins expression is observed in $\Delta exp5$ compared to wild type standing out Pse1 and Kap104 from group II, and Kap123 and Cse1 from group III. The overexpression of Pse1, Kap123 and Cse1 in the $\Delta exp5$ mutant in CM and MM-C suggests that they potentially share common cargoes with Exp5. Supporting this, we found that Exp5 immunoprecipitates with these three kaps as well as with Rsl1 (group II) and Crm1 (group III). The mechanism of cargo recognition by kaps is very flexible allowing two karyopherins with very low sequence identity to transport the same cargo (Mühlhäusser, *et al.*, 2001, Mosammaparast & Pemberton, 2004, Chook & Süel, 2011). Some cargoes, specifically those with essential cell functions normally have several routes of transport through the nuclear envelope. Thus, Exp5 is possibly regulating the nucleocytoplasmic transport of several cargoes associated to these kaps. The GDP-to-GTP exchange factor Gsp1/Ran (MGG_09952) and the nucleoporin Ndc1 also immunoprecipitate with Exp5, corroborating the strength of the assay. However, we still need to confirm if all these proteins are directly recognised by Exp5.

Karyopherins are involved in the assembly of nucleopore channels and regulate the S-phase during the cell cycle (Forbes, *et al.*, 2015). Msn5, the yeast orthologue of Exp5 is involved in the transition of cells from the G1 to the S phase of the cell cycle (Queralt & Igual, 2003). Entry into the S-phase is critical for the regulation of the initiation of appressorium development in *M. oryzae*, and completion of mitosis during appressorium development is an essential prerequisite to plant infection (Saunders, *et al.*, 2010). The inability of $\Delta exp5$ to form appressoria on hydrophobic surfaces and its hyperbranching phenotype on PS-PHIL surfaces associates this protein with its potential defects in cell cycle. Further experiments will need to confirm this hypothesis. Evidence supporting the significance of cell cycle control in morphogenesis and fungal plant virulence comes from studies on the cell cycle-regulated autophagy process in *M. oryzae* (Veneault-Fourrey, *et al.*, 2006) and on the cyclin-dependent kinase Cdk5 that is necessary for polarized growth and pathogenicity in *Ustilago maydis* (Castillo-Lluva, *et al.*, 2007).

Karyopherins are not only nucleocytoplasmic transport receptors but they can function in microtubule regulation. It has been demonstrated that some kaps bind microtubule-associated proteins promoting the

inactivation of microtubule aster production during interphase, a process that regulates the entry into mitosis (Mosammaparast & Pemberton, 2004, Forbes, *et al.*, 2015). The observed interaction of Exp5 with the tubulin alpha and beta chains (MGG_11412, MGG_00604 and MGG_06650) suggests potential links with microtubule-related processes and associated proteins. In fact, Exp5 immunoprecipitates with Cdc48, a protein that is required for mitotic spindle disassembly in yeast (Cao, *et al.*). The potential interaction of Exp5 with actin (MGG_03982) and other proteins involved in the exocytosis/endocytosis pathway point to an additional involvement of Exp5 in processes that required short distance transport in the cell.

Fungal respiration has been showed to play a relevant role in root-colonizing fungi (Tamasloukht, *et al.*, 2003, Sesma & Osbourn, 2004). The $\Delta exp5$ mutant also shows severe defects in root colonisation. This is possibly due to its transport defects in mitochondrial proteins, some of which are directly involved in fungal respiration (Table 3.1), including several subunits of the ATP synthase, the NADPH P450 reductase and the NADH cytochrome b5 reductase 1. Tps1 is a key protein involved in primary metabolism in *M. oryzae* and immunoprecipitates with Exp5. This protein is regulates the carbon catabolite repression pathway in *M. oryzae* and acts as a sensor of glucose. The interaction of Exp5 with Tps1 and the expression of Exp5 in CM and carbon-starved cells hint a role of Exp5 in sensing and regulating carbon metabolism in the fungal cell.

The RNA-binding properties of the Exp5 orthologues are evolutionary conserved from humans to budding yeast, although there are significant differences in their substrate preferences (Shibata, *et al.*, 2006). Since *M. oryzae* Exp5 orthologues can transport tRNAs, dsRNAs and pre-miRNAs, we decided to study and compared the sRNA profiles between wild type and $\Delta exp5$ mutant strains. The sequencing of sRNA libraries has demonstrated that Exp5 also participates in sRNA metabolism and tRNA transport. All the sRNA populations are down-regulated in $\Delta exp5$ with the exception of transcripts derived from introns, intergenic regions and transposable elements. The sRNAs derived from tRNAs are also down-regulated in $\Delta exp5$. In *S. cerevisiae*, Msn5 has preference for the export of mature tRNAs (spliced and aminoacylated) coming from retrograde pathway while the karyopherin Los1 is in charge of the first transport of tRNAs (precursors). Once tRNAs reach the cytoplasm, they are processed in the mitochondrial membranes and re-enter into the nucleus (Takano, *et al.*, 2005, Shibata, *et al.*, 2006, Murthi, *et al.*, 2010, Phizicky & Hopper,

2010). This reverse transport regulates the quantity and quality of tRNAs in the cytoplasm, which subsequently regulates the translation of new proteins by an unknown mechanism. The nuclear accumulation of the precursor of tRNA^{Lys(CUU)} and the mature form of tRNA^{Met} in $\Delta exp5$ suggest that Exp5 is required for their export to the cytoplasm. This pathway is possibly dependent on Los1 since we have observed a 13-fold increase in *LOS1* gene expression in the $\Delta exp5$ mutant background in CM.

Transposable elements are important determinants of fungal genome evolution since they are drivers of macro-micro rearrangements, inducers of point mutations in surrounding coding or noncoding regions, and modifiers of gene expression (Beare, *et al.*, 2009, Rouxel, *et al.*, 2011, Grandaubert, *et al.*, 2014, Chiapello, *et al.*, 2015, Ai, *et al.*, 2016, Yoshida, *et al.*, 2016). The control of their expression is crucial for the correct function of the cell. The RNAi silencing machinery has been partially characterized in *M. oryzae*. *M. oryzae* genome contains two Dicer-like proteins, Mdl-1 and Mdl-2, three Argonautes and three RNA-dependent RNA polymerases (Kadotani, *et al.*, 2004, Nunes, *et al.*, 2011). The lack of Mdl-1 and Mdl-2 does not altered disease symptoms production, suggesting that the canonical RNAi pathway in *M. oryzae* does not play a major role during infection. In $\Delta exp5$ sRNAs derived from MAGGY and RETRO5 are up-regulated. The size of these sRNAs oscillate between 30 to 50 nt, which indicates that these fragments either are sRNA precursors that can act in retrotransposon silencing pathways or they are just degradation products. Further characterisation of the mutants lacking each of the components of the RNAi machinery will help to dissect the sRNA pathways present in this organism and their contribution to *M. oryzae* virulence.

MATERIAL AND METHODS

Fungal Strains, Growth Conditions and infections assays.

M. oryzae strains used in this study were the wild type strain Guy11 (Leung, *et al.*, 1988) and the deletion mutant $\Delta exp5$ (Tucker, *et al.*, 2010). *M. oryzae* strains were incubated in a controlled temperature room at 25°C with a 16h light/8h dark cycle. Growth media (complete medium-CM, defined complex medium-DCM), and leaf and root infection assays were carried out as previously described (Foster, *et al.*, 2003, Tucker, *et al.*, 2010, Franceschetti, *et al.*, 2011).

Cellular fractionation, RNA extractions and sRNA library preparation.

M. oryzae cellular fractionation was performed using a modified protocol from *Arabidopsis thaliana* (Khang, *et al.*, 2005). Briefly, cellular fractionation was carried out using grinded, frozen mycelia obtained from liquid CM cultured during 48h at 25°C 120rpm in darkness. The same volume of cell wall-disrupting buffer (10 mM potassium phosphate/pH7.0; 0.1M NaCl; 10 mM β -mercaptoethanol; 1M hexylene glycol/Sigma M-1408) was added to the mycelia. After centrifugation at 1,500 x g for 10 min at 4°C both, the supernatant and the pellet were recovered. Supernatant was re-centrifuged at 13,000 x g for 15 min at 4°C. The supernatant after the second centrifugation was saved as the cytoplasmic fraction. Pellet from the first centrifugation was washed with nuclei preparation buffer (10mM potassium phosphate/pH7.0; 0.1M NaCl; 10mM β -mercaptoethanol, 1M hexylene glycol; 10mM $MgCl_2$; 0.5% Triton X-100) and centrifuged at 1,500 x g for 10 min at 4°C. Washing and centrifugation were repeated 4-5 times, and the final pellet was saved as the nuclear fraction.

Total RNA isolation was done using a modified protocol of the LiCl method (Franceschetti, *et al.*, 2011). Two to three additional washes with phenol:chloroform were implemented to avoid RNA degradation and increase RNA purity. Using this protocol RNAs more than 500nt in length were detected in the precipitate, while sRNAs <500nt remained in the supernatant. To precipitate these sRNAs 2x volume of ethanol 100%, 1.5x volume of 7.5M Ammonium acetate and 2 μ l glycogen (5mg/ml) were added. Samples then were incubated during 1h at -80°C and centrifuged at 13.000 x g for 30 min at 4°C. The pellet was washed with EtOH 70% and resuspended with RNases-free water. A cleaning step with phenol-

chlorophorm 1:1 volumes was carried out, incubating samples for three minutes at room temperature. After centrifugation at 13.000 x g 30 min at 4°C, supernatant was taken and added 0.1x volume 3M sodium acetate pH 5.2, 2.5x volumes of ethanol 100% and glycogen (5mg/ml), leaved at -80°C for an 1h, and centrifuged at 13.000 x g for 30min at 4°C. The final pellet was resuspended in 50µl RNases-free water. A second method for RNA isolation was carried out using Trizol extraction buffer (Invitrogen). After total RNA was extracted from the sample, RNAs were treated with the mirVana kit (Ambion) for enrichment in sRNAs up to 200nt length. sRNAs were enriched using at least 10 µg of total RNA. Small cDNA library was performed as previously described (Sorefan, *et al.*, 2012).

Quantitative polymerase chain reaction (qPCR).

One µg of total RNA was reverse transcribed using PrimeScript™ RT reagent Kit (Takara). The average threshold cycle (Ct) was normalized against actin transcript and relative quantification of gene expression was calculated using the $2^{\Delta\Delta C_t}$ method (Pfaffl, 2001). Primer efficiency was tested using dilutions of cDNA samples. qPCR reactions were carried out with 1 µl of reverse transcribed products and fast-start DNA master SYBR green I kit (Roche Diagnostics) in a final reaction of 10 µl using the following program: one cycle at 95°C for 4 min, followed by 40 cycles at 94°C for 30 s and 60°C for 30 s. The Ct (threshold cycle) provided a measure for the starting copy numbers of the target genes. Three technical repetitions from three independent biological experiments were used for each gene.

Bioinformatic analysis.

FASTQ files were processed using the FASTX-toolkit (Blankenberg, *et al.*, 2010). The sRNA TrueSeq adapter was removed with the fastx_clipper tool, reads were filtered based on quality score with fastq_quality_filter (arguments -q 30 -p 70) and artifacts were removed with fastx_artifacts_filter. HD adapters were removed from reads trimming 4 nt on both ends. Reads longer than 17 nt were aligned on *M. oryzae* reference genome (Dean, *et al.*, 2005) using bowtie (Langmead, *et al.*, 2009), allowing no mismatches. The sRNA transcriptome was assembled using the Inchworm software module from the Trinity package (Haas, *et al.*, 2013), setting 21 as kmer length. Differential expression was performed with DEseq2 (Love, *et al.*, 2014).

Northern blotting assays of sRNAs.

10 µg of non-fractionated and cytoplasmic sRNAs, and 7.5 µg of nuclear sRNAs obtained from both sRNA isolation methods used from Guy11 and *Δexp5* mutant were run on a denaturing 15% polyacrylamide gel with 7M urea, and transferred onto a Hybond-NX nylon membrane and fixed by chemical crosslinking. Complementary DNA oligonucleotide probes (Sigma) to targeted sRNAs were labelled with [γ -³²P]-ATP using T4 PNK (Roche) and detected using phosphorimager screens (Fujifilm). The Biorad molecular software, Image Lab. 3.0.1. (Beta 2), was used for signal visualization, and ImageJ software was used for quantification of signal strength and image processing (<http://rsb.info.nih.gov/ij/>).

Complementation of *Δexp5* with HA-FLAG tag.

Amino and carboxy translational fusion constructs of *Δexp5/EXP5:HA-FLAG* were generated by Multisite Gateway cloning as follows. A 1.21-kb fragment containing the 5'UTR of *EXP5* (exp5-GW1) was amplified using primers containing the attB4 and attB1R sites. A 5.14-kb fragment containing *EXP5* ORF and 3'-untranslated region (exp5-GW2) was amplified with primers containing attB2R and attB3 sites. A 5.62-kb fragment consisting of the 5' UTR and ORF (omitting the stop codon) of *EXP5* (exp5-GW3) was amplified with primers containing attB4 and attB1R sites, and a 0.73-kb fragment of the 3'-untranslated region (exp5-GW4) was amplified with primers containing attB2R and attB3 sites. Fragments exp5-GW1 and exp5-GW3 were cloned into pDONR P4-P1R (Invitrogen), and fragments exp5-GW2 and exp5-GW4 were cloned into pDONR P2R-P3 (Invitrogen). To complement the *Δexp5* with the *HA-FLAG:EXP5* amino fusion construct, plasmids containing exp5-GW1 (5' entry), exp5-GW2 (3' entry), and HA-FLAG without a stop codon (middle) were cloned into the binary vector pSULPH-R3R4 (Tucker, *et al.*, 2010). To generate the *EXP5:FLAG-HA* carboxy fusion construct, plasmids containing exp5-GW3 (5' entry), exp5-GW4 (3' entry), and HA-FLAG with a stop codon (middle) were cloned into the same binary vector. The two final plasmids were introduced into *A. tumefaciens* AGL1 and used to transform the *Δexp5* strain with *A. tumefaciens* mediated transformation (ATMT) (Tucker, *et al.*, 2010).

Protein extractions and tandem affinity protein purification.

10 days-old mycelia grown on CM plates were cut out from the agar, homogenised in a food Blender (Waring Commercial, USA) with 150 ml of liquid CM media, placed in 250 ml flasks and incubated in darkness on a shaker at 25°C/120 rpm for 2 days. Mycelia (1–3 g of wet weight) were collected by filtering through a double layer of miracloth, washed with sterile water and grounded in liquid nitrogen. Proteins were extracted from 300–400 mg of mycelia (wet weight) using 760 ml of extraction buffer (50 mM Tris pH 7.5, 5 mM EDTA, 1% Triton x-100, 10% glycerol, 2 mM Phenylmethanesulfonyl fluoride) and 20 ml/ml Protease Inhibitor Cocktail (PIC, Sigma). Cell debris were removed by centrifugation for 30 min at 4°C at 20000 x g. For Westerns, 40 µg of total protein extract were used per lane. The following antibodies and dilutions were used for Western blotting: anti-FLAG (Sigma; 1:10000); anti-mRFP (Abcam; 1:5000) and anti-HA (Abcam; 1:10000).

For purification of Exp5:FLAG-HA, 4 g of mycelia from liquid culture were homogenised in 15 ml of IP buffer (50 mM Tris HCl pH 7.5, 5 mM MgCl₂, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 2% PIC). Extracts were centrifuged at 20 x g for 30 min, at 4°C and 12 ml of supernatant were incubated with 0.350 ml of anti-FLAG M2 magnetic beads (Sigma) and rinsed with TBS (50 mM Tris–HCl, pH 7.4; 150 mM NaCl, 1 mM PMSF; 2% PIC) for 2 h at 4°C with rotation. Beads were collected using a magnetic stand and washed extensively with TBS. Bound proteins were eluted by competition with 0.875 ml of FLAG peptide (150 ng/ml, Sigma), incubated with 0.150 ml of HA resin (Sigma), and rinsed with RIPA buffer (150 mM NaCl, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris HCl pH 8, 1 mM PMSF, 2% PIC) for 1.5 h at 4°C with rotation. After transferring to SigmaPrep spin columns, resins were washed with RIPA buffer and proteins eluted by incubation with 0.180 ml of 50 mM Tris pH 7.5, 2% SDS at 65°C for 15 min. Eluted proteins were sent to the CNB proteomic facility for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Mass spectrometry data were aligned with the predicted set of *M. oryzae* proteins and parameters set to False discovery rate (FDR>20) from protein match and a minimum of two unique peptide matches.

REFERENCES

- Albertini M, Pemberton LF, Rosenblum JS & Blobel G (1998)** A novel nuclear import pathway for the transcription factor TFIIS. *Journal of Cell Biology* **143**: 1447-1455.
- Bakhrat A, Baranes-Bachar K, Reshef D, Voloshin O, Krichevsky O & Raveh D (2008)** Nuclear export of Ho endonuclease of yeast via Msn5. *Current Genetics* **54**: 271-281.
- Bardwell L, Cook JG, Voora D, Baggott DM, Martinez AR & Thorner J (1998)** Repression of yeast Ste12 transcription factor by direct binding of unphosphorylated Kss1 MAPK and its regulation by the Ste7 MEK. *Genes & Development* **12**: 2887-2898.
- Ben Chaabane S, Liu R, Chinnusamy V, et al. (2013)** STA1, an Arabidopsis pre-mRNA processing factor 6 homolog, is a new player involved in miRNA biogenesis. *Nucleic Acids Research* **41**: 1984-1997.
- Besi M, Tucker SL & Sesma A (2009)** *Magnaporthe* and its relatives. ed.^eds.), p.^pp. John Wiley & Sons, Ltd: Chichester.
- Blankenberg D, Gordon A, Von Kuster G, Coraor N, Taylor J, Nekrutenko A & Team tG (2010)** Manipulation of FASTQ data with Galaxy. *Bioinformatics* **26**: 1783-1785.
- Blondel M, Alepuz PM, Huang LS, Shaham S, Ammerer G & Peter M (1999)** Nuclear export of Far1p in response to pheromones requires the export receptor Msn5p/Ste21p. *Genes and Development* **13**: 2284-2300.
- Bohnsack MT, Czaplinski K & Gorlich D (2004)** Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. *RNA* **10**: 185-191.
- Bohnsack MT, Regener K, Schwappach B, Saffrich R, Paraskeva E, Hartmann E & Gorlich D (2002)** Exp5 exports eEF1A via tRNA from nuclei and synergizes with other transport pathways to confine translation to the cytoplasm. *Embo Journal* **21**: 6205-6215.
- Bollman KM, Aukerman MJ, Park M-Y, Hunter C, Berardini TZ & Poethig RS (2003)** HASTY, the Arabidopsis ortholog of exportin 5/MSN5, regulates phase change and morphogenesis. *Development* **130**: 1493-1504.
- Borukhov S, Sagitov V & Goldfarb A (1993)** Transcript cleavage factors from *E. coli*. *Cell* **72**: 459-466.
- Boustany LM & Cyert MS (2002)** Calcineurin-dependent regulation of Crz1p nuclear export requires Msn5p and a conserved calcineurin docking site. *Genes and Development* **16**: 608-619.
- Brownawell AM & Macara IG (2002)** Exportin-5, a novel karyopherin, mediates nuclear export of double-stranded RNA binding proteins. *Journal of Cell Biology* **156**: 53-64.
- Calado A, Treichel N, Muller EC, Otto A & Kutay U (2002)** Exportin-5-mediated nuclear export of eukaryotic elongation factor 1A and tRNA. *Embo Journal* **21**: 6216-6224.
- Castillo-Lluva S, Alvarez-Tabares I, Weber I, Steinberg G & Perez-Martin J (2007)** Sustained cell polarity and virulence in the phytopathogenic fungus *Ustilago maydis* depends on an essential cyclin-dependent kinase from the Cdk5/Pho85 family. *Journal of Cell Science* **120**: 1584-1595.

- Cautain B, Hill R, Pedro N & Link W (2015)** Components and regulation of nuclear transport processes. *Febs Journal* **282**: 445-462.
- Cook A, Bono F, Jinek M & Conti E (2007)** Structural Biology of Nucleocytoplasmic Transport. *Annual Review of Biochemistry* **76**: 647-822.
- Czech B & Hannon GJ (2011)** Small RNA sorting: matchmaking for Argonautes. *Nature Reviews Genetics* **12**: 19-31.
- Chen J, Zheng W, Zheng S, et al. (2008)** Rac1 Is Required for Pathogenicity and Chm1-Dependent Conidiogenesis in Rice Fungal Pathogen *Magnaporthe grisea*. *PLoS Pathogens* **4**: e1000202.
- Choi D-H, Kwon S-H, Kim J-H & Bae S-H (2012)** *Saccharomyces cerevisiae* Cmr1 protein preferentially binds to UV-damaged DNA in vitro. *Journal of Microbiology* **50**: 112-118.
- Chook YM & Süel KE (2011)** Nuclear import by Karyopherin- β s: recognition and inhibition. *Biochimica et biophysica acta* **1813**: 1593-1606.
- Dean RA, Talbot NJ, Ebbole DJ, et al. (2005)** The genome sequence of the rice blast fungus *Magnaporthe grisea*. *Nature* **434**: 980-986.
- DeVit MJ & Johnston M (1999)** The nuclear exportin Msn5 is required for nuclear export of the Mig1 glucose repressor of *Saccharomyces cerevisiae*. *Current Biology* **9**: 1231-1242.
- Doucet CM, Talamas JA & Hetzer MW (2010)** Cell Cycle-Dependent Differences in Nuclear Pore Complex Assembly in Metazoa. *Cell* **141**: 1030-1041.
- Dufresne M & Osbourn AE (2001)** Definition of tissue-specific and general requirements for plant infection in a phytopathogenic fungus. *Molecular Plant-Microbe Interactions* **14**: 300-307.
- Errede B & Ammerer G (1989)** STE12, a protein involved in cell-type-specific transcription and signal transduction in yeast, is part of protein-DNA complexes. *Genes & Development* **3**: 1349-1361.
- Farman ML, Tosa Y, Nitta N & Leong SA (1996)** MAGGY, a retrotransposon in the genome of the rice blast fungus *Magnaporthe grisea*. *Molecular and General Genetics MGG* **251**: 665-674.
- Farman ML, Eto Y, Nakao T, Tosa Y, Nakayashiki H, Mayama S & Leong SA (2002)** Analysis of the Structure of the AVR1-CO39 Avirulence Locus in Virulent Rice-Infecting Isolates of *Magnaporthe grisea*. *Molecular Plant-Microbe Interactions* **15**: 6-16.
- Ferrigno P, Posas F, Koepp D, Saito H & Silver PA (1998)** Regulated nucleo/cytoplasmic exchange of HOG1 MAPK requires the importin beta homologs NMD5 and XPO1. *The EMBO Journal* **17**: 5606-5614.
- Forbes DJ, Travesa A, Nord M & Bernis C (2015)** Nuclear Transport Factors: Global Regulation of Mitosis. *Current opinion in cell biology* **35**: 78-90.
- Foster AJ, Jenkinson JM & Talbot NJ (2003)** Trehalose synthesis and metabolism are required at different stages of plant infection by *Magnaporthe grisea*. *EMBO Journal* **22**: 225-235.
- Franceschetti M, Bueno E, Wilson RA, Tucker SL, Gómez-Mena C, Calder G & Sesma A (2011)** Fungal Virulence and Development Is Regulated by Alternative Pre-mRNA 3' End Processing in *Magnaporthe oryzae*. *PLoS Pathogens* **7**: e1002441.

- Gallina I, Colding C, Henriksen P, et al. (2015)** Cmr1/WDR76 defines a nuclear genotoxic stress body linking genome integrity and protein quality control. *Nature Communications* **6**: 6533.
- Gorlich D & Kutay U (1999)** Transport between the Cell Nucleus and the Cytoplasm. *Annual Review of Cell and Developmental Biology* **15**: 607-660.
- Gorner W, Durchschlag E, Wolf J, Brown EL, Ammerer G, Ruis H & Schuller C (2002)** Acute glucose starvation activates the nuclear localization signal of a stress-specific yeast transcription factor. *Embo Journal* **21**: 135-144.
- Gulshan K, Thommandru B & Moye-Rowley WS (2012)** Proteolytic Degradation of the Yap1 Transcription Factor Is Regulated by Subcellular Localization and the E3 Ubiquitin Ligase Not4. *The Journal of Biological Chemistry* **287**: 26796-26805.
- Gwizdek C, Ossareh-Nazari B, Brownawell AM, Evers S, Macara IG & Dargemont C (2004)** Minihelix-containing RNAs mediate exportin-5-dependent nuclear export of the double-stranded RNA-binding protein ILF3. *Journal of Biological Chemistry* **279**: 884-891.
- Haas BJ, Papanicolaou A, Yassour M, et al. (2013)** De novo transcript sequence reconstruction from RNA-Seq: reference generation and analysis with Trinity. *Nature protocols* **8**: 10.1038/nprot.2013.1084.
- Harel A & Forbes DJ (2004)** Importin Beta. *Molecular Cell* **16**: 319-330.
- Hood JK & Silver PA (1998)** Cse1p Is Required for Export of Srp1p/Importin- α from the Nucleus in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **273**: 35142-35146.
- Hopper AK (2013)** Transfer RNA Post-Transcriptional Processing, Turnover, and Subcellular Dynamics in the Yeast *Saccharomyces cerevisiae*. *Genetics* **194**: 43-67.
- Huang H-Y & Hopper AK (2015)** In vivo biochemical analyses reveal distinct roles of β -importins and eEF1A in tRNA subcellular traffic. *Genes & Development* **29**: 772-783.
- Izban MGaL, D S (1991)** Transcription on nucleosomal templates by RNA polymerase II in vitro: inhibition of elongation with enhancement of sequence-specific pausing. *Genes & Dev.* **5**: 683-696.
- Jeon J, Park S-Y, Chi M-H, et al. (2007)** Genome-wide functional analysis of pathogenicity genes in the rice blast fungus. *Nature genetics* **39**: 561-565.
- Kaffman A, Rank NM, O'Neill EM, Huang LS & O'Shea EK (1998)** The receptor Msn5 exports the phosphorylated transcription factor Pho4 out of the nucleus. *Nature* **482**: 482-485.
- Khang CH, Park SY, Lee YH & Kang S (2005)** A dual selection based, targeted gene replacement tool for *Magnaporthe grisea* and *Fusarium oxysporum*. *Fungal Genetics and Biology* **42**: 483-492.
- Kimura M & Imamoto N (2014)** Biological Significance of the Importin-beta Family-Dependent Nucleocytoplasmic Transport Pathways. *Traffic* **15**: 727-748.
- Komeili A, Wedaman KP, O Shea EK & Powers T (2000)** Mechanism of metabolic control: Target of rapamycin signaling links nitrogen quality to the activity of the Rtg1 and Rtg3 transcription factors. *Journal of Cell Biology* **151**: 863-878.
- Künzler M & Hurt EC (1998)** Cse1p functions as the nuclear export receptor for importin α in yeast. *FEBS Letters* **433**: 185-190.

- Langmead B, Trapnell C, Pop M & Salzberg SL (2009)** Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biology* **10**: R25-R25.
- Leung H, Borromeo E, Bernardo M & Notteghem JL (1988)** Genetic analysis of virulence in the rice blast fungus *Magnaporthe grisea*. *Phytopathology* **78**: 1227-1233.
- Lonhienne TG, Forwood JK, Marfori M, Robin G, Kobe B & Carroll BJ (2009)** Importin- β Is a GDP-to-GTP Exchange Factor of Ran: IMPLICATIONS FOR THE MECHANISM OF NUCLEAR IMPORT. *Journal of Biological Chemistry* **284**: 22549-22558.
- Love MI, Huber W & Anders S (2014)** Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology* **15**: 1-21.
- Lund E, Guttinger S, Calado A, Dahlberg JE & Kutay U (2004)** Nuclear export of microRNA precursors. *Science* **303**: 95-98.
- MacKinnon MA, Curwin AJ, Gaspard GJ, Suraci AB, Fernández-Murray JP & McMaster CR (2009)** The Kap60-Kap95 Karyopherin Complex Directly Regulates Phosphatidylcholine Synthesis. *Journal of Biological Chemistry* **284**: 7376-7384.
- Mahanty SK, Wang Y, Farley FW & Elion EA (1999)** Nuclear Shuttling of Yeast Scaffold Ste5 Is Required for Its Recruitment to the Plasma Membrane and Activation of the Mating MAPK Cascade. *Cell* **98**: 501-512.
- Mamnun YM, Pandjaitan R, Mahé Y, Delahodde A & Kuchler K (2002)** The yeast zinc finger regulators Pdr1p and Pdr3p control pleiotropic drug resistance (PDR) as homo- and heterodimers in vivo. *Molecular Microbiology* **46**: 1429-1440.
- Maurer P, Redd M, Solsbacher J, et al. (2001)** The Nuclear Export Receptor Xpo1p Forms Distinct Complexes with NES Transport Substrates and the Yeast Ran Binding Protein 1 (Yrb1p). *Molecular Biology of the Cell* **12**: 539-549.
- Mosammaparast N & Pemberton LF (2004)** Karyopherins: from nuclear-transport mediators to nuclear-function regulators. *Trends in Cell Biology* **14**: 547-556.
- Mühlhäusser P, Müller E-C, Otto A & Kutay U (2001)** Multiple pathways contribute to nuclear import of core histones. *EMBO Reports* **2**: 690-696.
- Muqbil I, Bao B, Abou-Samra AB, Mohammad RM & Azmi AS (2013)** Nuclear Export Mediated Regulation of MicroRNAs: Potential Target for Drug Intervention. *Current drug targets* **14**: 1094-1100.
- Murata T, Kadotani N, Yamaguchi M, Tosa Y, Mayama S & Nakayashiki H (2007)** siRNA-dependent and -independent post-transcriptional cosuppression of the LTR-retrotransposon MAGGY in the phytopathogenic fungus *Magnaporthe oryzae*. *Nucleic Acids Research* **35**: 5987-5994.
- Murthi A, Shaheen HH, Huang HY, Preston MA, Lai TP, Phizicky EM & Hopper AK (2010)** Regulation of tRNA Bidirectional Nuclear-Cytoplasmic Trafficking in *Saccharomyces cerevisiae*. *Molecular Biology of the Cell* **21**: 639-649.
- Park MY, Wu G, Gonzalez-Sulser A, Vaucheret H & Poethig RS (2005)** Nuclear processing and export of microRNAs in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **102**: 3691-3696.

- Park W, Li J, Song R, Messing J & Chen X (2005)** CARPEL FACTORY, a Dicer Homolog, and HEN1, a Novel Protein, Act in microRNA Metabolism in *Arabidopsis thaliana*. *Current Biology* **12**: 1484-1495.
- Pfaffl MW (2001)** A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29**: e45.
- Phizicky EM & Hopper AK (2010)** tRNA biology charges to the front. *Genes & Development* **24**: 1832-1860.
- Pierce JB, Chafe SC, Eswara MBK, van der Merwe G & Mangroo D (2014)** Chapter 19 - Strategies for Investigating Nuclear–Cytoplasmic tRNA Dynamics in Yeast and Mammalian Cells. *Methods in Cell Biology*, Vol. 122 (Valérie D, ed.) p. 415-436. Academic Press.
- Quan X, Tsoulos P, Kuritzky A, Zhang R & Stochaj U (2006)** The carrier Msn5p/Kap142p promotes nuclear export of the hsp70 Ssa4p and relocates in response to stress. *Molecular Microbiology* **62**: 592-609.
- Queralt E & Igual JC (2003)** Cell Cycle Activation of the Swi6p Transcription Factor Is Linked to Nucleocytoplasmic Shuttling. *Molecular and Cellular Biology* **23**: 3126-3140.
- Rappaport J, Cho K, Saltzman A, Prenger J, Golomb M & Weinmann R (1988)** Transcription Elongation Factor SII Interacts with a Domain of the Large Subunit of Human RNA Polymerase II. *Molecular and Cellular Biology* **8**: 3136-3142.
- Roberts RL & Fink GR (1994)** Elements of a single MAP kinase cascade in *Saccharomyces cerevisiae* mediate two developmental programs in the same cell type: mating and invasive growth. *Genes & Development* **8**: 2974-2985.
- Ryo Ueta AFaYY-I (2003)** Pse1p Mediates the Nuclear Import of the Iron-responsive Transcription Factor Aft1p in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **278**: 50120-50127.
- Saunders DG, Dagdas YF & Talbot NJ (2010)** Spatial uncoupling of mitosis and cytokinesis during appressorium-mediated plant infection by the rice blast fungus *Magnaporthe oryzae*. *Plant Cell* **22**: 2417-2428.
- Senger B, Simos G, Bischoff FR, Podtelejnikov A, Mann M & Hurt E (1998)** Mtr10p functions as a nuclear import receptor for the mRNA-binding protein Npl3p. *Embo Journal* **17**: 2196-2207.
- Sesma A (2016)** RNA metabolism and regulation of virulence programs in fungi. *Seminars in Cell & Developmental Biology* **57**: 120-127.
- Sesma A & Osbourn AE (2004)** The rice leaf blast pathogen undergoes developmental processes typical of root-infecting fungi. *Nature* **431**: 582-586.
- Shaheen HH & Hopper AK (2005)** Retrograde movement of tRNAs from the cytoplasm to the nucleus in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences of the United States of America* **102**: 11290-11295.
- Shibata S, Sasaki M, Miki T, Shimamoto A, Furuichi Y, Katahira J & Yoneda Y (2006)** Exportin-5 orthologues are functionally divergent among species. *Nucleic Acids Research* **34**: 4711-4721.
- Sloan KE, Gleizes P-E & Bohnsack MT (2016)** Nucleocytoplasmic Transport of RNAs and RNA–Protein Complexes. *Journal of Molecular Biology* **428**: 2040-2059.

- Solsbacher J, Maurer P, Bischoff FR & Schlenstedt G (1998)** Cse1p Is Involved in Export of Yeast Importin α from the Nucleus. *Molecular and Cellular Biology* **18**: 6805-6815.
- Sorefan K, Pais H, Hall AE, Kozomara A, Griffiths-Jones S, Moulton V & Dalmay T (2012)** Reducing ligation bias of small RNAs in libraries for next generation sequencing. *Silence* **3**: 4-4.
- Sydorsky Y, Dilworth DJ, Yi EC, Goodlett DR, Wozniak RW & Aitchison JD (2003)** Intersection of the Kap123p-Mediated Nuclear Import and Ribosome Export Pathways. *Molecular and Cellular Biology* **23**: 2042-2054.
- Takano A, Endo T & Yoshihisa T (2005)** tRNA Actively Shuttles Between the Nucleus and Cytosol in Yeast. *Science* **309**: 140-142.
- Tamasloukht M, Sejalou-Delmas N, Kluever A, Jauneau A, Roux C, Becard G & Franken P (2003)** Root factors induce mitochondrial-related gene expression and fungal respiration during the developmental switch from asymbiosis to presymbiosis in the arbuscular mycorrhizal fungus *Gigaspora rosea*. *Plant Physiology* **131**: 1468-1478.
- Terry LJ, Shows EB & Wente SR (2007)** Crossing the Nuclear Envelope: Hierarchical Regulation of Nucleocytoplasmic Transport. *Science* **318**: 1412-1416.
- Tkach JM, Yimit A, Lee AY, et al. (2012)** Dissecting DNA damage response pathways by analyzing protein localization and abundance changes during DNA replication stress. *Nature cell biology* **14**: 966-976.
- Tucker SL, Besi MI, Galhano R, et al. (2010)** Common Genetic Pathways Regulate Organ-Specific Infection-Related Development in the Rice Blast Fungus. *Plant Cell* **22**: 953-972.
- Turrà D, Segorbe D & Pietro AD (2014)** Protein Kinases in Plant-Pathogenic Fungi: Conserved Regulators of Infection. *Annual Review of Phytopathology* **52**: 267-288.
- Ueta R, Fujiwara N, Iwai K & Yamaguchi-Iwai Y (2012)** Iron-Induced Dissociation of the Aft1p Transcriptional Regulator from Target Gene Promoters Is an Initial Event in Iron-Dependent Gene Suppression. *Molecular and Cellular Biology* **32**: 4998-5008.
- Valent B & Chumley FG (1991)** Molecular Genetic Analysis of the Rice Blast Fungus, *Magnaporthe grisea*. *Annual Review of Phytopathology* **29**: 443-467.
- van den Bogaart G, Meinema AC, Krasnikov V, Veenhoff LM & Poolman B (2009)** Nuclear transport factor directs localization of protein synthesis during mitosis. *Nat Cell Biol* **11**: 350-356.
- Veneault-Fourrey C, Barooah M, Egan M, Wakley G & Talbot NJ (2006)** Autophagic Fungal Cell Death Is Necessary for Infection by the Rice Blast Fungus. *Science* **312**: 580-583.
- Whitney ML, Hurto RL, Shaheen HH & Hopper AK (2007)** Rapid and Reversible Nuclear Accumulation of Cytoplasmic tRNA in Response to Nutrient Availability. *Molecular Biology of the Cell* **18**: 2678-2686.
- Wild T, Horvath P, Wyler E, et al. (2010)** A Protein Inventory of Human Ribosome Biogenesis Reveals an Essential Function of Exportin 5 in 60S Subunit Export. *Plos Biology* **8**: e1000522.
- Wu J, Bao A, Chatterjee K, Wan Y & Hopper AK (2015)** Genome-wide screen uncovers novel pathways for tRNA processing and nuclear-cytoplasmic dynamics. *Genes & Development* **29**: 2633-2644.

- Xiao Z, McGrew JT, Schroeder AJ & Fitzgerald-Hayes M (1993)** CSE1 and CSE2, two new genes required for accurate mitotic chromosome segregation in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* **13**: 4691-4702.
- Xu JR, Staiger CJ & Hamer JE (1998)** Inactivation of the mitogen-activated protein kinase Mps1 from the rice blast fungus prevents penetration of host cells but allows activation of plant defense responses. *Proceedings of the National Academy of Sciences, USA* **95**: 12713-12718.
- Yoshida K & Blobel G (2001)** The karyopherin Kap142p/Msn5p mediates nuclear import and nuclear export of different cargo proteins. *Journal of Cell Biology* **152**: 729-739.
- Yuksel UM, Turker I, Dilek G, Dogan L, Gulcelik MA & Oksuzoglu B (2015)** Does CSE1L Overexpression Affect Distant Metastasis Development in Breast Cancer? *Oncology Research and Treatment* **38**: 431-434.
- Zeng Y & Cullen BR (2004)** Structural requirements for pre-microRNA binding and nuclear export by Exportin 5. *Nucleic Acids Research* **32**: 4776-4785.

Table 3.2. List of proteins that interact with Exp5:HA-Flag by immunoprecipitation (IP) assays. ^a IP experiments were carried out by triplicate; ^b peptide number; ^c percentage of the protein sequence covered by matching peptides; ^d The mean peptide number has been used to rank proteins that interact with Exp5:HA-Flag. This number is the result of subtracting the sum of the peptides of a given protein obtained from $\Delta exp5/Exp5:HA-FLAG$ and the sum of peptides obtained from the negative control (WT strain). *Proteins selected for further analysis.

EnsemBL_Acc	Neg. Cont.	Exp5-HA-FLAG (1) ^a		Neg. Cont.	Exp5-HA-FLAG (2)		Neg. Cont.	Exp5-HA-FLAG (3)		Mean Pept. Num. ^d	Function	Pathogenicity
	Pept. Num. ^b	Pept. Num.	Coverage ^c	Pept. Num.	Pept. Num.	Coverage	Pept. Num.	Pept. Num.	Coverage			
Nucleo-cytoplasmic transport (7+1)												
MGG_09560	5	90	48.3	2	82	42.4	2	110	48.6	91.0	Exp5; PHI:2167	Reduced virulence
MGG_11165	4	14	11.8	-	11	10.6	-	5	5.1	8.7	kap Cse1	
MGG_09952	5	9	37	3	6	30.6	3	7	31.5	3.7	RanGTP; GTP-binding nuclear protein Gsp1/Ran	
MGG_03537	1	7	5.9	-	4	4	-	1	1	3.6	kap Pse1	
MGG_02526	-	6	4.8	-	2	2.4	-	2	1.9	3.3	kap Crm1	
MGG_16711	4	8	8.1	1	3	4.1	1	4	3.8	3.0	Kap123	
MGG_17732	-	5	8.4	-	1	1.1	-	1	1.6	2.3	nucleoporin protein Ndc1	
MGG_03668	1	5	5.7	-	1	1.1	-	1	1.1	2.0	kap Rsl1	
General metabolism (95)												
MGG_10912	6	26	11.7	1	35	17.5	1	15	6.9	22.7	Mycocerosic acid synthase	
MGG_07613	3	20	8.7	-	12	5.5	-	4	2	11.0	Acetyl-CoA carboxylase	
MGG_04550	1	12	14.5	-	15	16.9	-	7	8.8	11.0	Calcium-translocating P-type ATPase	
MGG_02471	10	16	22.3	3	18	24.2	3	13	17	10.3	Transketolase	
MGG_05247	3	9	9	2	13	13	2	10	10.2	8.3	NAD-specific glutamate dehydrogenase	
MGG_04994	5	14	14.3	-	8	9.1	-	6	5.5	7.7	Plasma membrane H ⁺ -ATPase	
MGG_12805	3	9	14.9	-	9	17.9	-	7	15.2	7.3	Tryptophan synthase	
MGG_07216	8	14	49.3	1	11	43.1	1	6	24.1	7.0	Versicolorin reductase	
MGG_04503	-	11	8.6	-	4	4.3	-	4	4	6.3	Carbamoyl-phosphate synthase subunit arginine-specific large (metabolic process)	
MGG_06719	10	10	18.9	3	11	19.1	3	14	29.5	6.3	ATP-citrate synthase subunit 1	
MGG_07200	8	16	18.4	10	15	15.4	10	16	15.9	6.3	Plasma membrane ATPase	
MGG_09007	13	15	44.5	6	14	47.2	6	14	44.5	6.0	Xyl2p (1e-04); alcohol dehydrogenase zinc-type	
MGG_02503	14	16	29.8	6	15	25.9	6	13	24.2	6.0	Glucose-regulated protein	
MGG_10910	5	7	30.8	3	11	42	3	11	42.8	6.0	Aflatoxin biosynthesis ketoreductase nor-1	
MGG_15774	3	10	22	-	9	21.5	-	3	8	6.3	Ketol-acid reductoisomerase	
MGG_03915	-	6	31	-	4	23	-	7	39.1	5.7	Pyridoxamine phosphate oxidase	
MGG_01092	2	6	15.3	-	7	15.5	-	6	14	5.7	Homocitrate synthase; PHI:3234	Reduced virulence
MGG_07626	4	10	19.9	-	5	10.7	-	6	14.1	5.7	Cytochrome P450 monooxygenase	
MGG_11597	8	11	20	4	14	22	4	8	15	5.7	Glucosamine-fructose-6-phosphate aminotransferase	
MGG_02624	7	10	34	3	11	41.4	3	9	30.9	5.7	Transaldolase	
MGG_12752	-	8	20.9	-	4	14	-	4	12.6	5.3	Epidermal retinal dehydrogenase 2	
MGG_07756	5	12	11	1	7	6.8	1	4	3.8	5.3	Pyruvate carboxylase	
MGG_01566	2	8	28.1	-	6	17.2	-	3	8.3	5.0	Homoisocitrate dehydrogenase	
MGG_01790	4	8	16.6	-	6	10.5	-	5	12.4	5.0	Acyl-CoA desaturase	
MGG_10607	7	10	33.8	5	8	27.6	5	14	39.5	5.0	Enolase	
MGG_09471	4	10	13.3	-	6	9.6	-	3	4.5	5.0	NTH1; Neutral trehalase; PHI:123; PHI:775; PHI:794	Reduced virulence
MGG_03860	4	6	12.7	-	8	18	-	5	12.5	5.0	TPS1; trehalose-phosphate synthase 1; PHI:1064; PHI:2172; PHI:322	Reduced virulence
MGG_06868	5	9	11.6	-	6	8.2	-	5	9.1	5.0	Acetolactate synthase catalytic subunit	
MGG_05046	-	12	12.9	-	1	0.8	-	1	0.8	4.7	Adenine phosphoribosyltransferase (purin biosynthesis)	
MGG_00256	4	7	13.4	-	7	14.8	-	4	7.5	4.7	Cytosolic non-specific dipeptidase	
MGG_16813	7	12	34.4	1	5	15.2	1	6	20.1	4.7	NADPH-dependent oxidoreductase	

MGG_00383	7	9	22.8	1	9	20.8	1	5	14.5	4.7	S-adenosylmethionine synthase; PHI:877	Reduced virulence
MGG_16230	5	5	9.4	1	10	26.5	1	6	17.3	4.7	Succinyl-CoA ligase subunit beta	
MGG_04435	2	5	10.6	-	7	15.5	-	4	9.1	4.7	Bifunctional purine biosynthesis protein ADE17	
MGG_01288	2	4	12.6	-	5	17.9	-	6	22	4.3	Mannose-1-phosphate guanylttransferase	
MGG_02252	4	7	26.5	3	10	33.2	3	6	23.7	4.3	Tetrahydroxynaphthalene reductase; PHI:2022; PHI:685	Avirulent
MGG_00135	6	9	16.3	1	7	15.2	1	5	10.6	4.3	T-complex protein 1	
MGG_05078	-	10	9.9	-	1	1	-	1	1.3	4.0	Potassium/sodium efflux P-type ATPase	
MGG_01084	12	12	39	11	17	49.7	11	17	47	4.0	glyceraldehyde-3-phosphate deshydrogenase (65% identity with TDH1/2/3 of S. cerevisiae; cytoplasm and cell wall located)	
MGG_06639	7	11	20.5	-	6	12	-	2	4.8	4.0	T-complex protein 1 subunit gamma	
MGG_00923	5	5	12	2	10	23.7	2	6	13.3	4.0	Serine hydroxymethyltransferase	
MGG_08063	4	7	13.8	2	8	19.7	2	5	11.2	4.0	Pyruvate kinase	
MGG_01995	8	10	25.4	2	6	15.8	2	7	18.1	3.7	Isocitrate dehydrogenase subunit 1	
MGG_15700	1	5	9.6	-	4	8.9	-	3	5.6	3.7	glucose transporter; plasma membrane high glucose sensor that regulates glucose transport; Rgt2 / Snf3 (e-90)	
MGG_04432	2	6	13.5	-	5	10.3	-	2	4.4	3.7	Cytochrome P450 51; PHI:2191	Virulent
MGG_07268	6	9	17.6	1	5	14.2	1	5	9.6	3.7	Isocitrate dehydrogenase	
MGG_10569	3	6	18.3	1	5	11.7	1	5	14.4	3.7	Pyruvate dehydrogenase E1 component subunit beta	
MGG_03441	-	3	2.5	-	6	6.8	-	1	1.3	3.3	Trehalose-phosphatase	
MGG_01551	3	6	10.8	-	5	8.9	-	2	4.2	3.3	Long-chain-fatty-acid-CoA ligase 1	
MGG_06011	2	4	12.6	-	5	14.4	-	3	12.1	3.3	S-(Hydroxymethyl)glutathione dehydrogenase; PHI:4616	Reduced virulence
MGG_08895	2	5	22.1	1	5	18	1	4	14.8	3.3	Fructose-1,6-bisphosphatase	
MGG_08012	2	5	16.3	-	5	19	-	2	9.2	3.3	UDP-glucose 4-epimerase	
MGG_11927	3	4	13.5	-	5	18.2	-	4	17.2	3.3	3-oxoacyl	
MGG_02378	4	6	9.3	2	7	11.8	2	5	9.1	3.3	Glutamate decarboxylase	
MGG_06720	6	9	20.8	3	8	18.5	3	4	7.8	3.0	ATP-citrate synthase subunit 2	
MGG_07289	1	6	7.2	-	2	2.5	-	2	3.2	3.0	Glycogen synthase; PHI:3816	Reduced virulence
MGG_00832	-	6	13.5	-	1	2.6	-	2	4.7	3.0	Cytochrome P450	
MGG_06459	1	6	6.5	1	3	2.9	1	3	3.3	3.0	Hsp98-like protein	
MGG_10492	1	6	17.1	-	3	8.1	-	1	2.6	3.0	Carnitine/acyl carnitine carrier	
MGG_00805	2	6	10.4	-	2	3.8	-	3	5.3	3.0	Kynurenine 3-monooxygenase	
MGG_10334	1	5	11.6	-	4	10.2	-	1	2.8	3.0	Mannitol 2-dehydrogenase	
MGG_11794	2	3	10.2	-	5	14	-	3	10.6	3.0	D-3-phosphoglycerate dehydrogenase 2	
MGG_13781	1	6	10.9	-	3	4.8	-	1	2.1	3.0	Serine hydroxymethyltransferase; PHI:4491	Reduced virulence
MGG_06609	4	6	13.9	1	7	15.8	1	2	5.9	3.0	Acetyl-CoA hydrolase	
MGG_06276	3	6	10.1	-	5	9.4	-	1	2.2	3.0	FAD-NAD(P) binding domain; Nde1p (e-150)	
MGG_01582	-	5	11.3	-	2	6.4	-	1	2.3	2.7	T-complex protein 1 subunit delta	
MGG_08814	-	6	13.8	-	1	2.3	-	1	2.3	2.7	Dihydroorotate dehydrogenase	
MGG_01631	4	5	7.6	-	5	9.8	-	2	4.2	2.7	UTP-glucose-1-phosphate uridylyltransferase	
MGG_04156	2	6	17.1	-	2	4.8	-	2	4.6	2.7	Aspartate aminotransferase	
MGG_06371	4	5	12.7	-	5	8.9	-	2	4.6	2.7	Pyruvate dehydrogenase E1 component subunit alpha	
MGG_12767	3	4	4	-	4	5.8	-	3	3.4	2.7	2-oxoglutarate dehydrogenase	
MGG_17513	6	6	10.2	3	6	10.8	3	8	15.2	2.7	Delta-1-pyrroline-5-carboxylate dehydrogenase	
MGG_00901	5	5	10.6	1	6	13.7	1	4	10	2.7	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	
MGG_00969	-	4	4.8	-	3	4.8	-	1	1.2	2.7	Asparagine synthetase	
MGG_17677	-	6	13.9	-	1	3.2	-	1	2.1	2.7	Polyamine transporter; Tpo3 (2e-41)	
MGG_11786	-	3	6	-	3	6.6	-	2	5.4	2.7	Basic amino acid transporter	
MGG_14574	2	6	9.3	-	2	3.2	-	2	4.7	2.7	Pyridine nucleotide-disulphide oxidoreductase; FAD-binding domain	
MGG_09931	-	5	3.7	-	2	2.1	-	1	1.2	2.7	Lipid A export ATP-binding/permease MsbA	
MGG_00865	-	3	1.7	-	2	0.9	-	3	1.6	2.7	1,3-beta-glucan synthase component FKS1	
MGG_04404	-	2	4.4	-	3	7.7	-	2	5.6	2.3	Pisatin demethylase	

MGG_09501	-	4	11.4	-	1	4.5	-	2	8.7	2.3	Serine racemase	
MGG_13986	-	3	5.4	-	2	4.2	-	2	4.2	2.3	Delta(14)-sterol reductase	
MGG_01026	2	3	5.7	-	4	9.9	-	2	6.4	2.3	Hydroxymethylglutaryl-CoA synthase	
MGG_00175	7	9	22	6	9	23	6	8	16.5	2.3	6-phosphogluconate dehydrogenase	
MGG_03250	2	4	14.8	-	3	12.1	-	2	9.4	2.3	Enoyl reductase TSC13	
MGG_10568	1	3	9.5	-	3	6.8	-	2	4.5	2.3	Sterol 24-C-methyltransferase	
MGG_09550	1	3	8.6	-	4	10.5	-	1	3.9	2.3	Homoserine kinase; involved in glycine, serine and threonine metabolism	
MGG_03494	-	2	5	-	3	7.8	-	1	2.8	2.0	Aminotransferase	
MGG_05480	-	1	2.7	-	3	12.9	-	2	8.1	2.0	Succinyl-CoA ligase subunit alpha	
MGG_09901	-	2	5.6	-	3	7.7	-	1	2.7	2.0	NAD dependent epimerase/dehydratase required for UDP-galactose transport to the Golgi lumen; Hut1p (e-62)	
MGG_16886	1	3	8.8	-	2	4.8	-	2	4.8	2.0	Fructose-bisphosphate aldolase 1	
MGG_00223	1	2	4.4	-	3	9.7	-	2	7.2	2.0	Isovaleryl-CoA dehydrogenase 2	
MGG_02540	1	3	7.7	-	3	6.2	-	1	3	2.0	3-isopropylmalate dehydrogenase	
MGG_05223	4	4	12.6	-	4	10.1	-	2	5.7	2.0		
Mitochondria (33)												
MGG_01925	6	19	16.3	-	14	14.6	-	12	12.9	13.0	Bifunctional P-450/NADPH-P450 reductase	
MGG_03185	13	21	42	8	23	41.8	8	22	47.1	12.3	ATP synthase subunit beta	
MGG_07752	15	21	33.2	11	25	33	11	26	39.9	11.7	ATP synthase subunit alpha	
MGG_16149	15	19	35.9	7	18	39	7	18	39	8.7	ADP/ATP Mitochondrial carrier protein	
MGG_06289	5	10	33	-	11	35.3	-	9	34.6	8.3	NADH-cytochrome b5 reductase 1	
MGG_03244	2	8	18.9	-	9	23	-	6	16	7.0	V-type proton ATPase subunit B	
MGG_04752	2	8	27.2	-	8	28.9	-	5	22.8	6.3	ATP synthase subunit 4	
MGG_04469	-	8	13	-	6	9.6	-	5	7.6	6.3	Cytochrome P450 97B3	
MGG_03224	1	9	28.9	-	6	17.4	-	4	16.4	6.0	Mitochondrial GTP/GDP transporter; Ggc1p (1e-153)	
MGG_09906	4	11	33	-	5	16.2	-	4	13.1	5.3	Mitochondrial phosphate carrier protein 2	
MGG_03600	6	8	17.1	2	8	15	2	10	17.3	5.3	Mitochondrial-processing peptidase subunit beta	
MGG_02370	9	11	37.5	5	12	39.8	5	11	37.9	5.0	Mitochondrial phosphate carrier protein PHI:2038	Virulent
MGG_00968	4	8	24.7	2	5	13.8	2	8	26.9	4.3	Mitochondrial outer membrane protein porin	
MGG_11021	-	9	13.7	-	2	3.4	-	2	3.4	4.3	Mitochondrial ATPase; involved in mitochondrial protein sorting	
MGG_00488	1	6	23.4	-	6	31.4	-	1	5.1	4.0	Mic26; a component of the MICOS complex	
MGG_07307	9	12	41.6	1	5	21.4	1	5	21.1	3.7	NADH-cytochrome b5 reductase 2	
MGG_12983	1	5	16.2	-	4	13.4	-	3	9.5	3.7	Bifunctional triacylglycerol lipase and 1-acyl DHAP reductase; Ayr1 (e-41)	
MGG_04969	-	3	5.5	-	3	7.5	-	4	9.4	3.3	Electron transfer protein 1	
MGG_00979	1	6	13	-	3	4.8	-	2	3.4	3.3	Mitochondrial import receptor subunit tom-70	
MGG_12005	-	4	3.4	-	3	3.5	-	2	2.7	3.0	p-type ATPase; Spf1 (e0.0); PHI:2100	Reduced virulence
MGG_01210	1	4	17	-	3	11.1	-	3	11.1	3.0	Mitochondrial hypoxia responsive domain-containing protein	
MGG_01135	2	4	23.7	-	3	18.8	-	3	18.8	2.7	Cytochrome c oxidase polypeptide IV	
MGG_07117	1	7	10.6	-	1	1	-	1	2	2.7	NADPH-cytochrome P450 reductase/NADPH-cytochrome P450 reductase	
MGG_08087	4	7	14.5	2	5	9.4	2	4	6.6	2.7	V-type proton ATPase catalytic subunit A	
MGG_07201	3	5	15.1	2	5	13.2	2	5	13.2	2.7	Mitochondrial DNA replication protein YHM2	
MGG_11419	1	7	15.8	-	1	2	-	1	2	2.7	Mitochondrial inner membrane translocase subunit TIM44	
MGG_01232	1	3	17.9	-	4	21.4	-	2	9.2	2.7	Mitochondrial ATPase Atp20p (e-15)	
MGG_06878	-	4	4	-	2	2.2	-	2	1.9	2.7	Mitochondrial inner membrane half-type ABC transporter; Mdl2	
MGG_16462	4	5	19.8	-	3	11.1	-	3	11.1	2.3	Cytochrome c1	
MGG_16866	2	4	15.3	-	3	14.4	-	2	11	2.3	Cytochrome b-c1 complex subunit Rieske	
MGG_03154	1	5	9.4	-	1	3.7	-	2	3.7	2.3	mitochondrial PP2C superfamily Ptc5 (e-112)	
MGG_04483	-	4	10.8	-	1	4.4	-	1	4.1	2.0	Mitochondrial carrier protein	
MGG_03188	-	2	17.4	-	2	16.2	-	2	16.2	2.0	Cytochrome c oxidase polypeptide 5	
RNA metabolism and ribosome biogenesis (32)												
MGG_04042	19	28	29.5	3	22	24.3	3	19	20.2	14.7	Leucyl-tRNA synthetase	
MGG_12574	9	15	26.5	1	15	27.9	1	12	21	10.3	Aspartyl-tRNA synthetase	

MGG_03607	2	16	15.2	-	10	9.7	-	6	6.4	10.0	Alanyl-tRNA synthetase	
MGG_03641	12	13	35.7	6	13	32.1	6	16	40.8	6.0	Elongation factor Tef2; actions in the binding reaction of aminoacyl-tRNA (AA-tRNA) to ribosomes; may also have a role in tRNA re-export from nucleus	
MGG_05673	15	17	64.1	8	17	63.7	8	14	50.4	5.7	40S ribosomal protein S3	
MGG_01742	11	17	22.5	11	17	19.8	11	16	17.9	5.7	Elongation factor 2; Eft2; catalyzes ribosomal translocation during protein synthesis	
MGG_06936	9	10	25.7	4	12	24.5	4	11	26.5	5.3	Gamma subunit of translational elongation factor eEF1B; stimulates the binding of aminoacyl-tRNA (AA-tRNA) to ribosomes by releasing eEF1A (Tef1p/Tef2p) from the ribosomal complex	
MGG_04829	4	8	40.7	3	9	51	3	9	53.1	5.3	60S ribosomal protein L9-A	
MGG_04396	4	8	9.3	2	9	11	2	6	6.2	5.0	Valyl-tRNA synthetase	
MGG_10357	4	6	12	1	9	18.2	1	6	11.7	5.0	Prolyl-tRNA synthetase	
MGG_04710	-	10	3.8	-	3	1.3	-	1	0.4	4.7	Translational activator GCN1	
MGG_06913	3	4	11.5	-	8	17.3	-	5	11.5	4.7	Seryl-tRNA synthetase	
MGG_02952	7	11	47.6	2	6	23.6	2	8	34	4.7	40S ribosomal protein S9	
MGG_14971	15	19	18.5	10	16	14.1	10	13	12.5	4.3	Elongation factor 3; Hef3/Yef3; stimulates EF-1 alpha-dependent binding of aminoacyl-tRNA by the ribosome	
MGG_01046	1	8	11	-	4	6.6	-	2	3.6	4.3	Methionyl-tRNA synthetase	
MGG_02432	1	7	6.2	-	4	4.7	-	3	3	4.3	Leucyl-tRNA synthetase	
MGG_03220	13	19	16.8	-	2	2.9	-	4	3	4.0	Isoleucyl-tRNA synthetase	
MGG_12892	3	6	38.2	1	6	36.1	1	5	38.2	4.0	40S ribosomal protein S16	
MGG_04696	5	10	36.4	3	7	32.3	3	6	33.6	4.0	60S ribosomal protein L10a	
MGG_11776	6	9	31	5	8	22	5	10	27.9	3.7	40S ribosomal protein S0	
MGG_04444	3	8	36.1	2	6	27.2	2	3	12.9	3.3	60S ribosomal protein L6-B	
MGG_02747	7	11	35.1	1	6	22.5	1	2	9.2	3.3	40S ribosomal protein S4-A	
MGG_09540	3	5	10.9	-	4	8.3	-	3	6.7	3.0	Tryptophanyl-tRNA synthetase	
MGG_03653	4	7	43.1	2	6	34.5	2	4	24.1	3.0	60S ribosomal protein L20	
MGG_04966*	4	5	7.2	-	4	8.3	-	3	4.4	2.7	Lysyl-tRNA synthetase	
MGG_05449	6	8	29.2	3	6	25.2	3	6	24.8	2.7	60S ribosomal protein L16	
MGG_06044	1	3	25.8	-	3	28.9	-	3	28.9	2.7	60S ribosomal protein L40	
MGG_06919	8	10	37.1	3	6	21.1	3	6	21.1	2.7	40S ribosomal protein S1	
MGG_09194	3	6	33.3	2	4	23.7	2	4	23.1	2.3	60S ribosomal protein L17	
MGG_11547	2	3	5.7	-	2	3.1	-	3	4.7	2.0	Gcn20; forms a complex with Gcn1; Positive regulator of Gcn2	
MGG_06321	6	8	11.6	3	7	11.2	3	3	5.9	2.0	Glycyl-tRNA synthetase	
MGG_08640	1	3	9.4	-	2	5.1	-	2	5.1	2.0	Rbg2; Protein with a role in translation; forms a complex with Grr2p	
Endocytosis/secretion/cell wall (13)												
MGG_06860	7	11	15.1	-	13	16.3	-	10	11.4	9.0	Coatamer subunit beta	
MGG_03511	6	14	13.5	-	8	6.9	-	2	1.7	6.0	Coatamer alpha subunit	
MGG_04856	3	8	15.8	-	8	14.5	-	5	10.7	6.0	Protein transporter SEC61 subunit alpha	
MGG_06362	3	8	38.1	-	6	34.9	-	6	39.2	5.7	Small COPII coat GTPase SARI; anterograde transport	
MGG_06962	-	3	19.3	-	6	32.2	-	7	32.2	5.3	GTP-binding protein ypt1; Rab family GTPase; involved in the ER-to-Golgi step of the secretory pathway	
MGG_02634	-	9	10.3	-	5	6	-	2	2.7	5.3	Coatamer subunit gamma	
MGG_13014*	-	5	3.2	-	3	2.1	-	4	2.3	4.0	Class V chitin synthase; PHI:2302; Loss of pathogenesis	Avirulent
MGG_01802	1	7	8.1	-	4	4.7	-	1	1.5	3.7	Chitin synthase 1; PHI:2299	Reduced virulence
MGG_04438	1	4	23	-	4	21.9	-	4	31.1	3.7	ADP-ribosylation factor; involved in vesicle trafficking	
MGG_01185	3	4	20.4	-	3	14.5	-	4	19.5	2.7	GTP-binding protein ypt5; endosomal Rab family GTPase; required for endocytic transport and sorting of vacuolar hydrolases; required for endosomal localization of the CORVET complex; required with YPT52 for MVB biogenesis and sorting	
MGG_00506	-	3	3.5	-	2	1.8	-	2	1.7	2.3	Vacuolar protein sorting/targeting protein 10	
MGG_09517	3	4	4.7	-	2	3.2	-	3	4.4	2.0	dynamin; Vacuolar protein sorting-associated protein 1	
MGG_11279	1	3	3.8	-	3	4.8	-	1	1.4	2.0	Synaptotagmin-1; regulates levels of membrane phosphatidylinositol-4,5-bisphosphate and membrane trafficking	

Cytoskeleton (8)														
MGG_11412	6	12	21.6	-	8	21.1	-	11	29.5	8.3	Tubulin alpha chain			
MGG_00604	10	14	25.1	6	16	27.1	6	15	31.1	7.7	Tubulin beta chain			
MGG_06650	5	10	20.7	-	10	21.6	-	6	13.8	7.0	Tubulin alpha-B chain			
MGG_03982	13	19	46.9	9	17	35.5	9	16	41.1	7.0	Actin			
MGG_04143	2	3	14.4	-	6	38.8	-	3	16.3	3.3	Ras-like protein Rab-6A			
MGG_02731*	-	5	26.6	-	4	22.1	-	1	4	3.3	Rac1; Ras-like protein ced-10; PHI:2054	Avirulent		
MGG_01079	1	2	10.4	1	3	13.7	1	4	19.9	2.0	Ras-like protein Rab-11A			
MGG_06726	1	3	10.1	1	3	10.1	1	3	10.1	2.0	Septin like Spn2			
Signaling (7)														
MGG_06175	1	7	11.1	-	3	4.4	-	4	5.3	4.3	Thioredoxin Mgd1 (e-12); redox signalling			
MGG_00567	1	8	4.6	-	3	2	-	2	1.9	4.0	STAND N-terminal signal; signal transducing ATPase; Tetratricopeptide repeat			
MGG_09565*	2	4	9.8	-	5	12.4	-	3	7.9	3.3	Pmk1; MAPK protein kinase; PHI:2163;PHI:56; PHI: 690	Reduced virulence		
MGG_04943	2	8	16.4	1	2	4.3	1	3	4.6	3.0	Mps1; MAPK protein kinase; PHI:113	Reduced virulence		
MGG_04719	4	4	15.8	-	6	22.8	-	3	13	3.0	beta G protein subunit			
MGG_13806	4	7	25.9	1	3	10.8	1	2	7.2	2.0	14-3-3 family protein			
MGG_01722	-	1	2.1	-	3	5.6	-	2	3.9	2.0	Adenylyl cyclase-associated protein			
Other functions (53)														
MGG_07110	21	32	36.5	14	26	27.8	14	33	32.4	14.0	N-methyltransferase			
MGG_01581	9	16	40.3	-	11	27.1	-	12	30.3	10.0	26S protease regulatory subunit 7			
MGG_06958	20	23	37.6	5	18	29.2	5	16	27.6	9.0	Hsp70-like protein			
MGG_07120	6	15	17.5	-	10	10.8	-	7	8.9	8.7	26S proteasome regulatory subunit rpn-1			
MGG_06759	14	20	24.2	6	17	23.1	6	14	19.4	8.3	Heat shock protein 90			
MGG_14872	14	19	30.9	-	8	14.7	-	10	17.2	7.7	Calpain-9			
MGG_00884	6	13	25.7	-	8	13.8	-	7	12.4	7.3	26S proteasome non-ATPase regulatory subunit 3			
MGG_01014	6	12	12.1	2	14	15.6	2	6	7.4	7.3	C-1-tetrahydrofolate synthase			
MGG_04863	7	16	13	2	9	7.4	2	7	5.6	7.0	Cullin-associated NEDD8-dissociated protein 2; assembly factor of SCF (SKP1-CUL1-F-box protein) E3 ubiquitin ligase complexes			
MGG_05671	1	6	9.5	1	8	14	1	6	10.4	5.7	Protein phosphatase PP2A regulatory subunit A			
MGG_00908	5	13	29.5	-	4	9.7	-	4	9.7	5.3	26S protease regulatory subunit 6B			
MGG_11862	5	7	11.5	1	7	10.8	1	7	11.6	4.7	ABC transporter ATP-binding protein ARB1			
MGG_09941	-	7	4.4	-	3	2.7	-	4	2.8	4.7	ABC transporter; Nod factor export ATP-binding protein I			
MGG_01674	1	11	7.6	-	2	1.7	-	2	1.4	4.7	ABC transporter			
MGG_11141	-	7	8.3	-	5	5.9	-	2	2.5	4.7	Lon protease like protein			
MGG_12925	1	8	17.7	-	3	6	-	3	7.5	4.3	26S protease regulatory subunit 6A			
MGG_05193	8	13	16.8	4	9	12.6	4	7	10	4.3	Cdc48, Cell division control protein 48			
MGG_09287	1	10	21.5	-	2	3.3	-	2	3.7	4.3	Oligosaccharyltransferase alpha subunit			
MGG_03670	1	7	13.6	1	6	16	1	3	6	4.3	Subtilisin-like proteinase Spn1; PHI:2117	Reduced virulence		
MGG_06004	1	5	20.4	-	5	21.5	-	3	11.3	4.0	Prohibitin-1			
MGG_11513	14	14	25.4	11	18	29.3	11	16	30.8	4.0	Heat shock protein SSB1			
MGG_02954	-	6	5.6	-	2	2.8	-	3	3.5	3.7	Dolichyl-phosphate-mannose-protein mannosyltransferase 1			
MGG_06065	2	6	9.3	-	4	7.2	-	3	5.3	3.7	Hsp88-like protein			
MGG_00330	7	7	22.8	-	6	16.9	-	4	11.8	3.3	26S protease subunit rpt4			
MGG_03211*	-	3	7.1	-	5	11.6	-	2	4	3.3	26S proteasome non-ATPase regulatory subunit 11			
MGG_00133	-	3	4.9	-	4	6	-	2	4	3.0	Siderophore iron transporter mirC			
MGG_05686	-	5	10.8	-	2	3.4	-	2	3.6	3.0	Dolichyl pyrophosphate Glc1Man9GlcNAc2 alpha-1,3-glucosyltransferase~~			
MGG_07190	2	3	4.2	-	5	8.1	-	3	5.2	3.0	Dolichyl-phosphate-mannose-protein mannosyltransferase 2			
MGG_00500	-	5	8	-	2	3.1	-	1	1.1	2.7	Meiotically up-regulated 71 protein			
MGG_03524	2	3	7.9	-	3	6.5	-	4	9.9	2.7	26S proteasome regulatory subunit RPN9			
MGG_03039	2	5	6.9	-	4	4.6	-	1	0.9	2.7	Hsp70-like protein			
MGG_13438	2	4	7.4	1	7	13.5	1	1	2.4	2.7	Probable Xaa-Pro aminopeptidase PEPP			

MGG_00195	6	5	7.3	-	6	9.9	-	3	4.6	2.7	Clock-controlled-9 protein	
MGG_12775	-	3	9.3	-	4	7.9	-	1	3.4	2.7	Voltage-gated K ⁺ channel beta subunit	
MGG_08304	12	14	14.3	1	2	2.6	1	6	7	2.7	Mechanosensitive ion channel family protein	
MGG_02821	-	4	9.9	-	3	6.2	-	-	-	2.3	Dolichyl-di-phosphooligosaccharide-protein glycotransferase	
MGG_06951	1	4	7.8	-	3	7.2	-	1	2.2	2.3	CAAX prenyl protease 1; PHI:807	Reduced virulence
MGG_09766	4	3	8.6	-	6	15.7	-	2	5.5	2.3	3-deoxy-7-phosphoheptulonate synthase	
MGG_16743	3	8	13	-	1	1.7	-	1	1.4	2.3	Heterokaryon incompatibility protein (ascomycete fungi)	
MGG_00937	-	4	1.9	-	1	0.5	-	2	1.2	2.3	ABC transporter; PHI:1017; mutant phenotype mixed outcome	
MGG_03512	1	3	14.4	-	4	16.2	-	1	4.5	2.3	26S proteasome non-ATPase regulatory subunit 8	
MGG_04197	1	4	23.8	-	2	8	-	2	8	2.3	reticulon (ER protein); Rtn1 (e-9); Reticulon protein; stabilizes membrane curvature; involved in nuclear pore assembly and maintenance of tubular ER morphology	
MGG_03736	-	2	1.1	-	2	1.3	-	2	1.4	2.0	ABC transporter; Multidrug resistance-associated protein 1	
MGG_00106	-	2	3.6	-	1	1.9	-	3	4.7	2.0	Bud site selection protein 7	
MGG_00738	-	3	9.6	-	1	2.3	-	2	5.2	2.0	Zinc-binding alcohol dehydrogenase domain-containing protein 2	
MGG_01140	1	4	8.1	-	1	1.6	-	2	3.5	2.0	Alpha-1,2 mannosyltransferase KTR1	
MGG_11343	1	2	2.5	-	4	7.7	-	1	1.4	2.0	Phosphoribosylformylglycinamide cyclo-ligase	
MGG_02648	2	5	6.5	-	3	4.8	-	-	-	2.0	Interferon-induced GTP-binding protein Mx	
MGG_08795	-	1	1.5	-	4	7.4	-	1	2.4	2.0	Cytochrome P450 superfamily	
MGG_05271	-	2	5.3	-	2	7.2	-	2	7.2	2.0	Pex11 (Peroxisomal biogenesis factor 11); mutant not required for infection	
MGG_05991	2	5	4.9	-	2	2.3	-	1	1.5	2.0	26S proteasome regulatory subunit rpn2	
MGG_16706	1	2	6.8	-	3	10	-	2	5.9	2.0	26S proteasome regulatory subunit rpn11	
MGG_06504	2	6	10.7	1	2	5	1	2	3	2.0	26S proteasome regulatory subunit RPN7	
Unknown function (9)												
MGG_07470	-	9	15.6	-	4	6.6	-	3	5.4	5.3	Metallo-dependent phosphatase	
MGG_07792	-	4	12	-	3	11.7	-	4	12	3.7	4 transmembrane helix	
MGG_09167	-	7	12.4	-	2	5.6	-	2	4.7	3.7	3 transmembrane helix	
MGG_03197	-	4	16.9	-	2	7	-	3	9.1	3.0	6 transmembrane helix; eukaryotic DUF1751	
MGG_01461	1	2	7.4	-	4	9.8	-	3	9.8	2.7	6 transmembrane helix; fungal DUF1774	
MGG_05188	1	5	9.6	-	3	6.9	-	1	3.3	2.7	eukaryotic DUF1682	
MGG_17528	3	7	30.8	-	3	14.5	-	1	3.6	2.7	Metal-dependent hydrolase	
MGG_05849	1	3	14.3	-	3	15.8	-	2	9.8	2.3	2 transmembrane helix	
MGG_03327	1	3	8.3	-	2	4.8	-	2	4.8	2.0	4 transmembrane helix	

SUPPLEMENTAL INFORMATION

A

B

Supplemental Fig. S3.1. Mostly all the kaps show reduced expression levels in $\Delta exp5$ cells grown in MM-N. (A) Expression analysis of the thirteen karyopherins between wild type and $\Delta exp5$ strains in nitrogen starvation (MM-N). Gene expression is represented by fold change ($2^{-\Delta\Delta Ct}$) normalized against actin. Karyopherins show lower levels of expression than the actin gene (decimal values in Y-axis). (B) Gene expression ratio between $\Delta exp5$ and Guy11 in nitrogen starved cells.

A **B**

Supplemental Fig. S3.2. Expression relationship between each of the twelve karyopherins in the Guy11 strains in MM-N. (A) Expression analysis of the thirteen karyopherins (Exp5 included) between nitrogen starvation (MM-N) and complex medium (CM) in Guy11 strain. Gene expression is represented by fold change ($2^{-\Delta\Delta C_t}$) normalized against actin. (B) Karyopherins are lower expressed than actin to represent those data in an easily visual way we have decided represent the gene expression ratio between MM-N and CM in Guy11 strain.

A

B

Supplemental Fig. 3.3. *PSE1*, *EXP5*, *RSL1* and *KAP120* genes showed higher expression levels in carbon-starved cells in the wild type strain. (A) Wild type expression levels of all the karyopherins in complex medium (CM) or carbon-starved cells (MM-C). Gene expression is represented by fold change ($2^{-\Delta\Delta Ct}$) normalized against actin. **(B)** Gene expression ratios of karyopherin genes between CM and MM-C in the wild type strain.

Supplemental Fig. S3.4. Reads length distribution in the $\Delta exp5$ mutant changed with the disappearance of the 14-nt peak. After adapters removal the reads length is between 18 and 24 nt in both strains. Besides, there is a subpopulation of 14 nt reads in the wild type that is not present in $\Delta exp5$.

Supplemental Fig. S3.5. Polyacrilamide gel stained with ethidium bromide (EtBr) and loaded with total sRNA content and sRNAs extracted from nuclear and cytoplasmic fractions from wild type (WT) and $\Delta exp5$. Small RNAs were extracted with Trizol and enriched with the MirVana kit, data showed. Also northern blots with sRNA isolated with LiCl method were performed. Both data correlates. LiCl data were not shown.

Supplemental Table S3.1. β -Karyopherins described in *Magnaporthe oryzae* and *Saccharomyces cerevisiae*. Nmd5 and Sxm1 possibly derived from gene duplication since both proteins have MGG_02927.8 as a clear *M. oryzae* orthologue. Alternative names for each karyopherin are also shown. Asterisks denote karyopherins encoded by essential gene.

<i>M. oryzae</i>	<i>S. cerevisiae</i>	<i>S. cerevisiae</i> karyopherins functions	References
MGG_00744.8 Group I	Kap120 (Lph2) $4.2e^{-9}$	Responsible for the nuclear import of Rpf1p; Rpf1p is a ribosome maturation factor	(Caesar, <i>et al.</i> , 2006)
MGG_01449.8 Group IV	Mtr10 (Kap111) $3.4e^{-20}$	Nuclear import receptor; mediates the nuclear localization of proteins involved in mRNA-nucleus export; promotes dissociation of mRNAs from the nucleus- cytoplasm mRNA shuttling protein Npl3p; required for retrograde import of mature tRNAs; relocalizes from cytoplasm to the nuclear periphery upon DNA replication stress	(Windgassen, <i>et al.</i> , 2004, Murthi, <i>et al.</i> , 2010, Tkach, <i>et al.</i> , 2012)
MGG_02927.8 Group IV	Nmd5 (Kap119) $2.2e^{-20}$	Translocates transcription factors to the cytoplasm for its activation by phosphorylation as in the case of Ste12	(Albertini, <i>et al.</i> , 1998, Ferrigno, <i>et al.</i> , 1998)
	Sxm1 (Kap108) $9.4e^{-28}$	Involved in protein transport between the cytoplasm and nucleoplasm; similar to Nmd5p, Cse1p, Kap120, and the human cellular apoptosis susceptibility protein, CAS1	(Seedorf & Silver, 1997, Sydorsky, <i>et al.</i> , 2003)
MGG_03537.8 Group II	*Pse1 (Kap121) $7.1e^{-47}$	Interacts with the nuclear pore complex; acts as the nuclear import receptor for specific proteins, including Pdr1p, Yap1p, Ste12p, and Aft1p	(Agnès Delahodde, 2001, Takeshi Isoyama, 2001, Leslie, <i>et al.</i> , 2002, Ryo Ueta, 2003)
MGG_16711.8 Group III	Kap123 $1.7e^{-46}$	Mediates nuclear import of ribosomal proteins prior to assembly into ribosomes and import of histones H3 and H4; localizes to the nuclear pore, nucleus, and cytoplasm; exhibits genetic interactions with RAI1	(Sydorsky, <i>et al.</i> , 2003, Mosammaparast & Pemberton, 2004)
MGG_11165.8 Group III	Kap114 $3.3e^{-14}$	Responsible for nuclear import of Spt15p, Sua7p, histones H2A and H2B, and Nap1p; amino terminus shows similarity to those of other importins, particularly Cse1p; localization is primarily nuclear; function is regulated by sumoylation; protein abundance increases in response to DNA replication stress	(Mosammaparast, <i>et al.</i> , 2002, Hodges, <i>et al.</i> , 2005, Rothenbusch, <i>et al.</i> , 2012, Tkach, <i>et al.</i> , 2012)
MGG_00475.8 Group III	Pdr6 (Kap122) $1.2e^{-6}$	Responsible for import of the Toa1p-Toa2p complex into the nucleus; binds to nucleoporins Nup1p and Nup2p; may play a role in regulation of pleiotropic drug resistance	(Titov & Blobel, 1999)
MGG_03668.8 Group II	*Rsl1 (Kap95) $4.2e^{-52}$	Forms a complex with Kap α /Srp1p; interacts with nucleoporins to mediate nuclear import of NLS-containing cargo proteins via the nuclear pore complex; regulates pore complex biosynthesis; GDP-to-GTP exchange factor for RanGTP	(Cordula Enenkel, 1995, Aitchison, <i>et al.</i> , 1996, Rexach, 2003, Lonhienne, <i>et al.</i> , 2009, MacKinnon, <i>et al.</i> , 2009)
MGG_09208.8 Group II	Kap104 $2.4e^{-25}$	Mediates nuclear import/reimport of mRNA-binding proteins Nab2p and Hrp1p; regulates asymmetric protein synthesis in daughter cells during mitosis	(Aitchison, <i>et al.</i> , 1996, Seedorf & Silver, 1997, Lee, 1999, van den Bogaart, <i>et al.</i> , 2009)
MGG_09560.8 (Exp5) Group III	Msn5, Ste21 (Kap142) $1.2e^{-29}$	Involved in nuclear import and export of proteins, including import of replication protein A and export of Far1p and transcription factors Swi5p, Swi6p, Msn2p, and Pho4p; required for re-export of mature tRNAs. Associated with cell cycle	(Kaffman, <i>et al.</i> , 1998, Alepuz, <i>et al.</i> , 1999, Blondel, <i>et al.</i> , 1999, Yoshida & Blobel, 2001, Queralt & Igual, 2003, Shibata, <i>et al.</i> , 2006, Murthi, <i>et al.</i> , 2010, Taberner, <i>et al.</i> , 2012, Sunnåker, <i>et al.</i> , 2013)
MGG_03994.8 Group III	*Cse1 (Kap109) $1.8e^{-68}$	Mediates the nuclear export of Kap α /Srp1p back to the cytoplasm; required for accurate chromosome segregation and cell division	(Xiao, <i>et al.</i> , 1993, Hood & Silver, 1998, Schroeder AJ, 1999, Yuksel, <i>et al.</i> , 2015)
MGG_02526.8 Group III	*Crm1, Xpo1 (Kap124) 0.0	Involved in export of proteins, RNAs, and ribosomal subunits from the nucleus	(Maurer, <i>et al.</i> , 2001, Elson, <i>et al.</i>)

MGG_10127.8 Group III	Los1 2.1e ⁻⁴	Involved in nuclear export of pre-tRNA and in re-export of mature tRNAs; deletion mutation extends replicative lifespan, as does exclusion of Los1p from the nucleus in response to caloric restriction	(Hurt, <i>et al.</i> , 1987, Shen, 1993, Hellmuth, <i>et al.</i> , 1998, Murthi, <i>et al.</i> , 2010, McCormick, <i>et al.</i>)
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References supplemental Table S3.1

- Agnès Delahodde, R. P., Marisol Corral-Debrinski, Claude Jacq (2001).** Pse1/Kap121-dependent nuclear localization of the major yeast multidrug resistance (MDR) transcription factor Pdr1. *Molecular microbiology* **39**, 304–313.
- Ai, Y.-P., Zhong, J., Chen, C.-Y., Zhu, H.-J. & Gao, B.-D. (2016).** A novel single-stranded RNA virus isolated from the rice-pathogenic fungus *Magnaporthe oryzae* with similarity to members of the family Tombusviridae. *Archives of Virology* **161**, 725-729.
- Aitchison, J. D., Blobel, G. & Rout, M. P. (1996).** Kap104p: A Karyopherin Involved in the Nuclear Transport of Messenger RNA Binding Proteins. *Science* **274**, 624-627.
- Albertini, M., Pemberton, L. F., Rosenblum, J. S. & Blobel, G. (1998).** A novel nuclear import pathway for the transcription factor TFIIS. *Journal of Cell Biology* **143**, 1447-1455.
- Alepuz, P. M., Matheos, D., Cunningham, K. W. & Estruch, F. (1999).** The *Saccharomyces cerevisiae* RanGTP-binding protein Msn5p is involved in different signal transduction pathways. *Genetics* **153**, 1219-1231.
- Andersson, A., Jordan, D., Schneider, G. & Lindqvist, Y. (1996).** Crystal structure of the ternary complex of 1,3,8-trihydroxynaphthalene reductase from *Magnaporthe grisea* with NADPH and an active-site inhibitor. *Structure* **4**, 1161-1170.
- Badaruddin, M., Holcombe, L. J., Wilson, R. A., Wang, Z. Y., Kershaw, M. J. & Talbot, N. J. (2013).** Glycogen Metabolic Genes Are Involved in Trehalose-6-Phosphate Synthase-Mediated Regulation of Pathogenicity by the Rice Blast Fungus *Magnaporthe oryzae*. *Plos Pathogens* **9**.
- Bakhrat, A., Baranes-Bachar, K., Reshef, D., Voloshin, O., Krichevsky, O. & Raveh, D. (2008).** Nuclear export of Ho endonuclease of yeast via Msn5. *Current Genetics* **54**, 271-281.
- Bardwell, L., Cook, J. G., Voora, D., Baggott, D. M., Martinez, A. R. & Thorner, J. (1998).** Repression of yeast Ste12 transcription factor by direct binding of unphosphorylated Kss1 MAPK and its regulation by the Ste7 MEK. *Genes & Development* **12**, 2887-2898.
- Beare, P. A., Howe, D., Cockrell, D. C., Omsland, A., Hansen, B. & Heinzen, R. A. (2009).** Characterization of a *Coxiella burnetii* ftsZ Mutant Generated by Himar1 Transposon Mutagenesis. *Journal of Bacteriology* **191**, 1369-1381.
- Bell, W., Sun, W., Hohmann, S., Wera, S., Reinders, A., De Virgilio, C., Wiemken, A. & Thevelein, J. M. (1998).** Composition and Functional Analysis of the *Saccharomyces cerevisiae* Trehalose Synthase Complex. *Journal of Biological Chemistry* **273**, 33311-33319.
- Besi, M., Tucker, S. L. & Sesma, A. (2009).** *Magnaporthe* and its relatives. In *Encyclopedia of Life Sciences*: John Wiley & Sons, Ltd: Chichester.

- Blankenberg, D., Gordon, A., Von Kuster, G., Coraor, N., Taylor, J., Nekrutenko, A. & Team, t. G. (2010).** Manipulation of FASTQ data with Galaxy. *Bioinformatics* **26**, 1783-1785.
- Blondel, M., Alepuz, P. M., Huang, L. S., Shaham, S., Ammerer, G. & Peter, M. (1999).** Nuclear export of Far1p in response to pheromones requires the export receptor Msn5p/Ste21p. *Genes and Development* **13**, 2284-2300.
- Bohnsack, M. T., Regener, K., Schwappach, B., Saffrich, R., Paraskeva, E., Hartmann, E. & Gorlich, D. (2002).** Exp5 exports eEF1A via tRNA from nuclei and synergizes with other transport pathways to confine translation to the cytoplasm. *Embo Journal* **21**, 6205-6215.
- Bohnsack, M. T., Czaplinski, K. & Gorlich, D. (2004).** Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. *RNA* **10**, 185-191.
- Borukhov, S., Sagitov, V. & Goldfarb, A. (1993).** Transcript cleavage factors from *E. coli*. *Cell* **72**, 459-466.
- Boustany, L. M. & Cyert, M. S. (2002).** Calcineurin-dependent regulation of Crz1p nuclear export requires Msn5p and a conserved calcineurin docking site. *Genes and Development* **16**, 608-619.
- Brownawell, A. M. & Macara, I. G. (2002).** Exportin-5, a novel karyopherin, mediates nuclear export of double-stranded RNA binding proteins. *Journal of Cell Biology* **156**, 53-64.
- Caesar, S., Greiner, M. & Schlenstedt, G. (2006).** Kap120 Functions as a Nuclear Import Receptor for Ribosome Assembly Factor Rpf1 in Yeast. *Molecular and Cellular Biology* **26**, 3170-3180.
- Calado, A., Treichel, N., Muller, E. C., Otto, A. & Kutay, U. (2002).** Exportin-5-mediated nuclear export of eukaryotic elongation factor 1A and tRNA. *Embo Journal* **21**, 6216-6224.
- Cao, K., Nakajima, R., Meyer, H. H. & Zheng, Y. (2003).** The AAA-ATPase Cdc48/p97 Regulates Spindle Disassembly at the End of Mitosis. *Cell* **115**, 355-367.
- Castillo-Lluya, S., Alvarez-Tabares, I., Weber, I., Steinberg, G. & Perez-Martin, J. (2007).** Sustained cell polarity and virulence in the phytopathogenic fungus *Ustilago maydis* depends on an essential cyclin-dependent kinase from the Cdk5/Pho85 family. *Journal of Cell Science* **120**, 1584-1595.
- Cautain, B., Hill, R., Pedro, N. & Link, W. (2015).** Components and regulation of nuclear transport processes. *Febs Journal* **282**, 445-462.
- Cook, A., Bono, F., Jinek, M. & Conti, E. (2007).** Structural Biology of Nucleocytoplasmic Transport. *Annual Review of Biochemistry* **76**, 647-822.
- Cordula Enenkel, G. B. a. M. R. (1995).** Identification of a Yeast Karyopherin Heterodimer That Targets Import Substrate to Mammalian Nuclear Pore Complexes. *Journal of Biological Chemistry* **270**, 16499-16502.
- Cui, X., Wei, Y., Wang, Y.-H. & other authors (2015).** Proteins interacting with mitochondrial ATP-dependent Lon protease (MAP1) in *Magnaporthe oryzae* are involved in rice blast disease. *Molecular Plant Pathology* **16**, 847-859.
- Czech, B. & Hannon, G. J. (2011).** Small RNA sorting: matchmaking for Argonautes. *Nature Reviews Genetics* **12**, 19-31.
- Chen, J., Zheng, W., Zheng, S., Zhang, D., Sang, W., Chen, X., Li, G., Lu, G. & Wang, Z. (2008).** Rac1 Is Required for Pathogenicity and Chml1-Dependent

- Conidiogenesis in Rice Fungal Pathogen *Magnaporthe grisea*. *PLoS Pathogens* **4**, e1000202.
- Chen, R. E. & Thorner, J. (2007).** Function and regulation in MAPK signaling pathways: Lessons learned from the yeast *Saccharomyces cerevisiae*. *Biochimica Et Biophysica Acta* **1773**, 1311-1340.
- Chiappello, H., Mallet, L., Guerin, C. & other authors (2015).** Deciphering Genome Content and Evolutionary Relationships of Isolates from the Fungus *Magnaporthe oryzae* Attacking Different Host Plants. *Genome Biology Evolution* **7**, 2896-2912.
- Chook, Y. M. & Süel, K. E. (2011).** Nuclear import by Karyopherin- β s: recognition and inhibition. *Biochimica et biophysica acta* **1813**, 1593-1606.
- Dean, R. A., Talbot, N. J., Ebbole, D. J. & other authors (2005).** The genome sequence of the rice blast fungus *Magnaporthe grisea*. *Nature* **434**, 980-986.
- DeVit, M. J. & Johnston, M. (1999).** The nuclear exportin Msn5 is required for nuclear export of the Mig1 glucose repressor of *Saccharomyces cerevisiae*. *Current Biology* **9**, 1231-1242.
- Doucet, C. M., Talamas, J. A. & Hetzer, M. W. (2010).** Cell Cycle-Dependent Differences in Nuclear Pore Complex Assembly in Metazoa. *Cell* **141**, 1030-1041.
- Dufresne, M. & Osbourn, A. E. (2001).** Definition of tissue-specific and general requirements for plant infection in a phytopathogenic fungus. *Molecular Plant-Microbe Interactions* **14**, 300-307.
- Elson, S. L., Noble, S. M., Solis, N. V., Filler, S. G. & Johnson, A. D. (2009).** An RNA Transport System in *Candida albicans* Regulates Hyphal Morphology and Invasive Growth. *Plos Genetics* **5**, ARTN e1000664.
- Errede, B. & Ammerer, G. (1989).** STE12, a protein involved in cell-type-specific transcription and signal transduction in yeast, is part of protein-DNA complexes. *Genes & Development* **3**, 1349-1361.
- Farman, M. L., Tosa, Y., Nitta, N. & Leong, S. A. (1996).** MAGGY, a retrotransposon in the genome of the rice blast fungus *Magnaporthe grisea*. *Molecular and General Genetics MGG* **251**, 665-674.
- Farman, M. L., Eto, Y., Nakao, T., Tosa, Y., Nakayashiki, H., Mayama, S. & Leong, S. A. (2002).** Analysis of the Structure of the AVR1-CO39 Avirulence Locus in Virulent Rice-Infecting Isolates of *Magnaporthe grisea*. *Molecular Plant-Microbe Interactions* **15**, 6-16.
- Fernandez, J., Wright, J. D., Hartline, D., Quispe, C. F., Madayiputhiya, N. & Wilson, R. A. (2012).** Principles of Carbon Catabolite Repression in the Rice Blast Fungus: Tps1, Nmr1-3, and a MATE-Family Pump Regulate Glucose Metabolism during Infection. *PLoS Genetics* **8**, ARTN e1002673.
- Ferrigno, P., Posas, F., Koepp, D., Saito, H. & Silver, P. A. (1998).** Regulated nucleo/cytoplasmic exchange of HOG1 MAPK requires the importin beta homologs NMD5 and XPO1. *The EMBO Journal* **17**, 5606-5614.
- Forbes, D. J., Travesa, A., Nord, M. & Bernis, C. (2015).** Nuclear Transport Factors: Global Regulation of Mitosis. *Current opinion in cell biology* **35**, 78-90.
- Foster, A. J., Jenkinson, J. M. & Talbot, N. J. (2003).** Trehalose synthesis and metabolism are required at different stages of plant infection by *Magnaporthe grisea*. *EMBO Journal* **22**, 225-235.

- Franceschetti, M., Bueno, E., Wilson, R. A., Tucker, S. L., Gómez-Mena, C., Calder, G. & Sesma, A. (2011).** Fungal Virulence and Development Is Regulated by Alternative Pre-mRNA 3' End Processing in *Magnaporthe oryzae*. *PLoS Pathogens* **7**, e1002441.
- Gorlich, D. & Kutay, U. (1999).** Transport between the Cell Nucleus and the Cytoplasm. *Annual Review of Cell and Developmental Biology* **15**, 607-660.
- Gorner, W., Durchschlag, E., Wolf, J., Brown, E. L., Ammerer, G., Ruis, H. & Schuller, C. (2002).** Acute glucose starvation activates the nuclear localization signal of a stress-specific yeast transcription factor. *Embo Journal* **21**, 135-144.
- Grandaubert, J., Lowe, R. G., Soyer, J. L. & other authors (2014).** Transposable element-assisted evolution and adaptation to host plant within the *Leptosphaeria maculans*-*Leptosphaeria biglobosa* species complex of fungal pathogens. *BMC Genomics* **15**, 1-27.
- Gulshan, K., Thommandru, B. & Moye-Rowley, W. S. (2012).** Proteolytic Degradation of the Yap1 Transcription Factor Is Regulated by Subcellular Localization and the E3 Ubiquitin Ligase Not4. *The Journal of Biological Chemistry* **287**, 26796-26805.
- Gwizdek, C., Ossareh-Nazari, B., Brownawell, A. M., Evers, S., Macara, I. G. & Dargemont, C. (2004).** Minihelix-containing RNAs mediate exportin-5-dependent nuclear export of the double-stranded RNA-binding protein ILF3. *Journal of Biological Chemistry* **279**, 884-891.
- Haas, B. J., Papanicolaou, A., Yassour, M. & other authors (2013).** De novo transcript sequence reconstruction from RNA-Seq: reference generation and analysis with Trinity. *Nature protocols* **8**, 10.1038/nprot.2013.1084.
- Harbauer, A., Zahedi, R. P., Sickmann, A., Pfanner, N. & Meisinger, C. (2014).** The Protein Import Machinery of Mitochondria - A Regulatory Hub in Metabolism, Stress, and Disease. *Cell Metabolism* **19**, 357-372.
- Harel, A. & Forbes, D. J. (2004).** Importin Beta. *Molecular Cell* **16**, 319-330.
- Hellmuth, K., Lau, D. M., Bischoff, F. R., Künzler, M., Hurt, E. & Simos, G. (1998).** Yeast Los1p Has Properties of an Exportin-Like Nucleocytoplasmic Transport Factor for tRNA. *Molecular and Cellular Biology* **18**, 6374-6386.
- Hodges, J. L., Leslie, J. H., Mosammaparast, N., Guo, Y., Shabanowitz, J., Hunt, D. F. & Pemberton, L. F. (2005).** Nuclear Import of TFIIB Is Mediated by Kap114p, a Karyopherin with Multiple Cargo-binding Domains. *Molecular Biology of the Cell* **16**, 3200-3210.
- Hood, J. K. & Silver, P. A. (1998).** Cse1p Is Required for Export of Srp1p/Importin- α from the Nucleus in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **273**, 35142-35146.
- Hopper, A. K. (2013).** Transfer RNA Post-Transcriptional Processing, Turnover, and Subcellular Dynamics in the Yeast *Saccharomyces cerevisiae*. *Genetics* **194**, 43-67.
- Huang, H.-Y. & Hopper, A. K. (2015).** In vivo biochemical analyses reveal distinct roles of β -importins and eEF1A in tRNA subcellular traffic. *Genes & Development* **29**, 772-783.
- Hurt, D. J., Wang, S. S., Lin, Y. H. & Hopper, A. K. (1987).** Cloning and characterization of LOS1, a *Saccharomyces cerevisiae* gene that affects tRNA splicing. *Molecular and Cellular Biology* **7**, 1208-1216.

- Jeon, J., Park, S.-Y., Chi, M.-H. & other authors (2007).** Genome-wide functional analysis of pathogenicity genes in the rice blast fungus. *Nature genetics* **39**, 561-565.
- Kadotani, N., Nakayashiki, H., Tosa, Y. & Mayama, S. (2004).** One of the Two Dicer-like Proteins in the Filamentous Fungi *Magnaporthe oryzae* Genome Is Responsible for Hairpin RNA-triggered RNA Silencing and Related Small Interfering RNA Accumulation. *Journal of Biological Chemistry* **279**, 44467-44474.
- Kaffman, A., Rank, N. M., O'Neill, E. M., Huang, L. S. & O'Shea, E. K. (1998).** The receptor Msn5 exports the phosphorylated transcription factor Pho4 out of the nucleus. *Nature*, 482-485.
- Khang, C. H., Park, S. Y., Lee, Y. H. & Kang, S. (2005).** A dual selection based, targeted gene replacement tool for *Magnaporthe grisea* and *Fusarium oxysporum*. *Fungal Genetics and Biology* **42**, 483-492.
- Kimura, M. & Imamoto, N. (2014).** Biological Significance of the Importin-beta Family-Dependent Nucleocytoplasmic Transport Pathways. *Traffic* **15**, 727-748.
- Komeili, A., Wedaman, K. P., O Shea, E. K. & Powers, T. (2000).** Mechanism of metabolic control: Target of rapamycin signaling links nitrogen quality to the activity of the Rtg1 and Rtg3 transcription factors. *Journal of Cell Biology* **151**, 863-878.
- Langmead, B., Trapnell, C., Pop, M. & Salzberg, S. L. (2009).** Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biology* **10**, R25-R25.
- Lee, D. a. A., JD (1999).** Kap104p-mediated Nuclear Import NUCLEAR LOCALIZATION SIGNALS IN mRNA-BINDING PROTEINS AND THE ROLE OF RAN AND RNA. *Journal of Biological Chemistry* **274**, 29031-29037.
- Lee, Y. J., Kim, K. J., Kang, H. Y., Kim, H.-R. & Maeng, P. J. (2012).** Involvement of GDH3-encoded NADP(+)-dependent Glutamate Dehydrogenase in Yeast Cell Resistance to Stress-induced Apoptosis in Stationary Phase Cells. *The Journal of Biological Chemistry* **287**, 44221-44233.
- Leslie, D. M., Grill, B., Rout, M. P., Wozniak, R. W. & Aitchison, J. D. (2002).** Kap121p-Mediated Nuclear Import Is Required for Mating and Cellular Differentiation in Yeast. *Molecular and Cellular Biology* **22**, 2544-2555.
- Leung, H., Borromeo, E., Bernardo, M. & Notteghem, J. L. (1988).** Genetic analysis of virulence in the rice blast fungus *Magnaporthe grisea*. *Phytopathology* **78**, 1227-1233.
- Lonhienne, T. G., Forwood, J. K., Marfori, M., Robin, G., Kobe, B. & Carroll, B. J. (2009).** Importin- β Is a GDP-to-GTP Exchange Factor of Ran: IMPLICATIONS FOR THE MECHANISM OF NUCLEAR IMPORT. *Journal of Biological Chemistry* **284**, 22549-22558.
- Love, M. I., Huber, W. & Anders, S. (2014).** Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology* **15**, 1-21.
- Lund, E., Guttinger, S., Calado, A., Dahlberg, J. E. & Kutay, U. (2004).** Nuclear export of microRNA precursors. *Science* **303**, 95-98.

- Luse, M. G. I. a. D. S. (1991).** Transcription on nucleosomal templates by RNA polymerase II in vitro: inhibition of elongation with enhancement of sequence-specific pausing. *Genes & Dev* **5**, 683-696.
- MacKinnon, M. A., Curwin, A. J., Gaspard, G. J., Suraci, A. B., Fernández-Murray, J. P. & McMaster, C. R. (2009).** The Kap60-Kap95 Karyopherin Complex Directly Regulates Phosphatidylcholine Synthesis. *Journal of Biological Chemistry* **284**, 7376-7384.
- Mahanty, S. K., Wang, Y., Farley, F. W. & Elion, E. A. (1999).** Nuclear Shuttling of Yeast Scaffold Ste5 Is Required for Its Recruitment to the Plasma Membrane and Activation of the Mating MAPK Cascade. *Cell* **98**, 501-512.
- Mamnun, Y. M., Pandjaitan, R., Mahé, Y., Delahodde, A. & Kuchler, K. (2002).** The yeast zinc finger regulators Pdr1p and Pdr3p control pleiotropic drug resistance (PDR) as homo- and heterodimers in vivo. *Molecular Microbiology* **46**, 1429-1440.
- Maurer, P., Redd, M., Solsbacher, J. & other authors (2001).** The Nuclear Export Receptor Xpo1p Forms Distinct Complexes with NES Transport Substrates and the Yeast Ran Binding Protein 1 (Yrb1p). *Molecular Biology of the Cell* **12**, 539-549.
- McCormick, Mark A., Delaney, Joe R., Tsuchiya, M. & other authors (2015).** A Comprehensive Analysis of Replicative Lifespan in 4,698 Single-Gene Deletion Strains Uncovers Conserved Mechanisms of Aging. *Cell Metabolism* **22**, 895-906.
- Mosammamarast, N., Ewart, C. S. & Pemberton, L. F. (2002).** A role for nucleosome assembly protein 1 in the nuclear transport of histones H2A and H2B. *The EMBO Journal* **21**, 6527-6538.
- Mosammamarast, N. & Pemberton, L. F. (2004).** Karyopherins: from nuclear-transport mediators to nuclear-function regulators. *Trends in Cell Biology* **14**, 547-556.
- Mühlhäusser, P., Müller, E.-C., Otto, A. & Kutay, U. (2001).** Multiple pathways contribute to nuclear import of core histones. *EMBO Reports* **2**, 690-696.
- Murata, T., Kadotani, N., Yamaguchi, M., Tosa, Y., Mayama, S. & Nakayashiki, H. (2007).** siRNA-dependent and -independent post-transcriptional cosuppression of the LTR-retrotransposon MAGGY in the phytopathogenic fungus *Magnaporthe oryzae*. *Nucleic Acids Research* **35**, 5987-5994.
- Murthi, A., Shaheen, H. H., Huang, H. Y., Preston, M. A., Lai, T. P., Phizicky, E. M. & Hopper, A. K. (2010).** Regulation of tRNA Bidirectional Nuclear-Cytoplasmic Trafficking in *Saccharomyces cerevisiae*. *Molecular Biology of the Cell* **21**, 639-649.
- Nunes, C. C., Gowda, M., Sailsbery, J., Xue, M., Chen, F., Brown, D. E., Oh, Y., Mitchell, T. K. & Dean, R. A. (2011).** Diverse and tissue-enriched small RNAs in the plant pathogenic fungus, *Magnaporthe oryzae*. *BMC Genomics* **12**, 288.
- Park, M. Y., Wu, G., Gonzalez-Sulser, A., Vaucheret, H. & Poethig, R. S. (2005).** Nuclear processing and export of microRNAs in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 3691-3696.

- Pfaffl, M. W. (2001).** A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29**, e45.
- Phizicky, E. M. & Hopper, A. K. (2010).** tRNA biology charges to the front. *Genes & Development* **24**, 1832-1860.
- Polizotto, R. S. & Cyert, M. S. (2001).** Calcineurin-dependent nuclear import of the transcription factor Crz1p requires Nmd5p. *The Journal of Cell Biology* **154**, 951-960.
- Quan, X., Tsoulos, P., Kuritzky, A., Zhang, R. & Stochaj, U. (2006).** The carrier Msn5p/Kap142p promotes nuclear export of the hsp70 Ssa4p and relocates in response to stress. *Molecular Microbiology* **62**, 592-609.
- Queralt, E. & Igual, J. C. (2003).** Cell Cycle Activation of the Swi6p Transcription Factor Is Linked to Nucleocytoplasmic Shuttling. *Molecular and Cellular Biology* **23**, 3126-3140.
- Rexach, D. G. a. M. (2003).** Molecular Basis for the Rapid Dissociation of Nuclear Localization Signals from Karyopherin α in the Nucleoplasm. *Journal of Biological Chemistry* **278**, 51937-51949.
- Roberts, R. L. & Fink, G. R. (1994).** Elements of a single MAP kinase cascade in *Saccharomyces cerevisiae* mediate two developmental programs in the same cell type: mating and invasive growth. *Genes & Development* **8**, 2974-2985.
- Rothenbusch, U., Sawatzki, M., Chang, Y., Caesar, S. & Schlenstedt, G. (2012).** Sumoylation regulates Kap114-mediated nuclear transport. *The EMBO Journal* **31**, 2461-2472.
- Rouxel, T., Grandaubert, J., Hane, J. K. & other authors (2011).** Effector diversification within compartments of the *Leptosphaeria maculans* genome affected by Repeat-Induced Point mutations. *Nature Communications* **2**, 202.
- Ryo Ueta, A. F. a. Y. Y.-I. (2003).** Pselp Mediates the Nuclear Import of the Iron-responsive Transcription Factor Aft1p in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **278**, 50120-50127.
- Saitoh, H., Fujisawa, S., Ito, A., Mitsuoka, C., Berberich, T., Tosa, Y., Asakura, M., Takano, Y. & Terauchi, R. (2009).** SPM1 encoding a vacuole-localized protease is required for infection-related autophagy of the rice blast fungus *Magnaporthe oryzae*. *Fems Microbiology Letters* **300**, 115-121.
- Saunders, D. G., Dagdas, Y. F. & Talbot, N. J. (2010).** Spatial uncoupling of mitosis and cytokinesis during appressorium-mediated plant infection by the rice blast fungus *Magnaporthe oryzae*. *Plant Cell* **22**, 2417-2428.
- Schroeder AJ, C. X., Xiao Z, Fitzgerald-Hayes M. (1999).** Genetic evidence for interactions between yeast importin alpha (Srp1p) and its nuclear export receptor, Cse1p. *Molecular General Genetics* **261**, 788-795.
- Seedorf, M. & Silver, P. A. (1997).** Importin/karyopherin protein family members required for mRNA export from the nucleus. *Proceedings of the National Academy of Sciences* **94**, 8590-8595.
- Senger, B., Simos, G., Bischoff, F. R., Podtelejnikov, A., Mann, M. & Hurt, E. (1998).** Mtr10p functions as a nuclear import receptor for the mRNA-binding protein Npl3p. *Embo Journal* **17**, 2196-2207.
- Sesma, A. & Osbourn, A. E. (2004).** The rice leaf blast pathogen undergoes developmental processes typical of root-infecting fungi. *Nature* **431**, 582-586.
- Shen, S., Stanford, and Anita K. Hopper (1993).** The *Saccharomyces cerevisiae* LOSI Gene Involved in Pre-tRNA Splicing Encodes a Nuclear

- Protein That Behaves as a Component of the Nuclear Matrix. *Journal of Biological Chemistry* **268**, 19436-19444.
- Shibata, S., Sasaki, M., Miki, T., Shimamoto, A., Furuichi, Y., Katahira, J. & Yoneda, Y. (2006).** Exportin-5 orthologues are functionally divergent among species. *Nucleic Acids Research* **34**, 4711-4721.
- Sloan, K. E., Gleizes, P.-E. & Bohnsack, M. T. (2016).** Nucleocytoplasmic Transport of RNAs and RNA-Protein Complexes. *Journal of Molecular Biology* **428**, 2040-2059.
- Sorefan, K., Pais, H., Hall, A. E., Kozomara, A., Griffiths-Jones, S., Moulton, V. & Dalmay, T. (2012).** Reducing ligation bias of small RNAs in libraries for next generation sequencing. *Silence* **3**, 4-4.
- Sunnåker, M., Zamora-Sillero, E., Dechant, R., Ludwig, C., Busetto, A. G., Wagner, A. & Stelling, J. (2013).** Automatic Generation of Predictive Dynamic Models Reveals Nuclear Phosphorylation as the Key Msn2 Control Mechanism. *Science Signaling* **6**, ra41-ra41.
- Sydorsky, Y., Dilworth, D. J., Yi, E. C., Goodlett, D. R., Wozniak, R. W. & Aitchison, J. D. (2003).** Intersection of the Kap123p-Mediated Nuclear Import and Ribosome Export Pathways. *Molecular and Cellular Biology* **23**, 2042-2054.
- Taberner, F. J., Quilis, I., Sendra, J., Bañó, M. C. & Igual, J. C. (2012).** Regulation of cell cycle transcription factor Swi5 by karyopherin Msn5. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **1823**, 959-970.
- Takano, A., Endo, T. & Yoshihisa, T. (2005).** tRNA Actively Shuttles Between the Nucleus and Cytosol in Yeast. *Science* **309**, 140-142.
- Takeshi Ioyama, A. M., Akio Nomoto and Shusuke Kuge (2001).** Nuclear Import of the Yeast AP-1-like Transcription Factor Yap1p Is Mediated by Transport Receptor Pselp, and This Import Step Is Not Affected by Oxidative Stress. *Journal of Biological Chemistry* **276**, 21863-21869.
- Tamasloukht, M., Sejalon-Delmas, N., Kluever, A., Jauneau, A., Roux, C., Becard, G. & Franken, P. (2003).** Root factors induce mitochondrial-related gene expression and fungal respiration during the developmental switch from asymbiosis to presymbiosis in the arbuscular mycorrhizal fungus *Gigaspora rosea*. *Plant Physiology* **131**, 1468-1478.
- Terry, L. J., Shows, E. B. & Wente, S. R. (2007).** Crossing the Nuclear Envelope: Hierarchical Regulation of Nucleocytoplasmic Transport. *Science* **318**, 1412-1416.
- Titov, A. A. & Blobel, G. (1999).** The Karyopherin Kap122p/Pdr6p Imports Both Subunits of the Transcription Factor Iia into the Nucleus. *Journal of Cell Biology* **147**, 235-246.
- Tkach, J. M., Yimit, A., Lee, A. Y. & other authors (2012).** Dissecting DNA damage response pathways by analyzing protein localization and abundance changes during DNA replication stress. *Nature cell biology* **14**, 966-976.
- Tucker, S. L., Besi, M. I., Galhano, R., Franceschetti, M., Goetz, S., Lenhart, S., Osbourn, A. & Sesma, A. (2010).** Common Genetic Pathways Regulate Organ-Specific Infection-Related Development in the Rice Blast Fungus. *Plant Cell* **22**, 953-972.

- Turrà, D., Segorbe, D. & Pietro, A. D. (2014).** Protein Kinases in Plant-Pathogenic Fungi: Conserved Regulators of Infection. *Annual Review of Phytopathology* **52**, 267-288.
- Ueta, R., Fujiwara, N., Iwai, K. & Yamaguchi-Iwai, Y. (2012).** Iron-Induced Dissociation of the Aft1p Transcriptional Regulator from Target Gene Promoters Is an Initial Event in Iron-Dependent Gene Suppression. *Molecular and Cellular Biology* **32**, 4998-5008.
- Valent, B. & Chumley, F. G. (1991).** Molecular Genetic Analysis of the Rice Blast Fungus, *Magnaporthe grisea*. *Annual Review of Phytopathology* **29**, 443-467.
- van den Bogaart, G., Meinema, A. C., Krasnikov, V., Veenhoff, L. M. & Poolman, B. (2009).** Nuclear transport factor directs localization of protein synthesis during mitosis. *Nat Cell Biol* **11**, 350-356.
- Veneault-Fourrey, C., Barooah, M., Egan, M., Wakley, G. & Talbot, N. J. (2006).** Autophagic Fungal Cell Death Is Necessary for Infection by the Rice Blast Fungus. *Science* **312**, 580-583.
- Wild, T., Horvath, P., Wyler, E. & other authors (2010).** A Protein Inventory of Human Ribosome Biogenesis Reveals an Essential Function of Exportin 5 in 60S Subunit Export. *Plos Biology* **8**, e1000522.
- Wilson, R. A., Jenkinson, J. M., Gibson, R. P., Littlechild, J. A., Wang, Z. Y. & Talbot, N. J. (2007).** Tps1 regulates the pentose phosphate pathway, nitrogen metabolism and fungal virulence. *EMBO Journal* **26**, 3673-3685.
- Wilson, R. A., Gibson, R. P., Quispe, C. F., Littlechild, J. A. & Talbot, N. J. (2010).** An NADPH-dependent genetic switch regulates plant infection by the rice blast fungus. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 21902-21907.
- Windgassen, M., Sturm, D., Cajigas, I. J., Gonzalez, C. I., Seedorf, M., Bastians, H. & Krebber, H. (2004).** Yeast shuttling SR proteins Npl3p, Gbp2p, and Hrb1p are part of the translating mRNPs, and Npl3p can function as a translational repressor. *Molecular and Cellular Biology* **24**, 10479-10491.
- Wu, J., Bao, A., Chatterjee, K., Wan, Y. & Hopper, A. K. (2015).** Genome-wide screen uncovers novel pathways for tRNA processing and nuclear-cytoplasmic dynamics. *Genes & Development* **29**, 2633-2644.
- Xiao, Z., McGrew, J. T., Schroeder, A. J. & Fitzgerald-Hayes, M. (1993).** CSE1 and CSE2, two new genes required for accurate mitotic chromosome segregation in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* **13**, 4691-4702.
- Xu, J. R. & Hamer, J. E. (1996).** MAP kinase and cAMP signalling regulate infection structure formation and pathogenic growth in the rice blast fungus *Magnaporthe grisea*. *Genes and Development* **10**, 2696-2706.
- Xu, J. R., Staiger, C. J. & Hamer, J. E. (1998).** Inactivation of the mitogen-activated protein kinase Mps1 from the rice blast fungus prevents penetration of host cells but allows activation of plant defense responses. *Proceedings of the National Academy of Sciences, USA* **95**, 12713-12718.
- Yoshida, K. & Blobel, G. (2001).** The karyopherin Kap142p/Msn5p mediates nuclear import and nuclear export of different cargo proteins. *Journal of Cell Biology* **152**, 729-739.
- Yoshida, K., Saunders, D. G., Mitsuoka, C. & other authors (2016).** Host specialization of the blast fungus *Magnaporthe oryzae* is associated with

dynamic gain and loss of genes linked to transposable elements. *BMC Genomics* **17**, 370.

Yuksel, U. M., Turker, I., Dilek, G., Dogan, L., Gulcelik, M. A. & Oksuzoglu, B. (2015). Does CSE1L Overexpression Affect Distant Metastasis Development in Breast Cancer? *Oncology Research and Treatment* **38**, 431-434.

Zhang, H., Ma, H., Xie, X. & other authors (2014). Comparative proteomic analyses reveal that the regulators of G-protein signaling proteins regulate amino acid metabolism of the rice blast fungus *Magnaporthe oryzae*. *PROTEOMICS* **14**, 2508-2522.

Chapter 3: Exp5

Supplemental Table S3.2. Reads obtained from small cDNA libraries from WT and $\Delta exp5$ strains. FASTQ files were processed and filtered. Artifacts and HD adapters were removed. Reads longer than 17 nucleotides were aligned on the *Magnaporthe oryzae* 70-15 reference genome (Dean, *et al.*, 2005). The small RNA transcriptome was assembled using the Inchworm software module from the Trinity package (Haas, *et al.*, 2013).

Sample	Total reads	Filtered read	Filtered read %	Unique	Unique %
$\Delta exp5$ _1	25580902	23662841	7.50%	1962459	8.29%
$\Delta exp5$ _2	39423030	37346923	5.27%	2625645	7.03%
$\Delta exp5$ _3	16606918	12293728	25.97%	1242286	10.11%
WT_1	13826696	10577152	23.50%	1067465	10.09%
WT_2	21412622	17398315	18.75%	1559790	8.97%
WT_3	14168509	11826616	16.53%	1266876	10.71%

CHAPTER 4

Molecular characterization of a novel ourmia-like virus from the rice blast fungus *Magnaporthe oryzae*

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Molecular characterization of a novel ssRNA ourmia-like virus from the rice blast fungus *Magnaporthe oryzae*

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ABSTRACT

In this study we characterize a novel positive and single stranded RNA (ssRNA) mycovirus isolated from the rice field isolate of *Magnaporthe oryzae* Guy11. The ssRNA contains a single open reading frame (ORF) of 2,373 nucleotides in length and encodes an RNA-dependent RNA polymerase (RdRp) closely related to ourmiaviruses (plant viruses) and ourmia-like mycoviruses. Accordingly, we name this virus *Magnaporthe oryzae* ourmia-like virus 1 (MOLV1). Although phylogenetic analysis suggests that MOLV1 is closely related to ourmia and ourmia-like viruses, it has some features never reported before within the *Ourmiavirus* genus. 3' RLM-RACE (RNA ligase-mediated rapid amplification of cDNA ends) and extension poly(A) tests (ePAT) suggest that the MOLV1 genome contains a poly(A) tail whereas the three cysteine and the three guanine residues present in 5' and 3' untranslated regions (UTRs) of ourmia viruses are not observed in the MOLV1 sequence. The discovery of this novel viral genome supports the hypothesis that plant pathogenic fungi may have acquired this type of viruses from their host plants.

INTRODUCTION

Mycoviruses or fungal viruses are widespread in all taxonomic groups of fungi. Different outcomes result from the interaction between mycoviruses and their respective fungal hosts. Usually, a mycovirus infection remains latent and shows no symptoms in the fungal host (Ghabrial & Suzuki, 2009). Other mycoviruses are able to cause severe lesions and impair the vegetative growth of their fungal host, causing economic losses as in the case of the cultivated mushroom *Agaricus bisporus* (Ghabrial & Suzuki, 2009, Ghabrial, *et al.*, 2015). Presence of a mycovirus can also be beneficial for all the partners in the tripartite interaction of virus, fungus and plant host. This is the case described for a tropical panic grass and its fungal endophyte when infected by a virus, which increases its survival under stress temperature conditions (Márquez, *et al.*, 2007). The mycovirus can additionally cause fungal hypovirulence in the host plant making it a good option for biological control, as is the case for the well-known *Criphonectria hypovirus 1* (CHV1) and chestnut blight control in Europe (Nuss, 2005, Xie & Jiang, 2014).

Mycoviruses have a diverse genome organization. Most viruses have double stranded RNA (dsRNA) or linear positive (+) ssRNA genomes (Ghabrial & Suzuki, 2009). Unclassified linear negative ssRNAs genomes and circular ssDNA viruses have also been isolated (Liu, *et al.*, 2014, Ghabrial, *et al.*, 2015, Marzano & Domier, 2016, Marzano, *et al.*, 2016). Linear (+) ssRNA mycoviruses are classified into 5 families: *Alphaflexiviridae*, *Gammapflexiviridae*, *Hypoviridae*, *Narnaviridae* and *Barnaviridae* (Andrew King, 2012, Ghabrial, *et al.*, 2015). The *Narnaviridae* family includes the *Mitovirus* and *Narnavirus* genera.

The (+) ssRNA genomes from *Narnaviridae* family are the simplest mycoviruses. Members of this family have a 2-3 Kb genome size, which usually encodes an RNA-dependent RNA polymerase (RdRp) needed for their own replication. The Narnaviruses lack the coat protein (CP) that forms the capsid structure or the movement protein (MP) (Hillman & Cai, 2013). Phylogenetically, the narnaviruses are the closest relatives of the *Ourmiavirus* plant genus (Rastgou, *et al.*, 2009, Hillman & Cai, 2013). Plant ourmiaviruses contain a tripartite ssRNA genome that codes for three proteins, the RdRp, the CP and the MP (Rastgou, *et al.*, 2009). Phylogenetic analyses support the idea that the *Ourmiavirus* genus represents a link between mycoviruses and plant viruses (Hillman & Cai, 2013, Donaire, *et al.*, 2016).

The ascomycetous fungus *Magnaporthe oryzae* causes the damaging rice blast disease and results in approximately 30% yield loss in rice fields every year (Skamnioti & Gurr, 2009). *M. oryzae* was the first plant pathogenic fungus where the presence of a polyhedral virus was reported (Yamashita, *et al.*, 1971, Hunst PL., 1986). Several viruses from different families have been found in this fungal species. *Magnaporthe oryzae* virus 1, 2 and 3 (MoV1, MoV2 and MoV3) are dsRNA viruses belonging to *Totiviridae* family (Yokoi, *et al.*, 2007, Maejima, *et al.*, 2008, Tang, *et al.*, 2015). *Magnaporthe oryzae* chrysovirus 1A and B (MoCV1-A and MoCV1-B) also are dsRNA viruses that belong to the *Chrysoviridae* family. They can impair vegetative and invasive growth during *M. oryzae* host colonisation (Urayama, *et al.*, 2010, Urayama, *et al.*, 2012, Urayama, *et al.*, 2014). Recently, a (+) ssRNA virus has been described for the first time in this fungal species (Ai, *et al.*, 2016), *Magnaporthe oryzae* virus A (MoVA). The genome of MoVA is 3,246 nt in length and contains two in-frame ORFs, one of which encodes a protein with similarity to an RdRp observed in plant viruses of the *Tombusviridae* family.

In this work we describe a second and novel type of ssRNA virus found in the *M. oryzae* rice isolate Guy11, *Magnaporthe oryzae* ourmia-like virus 1 (MOLV1), a polyadenylated (+) ssRNA virus phylogenetically related to plant viruses from the *Ourmiavirus* genus.

RESULTS AND DISCUSSION

Discovery of a new mycovirus in *Magnaporthe oryzae*

During the analysis of sequences derived from small RNA libraries isolated from the rice field isolate Guy11 (Leung, *et al.*, 1988), we detected sequences that were not present in the published *M. oryzae* genome of the laboratory strain 70-15 (Chao & Ellingboe, 1991), a near-isogenic strain of Guy11. We additionally confirmed this by downloading EST sequences from *M. oryzae* Guy11 and aligning them to the reference genome. EST sequences that failed to align were collected and assembled using the Cap3 DNA sequence assembly program (Huang & Madan, 1999). Using these unknown ESTs, we predicted putative ORFs using the getorf tool from the EMBOSS suite (Rice, *et al.*, 2000), which were aligned against the NCBI non-redundant sequence collection (Pruitt, *et al.*, 2005) by blastp (Camacho, *et al.*, 2009). This allowed us to identify transcripts showing similarities with ourmia-like mycoviruses, and plant

viruses of the *Ourmiavirus* genus. Consequently, we named this viral genome *Magnaporthe oryzae ourmia-like virus 1* (MOLV1).

Presence of MOLV1 RNA virus in *M. oryzae* Guy11 strain was confirmed by Northern blotting using total RNA extracts (Fig. 4.1A). Almost all of the retrieved MOLV1 sequence was used as a DNA probe, and actin DNA was used as the control. A band of approximately 2.3 Kb in the *M. oryzae* Guy11 strain was observed, in addition to other smaller bands. These RNA molecules were not detected in the *M. oryzae* laboratory strain 70-15. This is consistent with the absence of these viral sequences from EST collections other than those of the fungal isolate Guy11.

3' RLM-RACE and ePAT experiments suggest that MOLV1 is a polyadenylated (+) ssRNA virus

To identify 5' and 3' untranslated regions (UTRs) of MOLV1 we carried out an RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) (Fig.4.1B). The 3' terminus is usually polyadenylated before conducting a 3' RLM-RACE in non-polyadenylated viruses. In our case we obtained a clear band without adding any adenosine to the RNA sample. We purified and sequenced the largest bands obtained from both 5' and 3' RLM-RACE experiments (asterisks, Fig. 4.1B). The 5' and 3' UTRs of MOLV1 are 116 nt and 430 nt in length, respectively. To confirm the presence of a poly(A) tail in the MOLV1 genome an extension poly(A) test (ePAT) experiment was carried out (Fig.4.1C). This method allows measurement of the poly(A) tail length of polyadenylated RNA molecules (Jänicke, *et al.*, 2012). The difference in band sizes between MOLV1 and the negative control TVN-PAT indicated that the poly(A) tail of MOLV1 is between 25-30 nt in length.

These experiments suggested that the MOLV1 genome is a positive and polyadenylated ssRNA molecule of 2,364 nt, excluding the poly(A) tail. We also performed the ePAT experiment to determine if the BOLV genome contained a poly(A) tail and found that it did not. With the exception of *S. sclerotiorum* mitovirus 1/KL-1 (SsMV-1/KL1) and 2a (CcMV-2a), no other members of the family *Narnaviridae* are polyadenylated at their 3' end (Xie & Ghabrial, 2012, Hillman & Cai, 2013). To our knowledge, this is the first describe ourmia-like mycovirus that has a poly(A) tail. However, this results should be treated cautiously since it is possible to find amplification products with oligodT primers in

Fig.4.1. Characterisation of a novel polyadenylated RNA virus in the rice blast fungus. (A) Northern blots using 30µg of total RNA extracted from *M. oryzae* Guy11 grown on complete media. MOLV1 and actin probes were PCR products amplified with MOLV1_fw (5'- GCTTCGCTTCTTTGGTCGT -3') / MOLV1_rv (5' -GCTCCCTAA CGTGGCGATAA -3'), and Actin_fw (5'- CTTCTCTCGGTGGACTTGG - 3') / Actin-rv (5'- TTCTACAACGAGCTGCGTGT -3') primers, respectively. The actin probe was used as hybridization control. Ethidium bromide gel staining of 28S ribosomal RNA is shown as a loading control. (B) 5' and 3' RLM-RACE reactions were carried out using 5' Outer (5'- ACTCTCAGATCAACCCTGAAGGT-3') / 5' Inner (5'-TCACTAAGCAGTCCAGAGAACGA -3') and 3' Outer (5'-AGGCGTATCGTCGAAAAGCCAA -3') / 3' Inner (5'- TTAAAG TTGTCGGTGAACGCGA -3') primers, respectively. PCR products were loaded into 2% agarose gel. Bands selected for cloning and further sequencing are indicated with asterisks. (C) ePAT experiment using total RNA from *M. oryzae* Guy11. PCR amplification of cDNA derived from 1 µg of RNA sample using gene-specific primers for the virus (ePAT_Fw 5'-GCCGGTCTGCTGTTGGGAT -3' and reverse 5'- TTTTTTTTTTTTCTTAA ACCTACATGCTGGTC -3'). This ePAT reaction generated a fragment of aprox. 468 bp, which reflects the size of the 3'UTR plus the length of the poly(A) tail. The TVN-PAT reaction was used with the same forward primer and the universal ePAT reverse primer (5'- GCGAGCTCCGCGGCCGCG-3'), which generated a fragment of aprox. 438 bp consistent with amplification of the 3'UTR without the poly(A) tail. PCR products from ePAT and TVN-PAT reactions were visualised in 2% agarose gels. (D) Schematic representation of MOLV1 RNA genome showing the ORF, UTRs and location of primers used for ePAT and Northern experiments.

non-polyadenylated RNA viruses due to the presence of small fractions of gRNA with poly(A) tails (Raju, *et al.*, 1999, Li, *et al.*, 2014) .

The MOLV1 genome has a unique open reading frame (ORF) from position 117 to 1931 (Fig.1D). The translation of this 818 nt ORF generates a polypeptide of 605 aa with a theoretical molecular weight of 67.12 kDa.

A

B

Fig.4.2. Sequence analysis of MOLV1. Leyend next page.

Fig.4.2. Sequence analysis of MOLV1. (A) Alignment showing RdRp conserved motifs I to VIII of MOLV1 (LT593139), RsOLV1 (ALD89131.1), BOLV (LN827955), SsOLV2 (ALD89139.1), RsOLV2 (ALD89132.1), SsOLV1 (ALD89138.1), SaOurV2 (KT598247.1) and SaOurV1 (KT598235.1). **(B)** Phylogenetic tree obtained by the Maximum Likelihood method with the RdRp sequences of 25 mycoviruses, including MOLV1, and 3 plant ourmiaviruses. The RdRp sequence of TMV (ABN79257.1) was included as an outgroup. The gray box shows ourmia-like fungal viruses. All bootstrap values (%) are represented at each node of the tree (test with 1000 replicates). Branch lengths are proportional to the number of amino acid substitutions and are measured by the scale bar. Sequence accession numbers of viruses used are: Botrytis ourmia-like virus (LN827955), Soybean-associated ourmiavirus 1 (KT598235.1), Soybean-associated ourmiavirus 2 (KT598247.1), Sclerotinia sclerotiorum ourmia-like virus 1 (ALD89138.1), Sclerotinia sclerotiorum ourmia-like virus 2 (ALD89139.1), Rhizoctonia solani ourmia-like virus 1 (ALD89131.1) Rhizoctonia solani ourmia-like virus 2 (ALD89132.1), Sclerotinia sclerotiorum mitovirus 6 (AHX84133.1), Ophiostoma mitovirus 3a (NP_660176.1), Sclerotinia sclerotiorum mitovirus 7 (AHE13866.1), Botrytis cinerea mitovirus 1 (YP_002284334.1), Cryphonectria parasitica mitovirus 1-NB631 (NP_660174.1), Cryphonectria cubensis mitovirus 2a (AAR01973.1), Ophiostoma mitovirus 1a (CAJ32466.1), Sclerotinia sclerotiorum mitovirus 2 (AEX91879.1), Cryphonectria cubensis mitovirus 1a (AAR01970.1), Sclerotinia sclerotiorum mitovirus 8 (AHE13867.1), Ophiostoma mitovirus 6 (NP_660181.1), Sclerotinia sclerotiorum mitovirus 5 (AHX84130.1), Ophiostoma mitovirus 4 (NP_660179.1), Phytophthora infestans RNA virus 4 (AEM89293.1), Saccharomyces 20S RNA narnavirus (NP_660178.1), Saccharomyces 23S RNA narnavirus (NP_660177.1), Cassava virus C (CsVC, YP_003104770.1), Ourmia melon virus (OuMV, YP_002019757.1) and Epirus cherry virus (EpCV, YP_002019754.1). Evolutionary analysis was conducted using the software MEGA6 (Tamura, *et al.*, 2013). Multiple sequence alignments of amino acid sequences of viral RdRps were obtained using MUSCLE (Multiple sequence comparison by log-expectation, (Edgar, 2004)) using default parameters. Phylogenetic relationships were inferred using the Maximum Likelihood method based on the WAG+G+F protein evolution model (Whelan & Goldman, 2001), and a bootstrap phylogeny test with 1,000 replicates. All positions with less than 50% site coverage were eliminated.

This protein has higher identity with the RdRp of *Rhizoctonia solani* although it is also similar to other ourmia-like mycoviruses and plant ourmiaviruses (Fig.4.2). The MOLV1 viral protein contains the conserved domains of the RdRp present in (+) ssRNA viruses (Koonin, 1991), including the highly conserved core domain GDD in motif VI (Fig.4.2A). These findings suggest that the ORF of MOLV1 encodes a putative RdRp. The amino acid alignment of MOLV1 with the BOLV sequence showed no conservation of the three cystein residues at the 5' UTR and the three guanine residues at the 3' UTR. The predicted secondary structure indicated the presence of a stable terminal stem-loop structure only for the first 40 nt of MOLV1 (ΔG value of -10.00 kcal/mol) (Fig.4.3A). The last 34 nt of the 3' UTR could be folded into a less stable stem-loop structure with ΔG value of -6.30 kcal/mol. These secondary structures at both

UTRs could play a role in the replication of MOLV1 by providing a recognition site for RdRp and/or protecting the genome from degradation as has been proposed for BOLV and mitoviruses (Hong, *et al.*, 1999, Xie & Ghabrial, 2012, Hillman & Cai, 2013).

Fig. 4.3. Predicted secondary structure of the 5' (left) and 3' (right) UTRs of MOLV1 with their corresponding ΔG in kcal/mol.

MOLV1 is closely related to plant ourmiaviruses and ourmia-like mycoviruses

Full-length amino acid sequences of published RdRps (29 proteins) were selected to construct a phylogenetic tree to infer the evolutionary history of MOLV1 (Fig.4.2B). The RdRp of the tobamovirus tobacco mosaic virus (TMV) was used as an outgroup in the tree. The maximum likelihood (ML) phylogenetic tree was inferred from the amino acid sequences of viral RdRps after removing positions with less than 50% site coverage. The phylogenetic analyses clearly showed two clades. The first clade grouped all mitoviruses. The second clade grouped the two *Saccharomyces* narnaviruses and *Phytophthora infestans* RNA virus (82% bootstrap support), and all the ourmia-like mycoviruses and the plant ourmiaviruses (97% bootstrap support). This ML analysis indicates that fungal ourmia-like viruses are more closely related to plant viruses of the *Ourmiavirus* genus than mycoviruses of the *Narnaviridae* family. As previously shown, this phylogenetic tree places ourmia-like viruses as

more closely related to members of the genus *Narnavirus* than to members of the genus *Mitovirus* (Rastgou, *et al.*, 2009, Cai, *et al.*, 2013, Donaire, *et al.*, 2016).

Different theories have been suggested for mycoviruses evolution (Ghabrial, 1998). The plant ourmiavirus genomes have three RNA molecules which encode three proteins, the RdRp, the CP and the MP (Rastgou, *et al.*, 2009). The identification of ourmia-like mycoviruses such as MOLV1, which contain a single ORF and being closely related to plant ourmia viruses suggest that they might have been originated by gene loss events during their adaptation to the fungal host as has been suggested for other ourmia-like mycoviruses (Donaire, *et al.*, 2016, Marzano & Domier, 2016, Marzano, *et al.*, 2016). However, we cannot discard the possibility of ourmia-like mycovirus being the ancestor of plant ourmiaviruses as it has been proposed (Rastgou, *et al.*, 2009).

In summary, MOLV1 is a novel (+) ssRNA virus isolated from the rice blast fungus closely related to ourmia-like mycoviruses and plant ourmia viruses that exhibits new features such as a poly(A) tail and showed no conservation of the three cystein residues at the 5' UTR and the three guanine residues at the 3' UTR.

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Compliance with ethical standards and conflict of interest

This paper is in compliance with ethical standards for research. The authors declare no conflicts of interest.

MATERIAL AND METHODS

Fungal Strains, Growth Conditions and infections assays

M. oryzae strains growth in this study were the wild-type strain Guy11 (Leung *et al.*, 1988) and the mutants Exp5, MGG_09560.8 which was generated previously (Tucker *et al.*, 2010) and Rbp35, MGG_02741.8 (Franceschetti, *et al.*, 2011, Rodríguez-Romero, *et al.*, 2015). *M. oryzae* strains were incubated in a controlled temperature room at 25°C with a 16h light/8h dark cycle. Media composition of complete medium (CM) was previously described (Sweigard, *et al.*, 1997, Foster, *et al.*, 2003). *M.*

oryzae leaf infection assays were carried out, as previously described (Tucker, *et al.*, 2010, Franceschetti, *et al.*, 2011)

RNA and cDNA isolation

Using a modified protocol of (TLES) LiCl method (Franceschetti, *et al.*, 2011), RNA was extracted from 8-day old fungal mycelia grown on CM agar plates. Two to three additional washes with phenol:chloroform were implemented to avoid RNA degradation. One µg of total RNA treated with DNase (Invitrogen) was reverse transcribed using PrimeScript™ RT reagent Kit (Takara)

Northern assay

Northern blot analyses were performed with total RNA (30 µg) from *Magnaporthe oryzae* Guy11 field isolate strain. This strain is considered as wild-type. 70-15 laboratory strain was used as negative control. Other mutant strains derived from Guy11 (*Δexp5* and *Δrbp35*) and their complemented strains (*Δexp5:Exp5-HA-FLAG* and *Δrbp35:Rbp35-HA-FLAG*) were analyzed to check the presence of MoOLV. Different DNA probes were obtained using as template total cDNA. Actin probe of 1700 nt was generated by Actin-fw (positive sense) (5' CTTCTCTCGGTTGGACTTGG 3') and Actin-rv (negative sense) (5' TTCTACAACGAGCTGCGTGT 3') primers. MoOLV probe of 2300 nt was generated by 5' PCR-MoOLV (5' GCTTTCGCTTCTTTGGTCGT3') and 3' PCR-MoOLV (5' GCTCCCTAACGTGGCGATAA3') primers. DNA probes were labeled with α-dCTP(³²p) 3000Ci/mmol using a High Prime kit (Roche) according to the manufacturer's instructions.

RLM-RACE

Total RNAs were used as templates to perform RNA Ligase Mediated Rapid Amplification of cDNA Ends (RLM-RACE). We used FirstChoice RLM-RACE kit according to the manufacturer's instructions (Life Technologies). PCRs were performed with specific primers and HighFidelity TaqDNA polymerase (Invitrogen). For 5' RACE MoOLV outer primer (negative sense) (5' ACTCTCAGATCAACCCTGAAGGT 3') and MoOLV inner primer (negative sense) (5' TCACTAA GCAGTCCAGAGAACGA 3') were used; and for 3' RACE MoOLV outer primer (positive sense) (5' AGGCGTATCGTCG AAAAGCCAA 3') and MoOLV inner primer (positive sense) (5' TTAAAGTTGTCGG

TGAACGCGA 3') were used. The PCR products were cloned using pGEM®series [Promega] which allow T: A cloning of PCR fragments. In this case is needed heat to 70°C for 30 minutes in the presence of 0.2 mM dATPs, 5 U *Taq* polymerase (Roche), 10 x *Taq* polymerase buffer and 2.5 mM MgCl₂ in a total volume of 10 µL before cloning in order to add a single overhanging A to facilitate cloning into the desired vector. Ligation reactions were performed at 4°C (Sambrook, *et al.*, 1989). After transformation in *Escherichia coli* DH10β competent cells, recombinant clones were selected using α-complementation of *lacZ* (Sambrook, *et al.*, 1989). Plasmid were amplified with M13 universal primers from Individual colonies and sequenced (<http://www.STAVIDA.com>).

Phylogenetic analysis

Evolutionary analysis was conducted using the software MEGA6 (Tamura, *et al.*, 2013). Multiple sequence alignments of amino acid sequences of viral RdRps were obtained using MUSCLE (Multiple sequence comparison by log-expectation, (Edgar, 2004) program, under the default parameters. The phylogenetic relationships was inferred by using the Maximum Likelihood method based on the WAG+G+F protein evolution model (Whelan & Goldman, 2001), using the bootstrap phylogeny test with 1000 replicates. All positions with less than 50% site coverage were eliminated. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

ePAT virus

The ePAT experiment was carried out with 1 µg of total RNA from Guy11 strain. RNA were combined with 1 µL of 100mM PAT-anchor primer (GCGAGCTCCGCGGCCGCGTTTTTTTTTTTTTT) or TVN-ePAT primer (GCGAGCTCCGCGGCCGCGTTTTTTTTTTTTTTVN) for TVN control and brought to a volume of 8 µL with dH₂O in a 200-µL PCR tube. The mixture was incubated at 80°C for 5 min and then cooled to room temperature. Once cooled, the sample was flash-centrifuged, and 12 µL of a master mix was added that contained 4 µL dH₂O, 4 µL Superscript III buffer (Life Technologies), 1 µL 100 mM DTT, 1 µL 10 mM dNTPs, 1 µL RNaseOUT (Life Technologies), and 1 µL (5 U) Klenow polymerase (NEB M0212S) per reaction. The sample was mixed thoroughly by inversion, flash-centrifuged, and then incubated at 37°C for 1 h. The polymerase was then inactivated by increasing the temperature to

80°C for 10 min prior to cooling the reaction to 55°C for 1 min. While maintaining the tubes at that temperature in the block, 1 µL (200 U) of Superscript III (Life Technologies) was added to the tubes. Incubation was then resumed at 55°C for 1 h, followed by inactivation of the reverse transcriptase by increasing the temperature to 80°C for 10 min. For the PCR reactions, cDNA was diluted 1:6 by the addition of 120 µL dH₂O. The PCR reactions were conducted in a total volume of 20 µL using 5 µL of diluted cDNA input and 105 µL OneTaq Hot Start Quick-Load 2X Master mix (NEB). PCR program: 94°C 5min 30X (94°C for 30 seg, 60°C for 30 seg, 72°C for 30 seg); 72°C 1 min. Primers: MoOLV inner primer (positive sense) (5′ TTAAAGTTGTCGGTGAACGCGA 3′) and universal reverse primer ePAT template oligo: (5′ GCGAGCTCCGCGGCCGCGTTTTTTTTTTTT 3′).

REFERENCES

- Ai Y-P, Zhong J, Chen C-Y, Zhu H-J & Gao B-D (2016)** A novel single-stranded RNA virus isolated from the rice-pathogenic fungus *Magnaporthe oryzae* with similarity to members of the family Tombusviridae. *Archives of Virology* 161: 725-729.
- Andrew King EL, Michael J. Adams and Eric B. Carstens (2012)** Virus Taxonomy, 1st Edition Ninth Report of the International Committee on Taxonomy of Viruses. *Elsevier* 1344.
- Cai G, Krychiw JF, Myers K, Fry WE & Hillman BI (2013)** A new virus from the plant pathogenic oomycete *Phytophthora infestans* with an 8 kb dsRNA genome: The sixth member of a proposed new virus genus. *Virology* 435: 341-349.
- Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K & Madden TL (2009)** BLAST+: architecture and applications. *BMC Bioinformatics* 10: 421-421.
- Chao CCT & Ellingboe AH (1991)** Selection for mating competence in *Magnaporthe grisea* pathogenic to rice. *Canadian Journal of Botany/Revue Canadienne de Botanique* 69: 130–134.
- Donaire L, Rozas J & Ayllón MA (2016)** Molecular characterization of Botrytis ourmia-like virus, a mycovirus close to the plant pathogenic genus Ourmiavirus. *Virology* 489: 158-164.
- Edgar RC (2004)** MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* 32: 1792-1797.
- Foster AJ, Jenkinson JM & Talbot NJ (2003)** Trehalose synthesis and metabolism are required at different stages of plant infection by *Magnaporthe grisea*. *EMBO Journal* 22: 225-235.
- Franceschetti M, Bueno E, Wilson RA, Tucker SL, Gómez-Mena C, Calder G & Sesma A (2011)** Fungal Virulence and Development Is Regulated by Alternative Pre-mRNA 3′ End Processing in *Magnaporthe oryzae*. *PLoS Pathogens* 7: e1002441.

- Ghabrial SA (1998)** Origin, adaptation and evolutionary pathways of fungal viruses. *Virus Genes* 16 (1): 119-131.
- Ghabrial SA & Suzuki N (2009)** Viruses of Plant Pathogenic Fungi. *Annual Review of Phytopathology* 47: 353-384.
- Ghabrial SA, Castón JR, Jiang D, Nibert ML & Suzuki N (2015) 50-plus years of fungal viruses. *Virology* 479-480: 356-368.
- Hillman BI & Cai G (2013)** Chapter Six - The Family Narnaviridae: Simplest of RNA Viruses. *Advances in Virus Research*, Vol. Volume 86 (Said AG, ed.^eds.), p.^pp. 149-176. Academic Press.
- Hong Y, Dover SL, Cole TE, Brasier CM & Buck KW (1999)** Multiple Mitochondrial Viruses in an Isolate of the Dutch Elm Disease Fungus *Ophiostoma novo-ulmi*. *Virology* 258: 118-127.
- Huang X & Madan A (1999)** CAP3: A DNA Sequence Assembly Program. *Genome Research* 9: 868-877.
- Hunst PL, LHF, Rossi AE (1986)** Variation in a double-stranded RNA from isolates of *Pyricularia oryzae*. *Phytopathology* 76: 674-678.
- Jänicke A, Vancuylenberg J, Boag PR, Traven A & Beilharz TH (2012)** ePAT: A simple method to tag adenylated RNA to measure poly(A)-tail length and other 3' RACE applications. *RNA* 18: 1289-1295.
- Koonin EV (1991)** The phylogeny of RNA-dependent RNA polymerases of positive-strand RNA viruses. *Journal of General Virology* 72: 2197-2206.
- Leung H, Borromeo E, Bernardo M & Notteghem JL (1988)** Genetic analysis of virulence in the rice blast fungus *Magnaporthe grisea*. *Phytopathology* 78: 1227-1233.
- Li W, Zhang Y, Zhang C, Pei X, Wang Z & Jia S (2014)** Presence of poly(A) and poly(A)-rich tails in a positive-strand RNA virus known to lack 3' poly(A) tails. *Virology* 454-455: 1-10.
- Liu L, Xie J, Cheng J, Fu Y, Li G, Yi X & Jiang D (2014)** Fungal negative-stranded RNA virus that is related to bornaviruses and nyaviruses. *Proceedings of the National Academy of Sciences of the United States of America* 111: 12205-12210.
- Maejima K, Himeno M, Komatsu K, Kakizawa S, Yamaji Y, Hamamoto H & Namba S (2008)** Complete nucleotide sequence of a new double-stranded RNA virus from the rice blast fungus, *Magnaporthe oryzae*. *Archives of Virology* 153: 389-391.
- Márquez LM, Redman RS, Rodriguez RJ & Roossinck MJ (2007)** A Virus in a Fungus in a Plant: Three-Way Symbiosis Required for Thermal Tolerance. *Science* 315: 513-515.
- Marzano S-YL & Domier LL (2016)** Novel mycoviruses discovered from metatranscriptomics survey of soybean phyllosphere phytobiomes. *Virus Research* 213: 332-342.
- Marzano S-YL, Nelson BD, Ajayi-Oyetunde O, et al. (2016)** Identification of diverse mycoviruses through metatranscriptomics characterization of the viromes of five major fungal plant pathogens. *Journal of Virology*.
- Nuss DL (2005)** Hypovirulence: Mycoviruses at the fungal-plant interface. *Nat Rev Micro* 3: 632-642.
- Pruitt KD, Tatusova T & Maglott DR (2005)** NCBI Reference Sequence (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins. *Nucleic Acids Research* 33: D501-D504.

- Raju R, Hajjou M, Hill KR, Botta V & Botta S (1999)** In Vivo Addition of Poly(A) Tail and AU-Rich Sequences to the 3' Terminus of the Sindbis Virus RNA Genome: a Novel 3'-End Repair Pathway. *Journal of Virology* 73: 2410-2419.
- Rastgou M, Habibi MK, Izadpanah K, et al. (2009)** Molecular characterization of the plant virus genus Ourmiavirus and evidence of inter-kingdom reassortment of viral genome segments as its possible route of origin. *Journal of General Virology* 90: 2525-2535.
- Rice P, Longden I & Bleasby A (2000)** EMBOSS: The European Molecular Biology Open Software Suite. *Trends in Genetics* 16: 276-277.
- Rodríguez-Romero J, Franceschetti M, Bueno E & Sesma A (2015)** Multilayer Regulatory Mechanisms Control Cleavage Factor I Proteins in Filamentous Fungi. *Nucleic Acids Research* 43: 179-195.
- Sambrook J, Fritsch EF & Maniatis T (1989)** *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N Y.
- Skamnioti P & Gurr SJ (2009)** Against the grain: safeguarding rice from rice blast disease. *Trends in Biotechnology* 27: 141-150.
- Sweigard JA, Chumley F, Carroll A, Farrall L & Valent B (1997)** A series of vectors for fungal transformation. *Fungal Genetics Newsletter* 44: 52-53.
- Tamura K, Stecher G, Peterson D, Filipski A & Kumar S (2013)** MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution* 30: 2725-2729.
- Tang L, Hu Y, Liu L, et al. (2015)** Genomic organization of a novel victorivirus from the rice blast fungus *Magnaporthe oryzae*. *Arch Virol*.
- Tucker SL, Besi MI, Galhano R, et al. (2010)** Common Genetic Pathways Regulate Organ-Specific Infection-Related Development in the Rice Blast Fungus. *Plant Cell* 22: 953-972.
- Urayama S, Ohta T, Onozuka N, et al. (2012)** Characterization of *Magnaporthe oryzae* Chrysovirus 1 Structural Proteins and Their Expression in *Saccharomyces cerevisiae*. *Journal of Virology* 86: 8287-8295.
- Urayama S, Kato S, Suzuki Y, et al. (2010)** Mycoviruses related to chrysovirus affect vegetative growth in the rice blast fungus *Magnaporthe oryzae*. *Journal of General Virology* 91: 3085-3094.
- Urayama S, Sakoda H, Takai R, et al. (2014)** A dsRNA mycovirus, *Magnaporthe oryzae* chrysovirus 1-B, suppresses vegetative growth and development of the rice blast fungus. *Virology* 448: 265-273.
- Whelan S & Goldman N (2001)** A General Empirical Model of Protein Evolution Derived from Multiple Protein Families Using a Maximum-Likelihood Approach. *Molecular Biology and Evolution* 18: 691-699.
- Xie J & Ghabrial SA (2012)** Molecular characterizations of two mitoviruses co-infecting a hyovirulent isolate of the plant pathogenic fungus *Sclerotinia sclerotiorum*. *Virology* 428: 77-85.
- Xie J & Jiang D (2014)** New Insights into Mycoviruses and Exploration for the Biological Control of Crop Fungal Diseases. *Annual Review of Phytopathology* 52: 45-68.
- Yamashita S, Doi Y & Yora K (1971)** A Polyhedral Virus Found in Rice Blast Fungus, *Pyricularia oryzae* Cavara. *Japanese Journal of Phytopathology* 37: 356-359.

Chapter 4: MOLV1

Yokoi T, Yamashita S & Hibi T (2007) The nucleotide sequence and genome organization of Magnaporthe oryzae virus 1. *Archives of Virology* 152: 2265-2269

GENERAL DISCUSSION

GENERAL DISCUSSION

In this study, we sought to characterise novel genetic determinants for successful colonisation of plant organs by *M. oryzae*. The two major outcomes of this research project have been the identification and characterisation of two genes associated with *M. oryzae* pathogenicity. These two genes were previously identified during the screening of a *M. oryzae* T-DNA insertional library looking for pathogenicity-deficient mutants. The *TPC1* and *EXP5* genes were disrupted in the T-DNA mutants M1422 and M1373, respectively. The initial screening was carried out on hydrophilic polystyrene surfaces (PS-PHIL) and rice roots. Both genes were found to be required for root infection as well as aerial infection.

The T-DNA insertion of M1422 was located within the open reading frame of a gene (MGG_01285.8) encoding a Zn(II)₂Cys₆ binuclear cluster protein, a domain only found in fungi. We named the gene *TPC1* for Transcription factor for Polarity Control 1. Our results indicate that TPC1 plays an important role in vegetative fungal growth and *in planta* fungal colonisation. This study also shows that the establishment of polarity in *M. oryzae* is intimately linked to general developmental processes. Autophagic cell death is linked with appressorium function and penetration in *M. oryzae* (Veneault-Fourrey, *et al.*, 2006) and it is an essential prerequisite for appressorium function (Talbot & Kershaw, 2009). Tpc1 is required for penetration, and is also required for infection-associated autophagy. Although M1422 conidia are able to undergo conidial cell death, the cellular localization of autophagosomes and glycogen deposits suggests that the process is delayed. Consequently, Tpc1 seems to be an upstream positive regulator of infection-associated autophagy during appressorium maturation. The formation of a penetration peg at the base of the appressorial pore is a cellular process intrinsically linked to polar growth (Macara & Mili, 2008, Riquelme, 2013). The F-actin cytoskeleton plays a crucial role during germ tube repolarisation and penetration peg emergence, and it is known to be regulated by the Nox proteins (Ryder, *et al.*, 2013).

The studies on Tpc1 lead us to the discovery and characterisation of NoxD, a protein component of the fungal NADPH complex. The oxidation-reduction processes are significantly affected in $\Delta tpc1$, likely due in part to the down-regulation of the fungal homologue of the p22phox NADPH subunit, the *NOXD* gene (Lacaze, *et al.*, 2014, Siegmund, *et al.*, 2015). The deletion of this gene makes the fungus

unable to infect leaves and roots of the rice host. We have confirmed that NoxD is part of the NADPH complex based on its interaction with Nox1. However, NoxD does not interact with NoxR or Nox2, the other two subunits of the NADPH complex. We have also confirmed the requirement of NoxD for superoxide generation and sexual reproduction in *M. oryzae*, consistent with NoxD functions in *B. cinerea* and *P. anserine* (Lacaze, *et al.*, 2014, Siegmund, *et al.*, 2015). Nox2-dependent process initiates septin ring formation, while Nox1 is necessary for maintenance of this conformation. NoxD may therefore be associated at a relatively early stage in recruiting Nox1 to the appressorium pore. This can explain why its absence results in a more severe phenotype with respect to actin and septin assembly at the pore. Since the NADPH oxidase complex regulates septin assembly and F-actin remodelling at the base of the appressorium in *M. oryzae*, Tpc1 orchestrates cytoskeleton remodeling during penetration peg formation by regulating NoxD expression. It is worth noting that *B. cinerea* $\Delta noxD$ mutant shows growth defects in the presence of oxidative stressors in contrast to the wild type growth that *M. oryzae* $\Delta noxD$ exhibits. This suggests that NoxD can play different roles in fungi. Consequently, a diversification of the cellular functions of NoxD has occurred during fungal evolution. This result also suggests differences in ROS-mediated signalling pathways in the fungal kingdom.

The second T-DNA mutant M1373 was inserted in the coding sequence of the *M. oryzae* protein orthologue of the human exportin-5 and the yeast Msn5 (Exp5, MGG_09560.8). Exp5 is one of the 13 karyopherins present in *M. oryzae* and it is able to transport cargoes between nucleus and cytoplasm in a Ran-GTP dependent manner (Tucker, *et al.*, 2010, Sloan, *et al.*, 2016). Exp5 is required for full virulence in *M. oryzae*. The $\Delta exp5$ mutants barely produce any disease symptoms on roots. However, they are still able to cause disease symptoms on leaves, producing similar lesion numbers at penetration points although their perimeter is significantly smaller compared to the wild type strain, which suggests deficiencies in invasive growth (Tucker, *et al.*, 2010). These differences indicate that some of its cargoes can confer the ability to infect more efficiently roots. Based on these results, the role of Exp5 in *M. oryzae* has been studied in more detail to identify cargoes and RNAs associated with this karyopherin.

First, we have looked at the expression levels of the rest of karyopherins in wild type and $\Delta exp5$ to understand their relevance and

potential links with Exp5 under different nutritional conditions. The expression of all the karyopherins increases in the background of the $\Delta exp5$ mutant in CM. The most highly expressed kaps are Kap123 and Pse1 (group III) and Nmd5 (group IV). They show a 95-, 61- and 32-fold increase in gene expression in $\Delta exp5$. In yeast, Kap123 exports ribosomal proteins and histones, Pse1 is an essential karyopherin that exports transcription factors and ribosomal proteins and Nmd5 translocates primarily transcription factors for its activation by phosphorylation in the cytoplasm. The overexpression of Kap123 and Pse1 correlates with the need of the cell to maintain fast growth rates, i.e. a high number of ribosomal proteins and histones in the proper cellular compartment (Sydorsky, *et al.*, 2003, Kobayashi & Matsuura, 2013).

The lack of carbon and nitrogen sources reduces drastically cell growth, and canonical protein translation is drastically reduced, except for the mRNAs and proteins required for helping the fungus to survive under these conditions (Spriggs, *et al.*, 2008). Very low expression level of the kaps has been detected in carbon and nitrogen starved cells even in the absence of Exp5. The reduction of nucleo-cytoplasmic receptors is in accordance with the drastic reduction of protein levels in the cell. In the background of the mutant in carbon starved cells, in addition to Kap123 and Pse1 two new karyopherins are overexpressed, Kap104 (group II) and Cse1 (group III). The yeast Kap104 is involved in export of mRNA binding proteins whereas Cse1 is an essential karyopherin involved in cell cycle, cell division and mitotic chromosome segregation. Cse1 also mediates the nuclear export of Kap α and Pse1 in *S. cerevisiae* (Hood & Silver, 1998, Künzler & Hurt, 1998, Solsbacher, *et al.*, 1998, Yuksel, *et al.*, 2015). Kap α is the α -karyopherin that forms a dimer with Rsl1 to mediate import of different nuclear proteins. Additionally, Kap α is involved in cotranslational protein degradation and binds ribosome-bound nascent polypeptides (Ha, *et al.*, 2014). In nitrogen starved cells, not only Nmd5, Cse1 and Pse1 but also Crm1 (group III) increased their expression in $\Delta exp5$. Crm1 is an essential yeast protein that can bind DNA and is involved in DNA damage checkpoint regulation and protein quality control (Choi, *et al.*, 2012, Tkach, *et al.*, 2012, Gallina, *et al.*, 2015). Taking in account the compensation effects at expression level of Kap123, Pse1, Cse1 and Nmd5 in the $\Delta exp5$ background, and the observed interaction of Exp5 with the kaps Rsl1, Crm1 and Pse1, our results suggest that Exp5

participates at least in two important processes previously associated with infection in *M. oryzae*, i.e. cell cycle control (Saunders, *et al.*, 2010) and protein quality control (Yi, *et al.*, 2009). Twelve components of the 26S proteasome and Cdc48 immunoprecipitate with Exp5 and corroborate the potential links of Exp5 with these cellular processes. The hyperbranching phenotype associated with $\Delta exp5$ growth in PS-PHIL surfaces, the inability to develop appressoria in hydrophobic surfaces and the interaction of Exp5 with microtubule-associated proteins further support the potential role of Exp5 in cell cycle control.

The *M. oryzae* $\Delta rac1$ mutant is not able to infect leaves or roots indicating that maintenance of polarized cell growth is important for infection in both tissues (Chen, *et al.*, 2008). The $\Delta exp5$ hyperbranching phenotype is also an indicative of polarity-associated defects (Tucker, *et al.*, 2010). *M. oryzae* Rac1 interacts with Nox1 and Nox2 NADPH oxidases (Chen, *et al.*, 2008), and Rac1 also immunoprecipitates with Exp5. It is possible that Exp5 facilitates the transport of Rac1 to regulate the NADPH complex. Interestingly, our results converge at this point. The transcription factor Tpc1 also controls the NADPH complex by regulating NoxD expression. Further experiments will help to confirm if Exp5 impose other level of regulation in the NADPH complex by participating in the nucleo-cytoplasmic transport of Tpc1. Tpc1:GFP localisation in the background of the $\Delta exp5$ mutant will help to confirm or exclude this hypothesis.

The up-regulation of *M. oryzae* Nmd5 suggests that Exp5 can export certain type of transcription factors. In yeast, Ste12 is found among Nmd5 cargoes. This transcription factor is activated by the Fus3/Kss1 MAPK signalling cascade. In *M. oryzae* the transcription factor Mst12 is the orthologue of the yeast Ste12. Mst12 plays an important role in appressorium-mediated leaf penetration and invasive growth (Errede & Ammerer, 1989, Roberts & Fink, 1994, Bardwell, *et al.*, 1998). The reduction on symptoms observed in $\Delta exp5$ can be due to an impaired transport of the active form of Mst12. Alternatively, the localisation of Mst12 is possibly regulated by its phosphorylation status, which is controlled by the MAPK Pmk1 in *M. oryzae*. Overexpression of Nmd5 in the absence of Exp5 can be linked to nuclear transport defects of Pmk1, one of the two MAPKs that immunoprecipitate with Exp5 and the orthologue of the yeast Fus3/Kss1 kinases. This signalling pathway is conserved in fungi and controls the infection process of all the fungal pathogenic species tested (Turrà, *et al.*, 2014). During *M. oryzae*

vegetative growth Pmk1 is located in the cytoplasm while Pmk1 localises in the nucleus of the appressoria (Bruno *et al.*, 2004). Exp5 can be involved in the translocation of Pmk1 during appressorium development. The defects in appressorium formation displayed by the $\Delta exp5$ mutant on coverslips supports this hypothesis.

To address if Exp5 is involved in sRNA metabolism, sRNA libraries from wild type and mutant were generated and sequenced. These experiments have shown us that overall all the transcripts are down-regulated in $\Delta exp5$ except those derived from introns, intergenetic regions and transposable elements. Specifically, tRNAs are down-regulated in $\Delta exp5$. The northern experiments carried out using the tRNA^{Lys} and tRNA^{Met} suggest that Exp5 is involved in their export to the cytoplasm. The transport of tRNAs was considered one-way movement from nucleus to the cytoplasm until the discovery of the tRNA retrograde pathways (Shaheen & Hopper, 2005, Takano, *et al.*, 2005, Whitney, *et al.*, 2007, Hopper, 2013). In *S. cerevisiae*, Msn5 is implicated in re-export of tRNAs to the cytoplasm after a carbon starvation period. Msn5 is also required for the retrograde import of tRNAs into the nucleus (Takano, *et al.*, 2005, Shibata, *et al.*, 2006, Murthi, *et al.*, 2010). In *M. oryzae*, the involvement of Exp5 in this process might also be relevant since Los1, an important kap highly conserved required for the first transport of immature tRNAs into cytoplasm showed 13-fold increase in expression levels in the mutant background.

The analysis of sRNA populations has provided two additional results. First, we have observed a nuclear accumulation of sRNAs derived from retrotransposons in Exp5, which suggests an involvement of this karyopherin in their transport. Second, it allowed us to discover the presence of the ssRNA virus MOLV1. The protein sequence of the viral RNA polymerase shows similarities with *Ourmia*-like mycoviruses, and plant viruses from *Ourmiavirus* genus. MOLV1 is a single-stranded polyadenylated (+) ssRNA virus, and it is the first *Ourmia*-like virus whose RNA is polyadenylated. It is at least surprising that this virus was also not found earlier since this is a reference strain and several labs work with this fungal strain worldwide. Based on our preliminary infection tests, the comparison of the wild type strain Guy11 with the near isogenic strain 70-15 suggested that the presence of the virus does not limit Guy11 infection ability although it seems to affect moderately *in vitro* fungal

growth. The introduction of the MOLV1 virus into 70-15 strain will help to confirm this effect in Guy11 fitness.

In this study, we aimed to better understand *M. oryzae* infection-related processes. We have achieved this by expanding our knowledge in *M. oryzae* infection processes through the characterisation of the transcription factor Tpc1 and the karyopherin Exp5. Pathogenicity pathways related to leaf infection have been studied in exquisite detail although other pathways of entry into the plant such as neck, panicle or roots are still largely uncharacterized. New studies towards understanding these novel pathways of entry in the host plant looking at the participation in these pathways of the NADPH oxidases regulated by Tpc1 and the proteins translocated by Exp5 will provide new data for a more sustainable and durable control methods against this devastating rice disease.

REFERENCES

- Ameres SL & Zamore PD (2013)** Diversifying microRNA sequence and function. *Nature Reviews Molecular Cell Biology* 14: 475-488.
- Bardwell L, Cook JG, Voora D, Baggott DM, Martinez AR & Thorner J (1998)** Repression of yeast Ste12 transcription factor by direct binding of unphosphorylated Kss1 MAPK and its regulation by the Ste7 MEK. *Genes & Development* 12: 2887-2898.
- Bartel DP (2009)** MicroRNAs: Target Recognition and Regulatory Functions. *Cell* 136: 215-233.
- Baulcombe DC (2015)** VIGS, HIGS and FIGS: small RNA silencing in the interactions of viruses or filamentous organisms with their plant hosts. *Current Opinion in Plant Biology* 26: 141-146.
- Borges F & Martienssen RA (2015)** The expanding world of small RNAs in plants. *Nature Reviews Molecular Cell Biology* 16: 727-741.
- Chen J, Zheng W, Zheng S, et al. (2008)** Rac1 Is Required for Pathogenicity and Chm1-Dependent Conidiogenesis in Rice Fungal Pathogen *Magnaporthe grisea*. *PLoS Pathogens* 4: e1000202.
- Choi D-H, Kwon S-H, Kim J-H & Bae S-H (2012)** *Saccharomyces cerevisiae* Cmr1 protein preferentially binds to UV-damaged DNA in vitro. *Journal of Microbiology* 50: 112-118.
- Errede B & Ammerer G (1989)** STE12, a protein involved in cell-type-specific transcription and signal transduction in yeast, is part of protein-DNA complexes. *Genes & Development* 3: 1349-1361.
- Gallina I, Colding C, Henriksen P, et al. (2015)** Cmr1/WDR76 defines a nuclear genotoxic stress body linking genome integrity and protein quality control. *Nature Communications* 6: 6533.
- Gowda M, Nunes CC, Sailsbery J, et al. (2010)** Genome-wide characterization of methylguanosine-capped and polyadenylated small RNAs in the rice blast fungus *Magnaporthe oryzae*. *Nucleic Acids Research* 38: 7558-7569.

- Ha S-W, Ju D & Xie Y (2014)** Nuclear Import Factor Srp1 and Its Associated Protein Sts1 Couple Ribosome-bound Nascent Polypeptides to Proteasomes for Cotranslational Degradation. *The Journal of Biological Chemistry* 289: 2701-2710.
- Han BW & Zamore PD (2014)** piRNAs. *Current Biology* 24: R730-R733.
- Hood JK & Silver PA (1998)** Cse1p Is Required for Export of Srp1p/Importin- α from the Nucleus in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* 273: 35142-35146.
- Hopper AK (2013)** Transfer RNA Post-Transcriptional Processing, Turnover, and Subcellular Dynamics in the Yeast *Saccharomyces cerevisiae*. *Genetics* 194: 43-67.
- Kadotani N, Nakayashiki H, Tosa Y & Mayama S (2004)** One of the Two Dicer-like Proteins in the Filamentous Fungi *Magnaporthe oryzae* Genome Is Responsible for Hairpin RNA-triggered RNA Silencing and Related Small Interfering RNA Accumulation. *Journal of Biological Chemistry* 279: 44467-44474.
- Kadotani N, Murata T, Quoc NB, Adachi Y & Nakayashiki H (2008)** Transcriptional Control and Protein Specialization Have Roles in the Functional Diversification of Two Dicer-Like Proteins in *Magnaporthe oryzae*. *Genetics* 180: 1245-1249.
- Kobayashi J & Matsuura Y (2013)** Structural Basis for Cell-Cycle-Dependent Nuclear Import Mediated by the Karyopherin Kap121p. *Journal of Molecular Biology* 425: 1852-1868.
- Künzler M & Hurt EC (1998)** Cse1p functions as the nuclear export receptor for importin α in yeast. *FEBS Letters* 433: 185-190.
- Lacaze I, Lalucque H, Siegmund U, Silar P & Brun S (2014)** Identification of NoxD/Pro41 as the homologue of the p22(phox) NADPH oxidase subunit in fungi. *Molecular Microbiology* 95: 1006-1024.
- Lambou K, Malagnac F, Barbisan C, Tharreau D, Lebrun MH & Silar P (2008)** The Crucial Role of the Pls1 Tetraspanin during Ascospore Germination in *Podospora anserina* Provides an Example of the Convergent Evolution of Morphogenetic Processes in Fungal Plant Pathogens and Saprobes. *Eukaryotic Cell* 7: 1809-1818.
- Lee HC, Chang SS, Choudhary S, Aalto AP, Maiti M, Bamford DH & Liu Y (2009)** qiRNA is a new type of small interfering RNA induced by DNA damage. *Nature* 459: 274-U163.
- Lee HC, Li LD, Gu WF, et al. (2010)** Diverse Pathways Generate MicroRNA-like RNAs and Dicer-Independent Small Interfering RNAs in Fungi. *Molecular Cell* 38: 803-814.
- Lim RSM & Kai T (2015)** A piece of the pi(e): The diverse roles of animal piRNAs and their PIWI partners. *Seminars in Cell & Developmental Biology* 47-48: 17-31.
- Macara IG & Mili S (2008)** Polarity and Differential Inheritance-Universal Attributes of Life? *Cell* 135: 801-812.
- Murthi A, Shaheen HH, Huang HY, Preston MA, Lai TP, Phizicky EM & Hopper AK (2010)** Regulation of tRNA Bidirectional Nuclear-Cytoplasmic Trafficking in *Saccharomyces cerevisiae*. *Molecular Biology of the Cell* 21: 639-649.

- Nicolas FE, Torres-Martinez S & Ruiz-Vazquez RM (2003)** Two classes of small antisense RNAs in fungal RNA silencing triggered by non-integrative transgenes. *Embo Journal* 22: 3983-3991.
- Nicolas FE, Torres-Martinez S & Ruiz-Vazquez RM (2013)** Loss and Retention of RNA Interference in Fungi and Parasites. *Plos Pathogens* 9: ARTN e1003089.
- Nicolas FE, de Haro JP, Torres-Martinez S & Ruiz-Vazquez RM (2007)** Mutants defective in a *Mucor circinelloides* dicer-like gene are not compromised in siRNA silencing but display developmental defects. *Fungal Genet Biol* 44: 504-516.
- Nicolas FE, Vila A, Moxon S, Cascales MD, Torres-Martinez S, Ruiz-Vazquez RM & Garre V (2015)** The RNAi machinery controls distinct responses to environmental signals in the basal fungus *Mucor circinelloides*. *BMC Genomics* 16: 237.
- Nunes CC, Gowda M, Sailsbery J, et al. (2011)** Diverse and tissue-enriched small RNAs in the plant pathogenic fungus, *Magnaporthe oryzae*. *BMC Genomics* 12: 288.
- Raman V, Simon SA, Romag A, et al. (2013)** Physiological stressors and invasive plant infections alter the small RNA transcriptome of the rice blast fungus, *Magnaporthe oryzae*. *BMC Genomics* 14: 326.
- Riquelme M (2013)** Tip Growth in Filamentous Fungi: A Road Trip to the Apex. *Annual Review of Microbiology* 67: 587-609.
- Roberts RL & Fink GR (1994)** Elements of a single MAP kinase cascade in *Saccharomyces cerevisiae* mediate two developmental programs in the same cell type: mating and invasive growth. *Genes & Development* 8: 2974-2985.
- Ryder LS, Dagdas YF, Mentlak TA, et al. (2013)** NADPH oxidases regulate septin-mediated cytoskeletal remodeling during plant infection by the rice blast fungus. *Proc Natl Acad Sci U S A* 110: 3179-3184.
- Saunders DG, Dagdas YF & Talbot NJ (2010)** Spatial uncoupling of mitosis and cytokinesis during appressorium-mediated plant infection by the rice blast fungus *Magnaporthe oryzae*. *Plant Cell* 22: 2417-2428.
- Sesma, A. (2016).** RNA metabolism and regulation of virulence programs in fungi. *Seminars in Cell & Developmental Biology* 57, 120-127.
- Shaheen HH & Hopper AK (2005)** Retrograde movement of tRNAs from the cytoplasm to the nucleus in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences of the United States of America* 102: 11290-11295.
- Shibata S, Sasaki M, Miki T, Shimamoto A, Furuichi Y, Katahira J & Yoneda Y (2006)** Exportin-5 orthologues are functionally divergent among species. *Nucleic Acids Research* 34: 4711-4721.
- Siegmund U, Marschall R & Tudzynski P (2015)** BcNoxD, a putative ER protein, is a new component of the NADPH oxidase complex in *Botrytis cinerea*. *Molecular Microbiology* 95: 988-1005.
- Sloan KE, Gleizes P-E & Bohnsack MT (2016)** Nucleocytoplasmic Transport of RNAs and RNA-Protein Complexes. *Journal of Molecular Biology* 428: 2040-2059.
- Solsbacher J, Maurer P, Bischoff FR & Schlenstedt G (1998)** Cse1p Is Involved in Export of Yeast Importin α from the Nucleus. *Molecular and Cellular Biology* 18: 6805-6815.

- Spriggs, K. A., Stoneley, M., Bushell, M. & Willis, A. E. (2008).** Re-programming of translation following cell stress allows IRES-mediated translation to predominate. *Biology of the Cell* **100**, 27-38.
- Sydorskyy Y, Dilworth DJ, Yi EC, Goodlett DR, Wozniak RW & Aitchison JD (2003)** Intersection of the Kap123p-Mediated Nuclear Import and Ribosome Export Pathways. *Molecular and Cellular Biology* **23**: 2042-2054.
- Takano A, Endo T & Yoshihisa T (2005)** tRNA Actively Shuttles Between the Nucleus and Cytosol in Yeast. *Science* **309**: 140-142.
- Talbot NJ & Kershaw MJ (2009)** The emerging role of autophagy in plant pathogen attack and host defence. *Current Opinion in Plant Biology* **12**: 444-450.
- Tkach JM, Yimit A, Lee AY, et al. (2012)** Dissecting DNA damage response pathways by analyzing protein localization and abundance changes during DNA replication stress. *Nature cell biology* **14**: 966-976.
- Trieu TA, Calo S, Nicolas FE, et al. (2015)** A Non-canonical RNA Silencing Pathway Promotes mRNA Degradation in Basal Fungi. *Plos Genetics* **11**: e1005168.
- Tucker SL, Besi MI, Galhano R, et al. (2010)** Common Genetic Pathways Regulate Organ-Specific Infection-Related Development in the Rice Blast Fungus. *Plant Cell* **22**: 953-972.
- Veneault-Fourrey C, Barooah M, Egan M, Wakley G & Talbot NJ (2006)** Autophagic Fungal Cell Death Is Necessary for Infection by the Rice Blast Fungus. *Science* **312**: 580-583.
- Weiberg A & Jin HL (2015)** Small RNAs - the secret agents in the plant-pathogen interactions. *Current Opinion in Plant Biology* **26**: 87-94.
- Weiberg A, Wang M, Bellinger M & Jin H (2014)** Small RNAs: a new paradigm in plant-microbe interactions. *Annu Rev Phytopathol* **52**: 495-516.
- Whitney ML, Hurto RL, Shaheen HH & Hopper AK (2007)** Rapid and Reversible Nuclear Accumulation of Cytoplasmic tRNA in Response to Nutrient Availability. *Molecular Biology of the Cell* **18**: 2678-2686.
- Yi M, Chi MH, Khang CH, Park SY, Kang S, Valent B & Lee YH (2009)** The ER Chaperone LHS1 Is Involved in Asexual Development and Rice Infection by the Blast Fungus *Magnaporthe oryzae*. *Plant Cell* **21**: 681-695.
- Yuksel UM, Turker I, Dilek G, Dogan L, Gulcelik MA & Oksuzoglu B (2015)** Does CSE1L Overexpression Affect Distant Metastasis Development in Breast Cancer? *Oncology Research and Treatment* **38**: 431-434.

CONCLUSIONS

CONCLUSIONS

1. Tpc1 is a transcription factor with a Zn₂Cys₆ binuclear cluster domain involved in the regulation of autophagy, F-actin network and glycogen degradation during appressorium development.
2. Tpc1 controls the spatial and temporal organization of F-actin cytoskeleton through the regulation of the *NOXD* gene and the NADPH complex during appressorium repolarization.
3. *M. oryzae* NoxD is an ER- and plasma membrane-localised protein that interacts with Nox1, a component of the fungal NADPH oxidase complex. In *M. oryzae* NoxD is required for superoxide production, sexual development and plant penetration.
4. Nuclear accumulation of sRNAs derived from retrotransposons and tRNAs in the $\Delta exp5$ mutant suggest the involvement of Exp5 in their export to the cytoplasm.
5. Karyopherins Pse1, Cse1 and Kap123 immunoprecipitate with Exp5 and are overexpressed in the mutant background in several media. These evidences suggest that Exp5 either shares cargoes with these three karyopherins or participates in common cellular processes.
6. *In vivo* immunoprecipitation assays allowed us to identify potential candidates translocated by Exp5, including proteins involved in several cellular processes such as mitochondrial protein import, primary metabolism, protein homeostasis and MAPK signaling.
7. MOLV1 is a novel polyadenylated, positive and single stranded RNA virus closely related to ourmia plant viruses isolated from the *M. oryzae* field isolated Guy11.

ANNEXES

ANNEXES

Annex Table A2.1. List of characterized transcriptional regulators in *M. oryzae*.

Protein name	Gene locus	aa	Function	DNA-binding domain	References
MSTU1/ MoSTU1	MGG_00692.8	618	orthologue of <i>Aspergillus nidulans</i> StuA; involved in conidiation, growth, appressorium development and pathogenicity (mobilization of lipids and glycogen required for appressorial turgor generation).	APSES	(Nishimura et al., 2009)
MoSwi6	MGG_09869.8	895	homologue of <i>S. cerevisiae</i> Swi6; a downstream effector of MAPK Sit2 signaling; required for hyphal and conidial morphogenesis, appressorial function and pathogenicity. Interacts in vivo and in vitro with <i>M. oryzae</i> MAPK Mps1.	APSES	(Qi et al., 2012)
NUT1	MGG_02755.8	956	orthologue of <i>Neurospora crassa</i> NIT-2 and <i>A. nidulans</i> AREA; fully complements Δ area mutant of <i>A. nidulans</i> ; required for expression of nitrogen-regulated genes; on leaves, infection efficiency of Δ nut1 is not affected although Δ nut1 produces smaller lesions; on roots Δ nut1 is non pathogenic [Dufresne and Osbourn, 2001; Tucker et al., 2010].	GATA Zn-F	(Froeliger and Carpenter, 1996)
MoWC-1*	MGG_03538.8	1101	homologue of <i>N. crassa</i> white collar-1; involved in light regulation	GATA Zn-F	(Lee et al., 2006)
MoMCM1	MGG_02773.8	224	Mst12-interacting protein and orthologous to yeast Mcm1; required for male fertility, microconidium production and virulence.	MADS-box	(Zhou et al., 2011)
MIG1	MGG_01204.8	702	yeast RLM1 homologue; nuclear localised; interacts with MAPK MPS1 in Y2H; Δ mig1 has normal growth rate, forms normal appressoria, penetrates developing primary infectious hyphae but it is nonpathogenic and fails to infect leaves through wounds.	MADS-box	(Mehrabani et al., 2008)
COO1 = CON1	MGG_03977.8	491	Conidiophore stalk-less1; involved in conidiation and mycelial infection.	C2H2 Zn-F	(Zhou et al., 2009)
MoCrz1*	MGG_05133.8	726	calcineurin-responsive transcription factor; involved in fungal growth and pathogenicity. MoCrz1 regulates expression of genes implicated in calcium signaling, small molecule transport, ion homeostasis, cell wall synthesis/maintenance, and fungal virulence.	C2H2 Zn-F	(Choi et al., 2009; Zhang et al., 2009; Kim et al., 2010)

CON7*	MGG_05287.8	412	nuclear localised protein; involved in appressorium formation, proper cell wall formation, and in planta growth. CON7 gene was missannotated and consequently not present in the Fungal Transcription Factor Database.	C2H2 Zn-F	(Odenbach et al., 2007)
TDG2/MoMsn2	MGG_00501.8	548	orthologue of AAB04132 <i>Nectria haematococca</i> ; regulated by Tra1; necessary for adhesion, germination, aerial hyphal growth, conidial production, and plant infection.	C2H2 Zn-F	(Breth et al., 2013; Zhang et al., 2014)
MoCMTF1	MGG_11346.8	1456	no homologue in yeast; nuclear localised; regulated by cAMP/PKA; required for growth, pigmentation, conidia and conidiophore formation, sexual development, appressorium formation from mycelium, and pathogenicity.	C2H2 Zn-F-like	(Yan et al., 2011)
PIG1	MGG_07215.8	973	required for transcription of melanin biosynthesis genes; developmentally regulated.	C2H2 Zn-F, Zn2Cys6	(Tsuiji et al., 2000)
TPC1*	MGG_01285.8	839	required for polarised growth, cell patterning and virulence.	Zn2Cys6	This study
TDG3	MGG_06832.8	639	orthologue of Yrm1p Q12340 <i>S. cerevisiae</i> ; regulated by Tra1; no phenotype found.	Zn2Cys6	(Breth et al., 2013)
Tra1*	MGG_10197.8	726	regulated by Con7; involved in attachment, germination, appressorium formation and virulence; interacts with MoOsm1 kinase in vivo	Zn2Cys6; Myb	(Breth et al., 2013)
Far1; Far2	MGG_01836.8 ; MGG_08199.8	957; 1009	involved in growth on long chain fatty acid, acetate and short chain fatty acids; required for differential expression of genes involved in fatty acid β -oxidation, acetyl-CoA translocation, peroxisomal biogenesis, and the glyoxylate cycle in response to the presence of lipids	Zn2Cys6	(bin Yusof et al., 2014)
Gpf1*	MGG_17841.8	700	necessary for growth, conidial germination, appressorium formation, stress response and plant infection	Zn2Cys6	(Lu et al., 2014)
Cnf2*	MGG_15023.8	963	involved in plant infection and stress response	Zn2Cys6	(Lu et al., 2014)

MoCod1	MGG_05343.8	753	expression was notably induced during conidiation; necessary for conidiation and pathogenicity due to defects in appressorium formation and invasive growth	Zn2Cys6	(Chung et al., 2013)
MoCod2	MGG_09263.8	1226	expression was notably induced during conidiation; critical regulator in conidiation and pathogenicity, not required for conidial germination and appressorium formation.	Zn2Cys6	(Chung et al., 2013)
Xlr1	MGG_01414.8	1009	XlnR homologue; involved in the transcriptional control of the pentose catabolic pathway, but not hemicellulolytic enzymes; M. oryzae Xlr1 regulates growth on D-xylose and xylan, but not on L-arabinose and arabinan.	Zn2Cys6; Myb	(Battaglia et al., 2013)
MoMyb1	MGG_06898.8	395	involved in vegetative growth, cell wall biogenesis, conidiation and conidial germination; mutant is pathogenic on leaves but not on roots	Myb	(Dong et al., 2015)
HTF1= MoHOX2	MGG_00184.8	692	involved in conidogenesis and regulation of the expression of conidiation-related genes	Homeobox	(Kim et al., 2009; Liu et al., 2010)
HTF2= MoHOX3	MGG_01730.8	1185	Δ MoHox3 has no defects in growth, conidiation, conidium size, conidial germination, appressorium formation, and pathogenicity.	C2H2 Zn-F, Homeobox	(Kim et al., 2009; Liu et al., 2010)
MST12=MoH OX8	MGG_12958.8	715	required for penetration and invasive growth; SteA	C2H2 Zn-F, Homeobox	(Park et al., 2002; Park et al., 2004)
HTF3= MoHOX1	MGG_04853.8	625	required for hyphal growth. The Δ MoHox1 mutant presents increase melanin pigmentation compared to wild type.	Homeobox	(Kim et al., 2009; Liu et al., 2010)
HTF4= MoHOX4	MGG_06285.8	977	involved in conidial size and hyphal growth.	Homeobox	(Kim et al., 2009; Liu et al., 2010)
HTF5= MoHOX5	MGG_07437.8	714	Δ MoHox5 has no defects in growth, conidiation, conidium size, conidial germination, appressorium formation, and pathogenicity.	Homeobox	(Kim et al., 2009; Liu et al., 2010)

HTF6=MoHOX6	MGG_11712.8	685	Involved in conidial size and hyphal growth.	Homeobox	(Kim et al., 2009; Liu et al., 2010)
PTH12=MoHOX7	MGG_12865.8	470	essential for appressorium formation.	Homeobox	(Sveigard et al., 1998; Kim et al., 2009; Liu et al., 2010)
MoBZIP13/MoHAPX	MGG_05959.8	639	hap4 domain, orthologue of A. nidulans HapX; involved in iron homeostasis and infection	bZIP	(Kong et al., 2015)
MoATF1	MGG_08212.8	526	homologue of S. pombe ATF/GREB; involved in regulation of oxidative stress response. The mutant is severely impaired in enzymatic activity of extracellular laccases and peroxidases.	bZIP	(Guo et al., 2010)
MoAPI1*	MGG_12814.8	576	orthologue of S. cerevisiae Yap1; involved in oxidative stress response and pathogenicity; MoAPI1 localises in the nucleus upon exposure to H ₂ O ₂ .	bZIP	(Guo et al., 2011)
MoHac1	MGG_09010.8	31/556	involved in the endoplasmic reticulum stress response through a conserved unfolded protein response pathway; two alternatively spliced transcripts.	bZIP	(Tang et al., 2015)
MoBZIP22/MoMETR	MGG_14561.8	302	orthologue of AnMetR (48%) and NcCyc3 (51%); involved in amino acid metabolism, sulfate assimilation, growth and differentiation, and plant infection	bZIP	(Kong et al., 2015; Tang et al., 2015)
MoBzip10	MGG_06131.8	240/324	required for appressorium function and invasive hyphal growth.	bZIP	(Tang et al., 2015)
MoBzip5	MGG_03288	616	required for appressorium formation	bZIP	(Tang et al., 2015)
MoMeaB	MGG_05306	414	Involved in nutrient uptake and nitrogen utilization.	bZIP	(Tang et al., 2015)

Other transcriptional regulators					
MoSOM1*	MGG_04708.8	737/762	weak homology with <i>S. cerevisiae</i> Flo8 (e-04); interacts with MoSTU1 and MoCDTF1 in yeast two hybrid; nuclear localised; regulated by cAMP/PKA; five spliced variants; complements flo8 yeast mutant required for growth, pigmentation, conidia and conidiophore formation, sexual development, appressorium formation from mycelium, and pathogenicity.	LisH (e0.08)	(Yang et al., 2011)
MoSFL1	MGG_06971.8	600	Phosphorylated in vitro by PMK1; it complements the flocculation defects of the yeast Δ sfl1 mutant; required for invasive growth in penetration assays with rice leaf sheaths; transcription repression domain.	HSF-like domain	(Li et al., 2011)
MoLDB1	MGG_01057.6	806	necessary for vegetative growth, infection-related morphogenesis and pathogenicity	LIM	(Li et al., 2010)
MoTup1	MGG_08829.8	607	homologue of <i>S. cerevisiae</i> Tup1 transcriptional repressor; involved in growth, conidiogenesis and plant infection.	Tup1 domain	(Chen et al., 2015)
Mnh6	MGG_04489.8	101	non-histone protein involved in mycelial growth, conidiation, appressorium development, plant penetration, and infectious growth in host cells.	HMG1 box	(Lu et al., 2007)
COM1	MGG_01215.8	774	putative transcriptional regulator present in ascomycetes; involved in conidial development and invasive growth.	RNA Pol II elongation factor domain	(Yang et al., 2010)
MoATH10	MGG_05185.8	562	Involved in pigmentation, conidiation and virulence; the mutant shows defects in invasive growth inside plant cells, but not in appressorium-mediated penetration	AT-hook	(Shin et al., 2014)

MoGti1*	MGG_08850.8	464	necessary for conidiogenesis, appressorial formation, invasive hyphae growth and pathogenicity; regulated by MoAP1; nuclear localisation regulated by Pmk1; involved in suppression of plant defense responses	Gti1/Pac2	(Chen et al., 2013)
MoPac2*	MGG_06564.8	463	regulated by MoAP1; mainly cytoplasmic protein; required for conidiogenesis and full pathogenicity	Gti1/Pac2	(Chen et al., 2013)
Nmr1-3	MGG_00156; MGG_02860; MGG_09705	321; 317; 321	transcriptional inhibitors; regulate carbon catabolite repression in <i>M. oryzae</i>	NmrA-like	(Wilson et al., 2010; Fernandez et al., 2012)
ACR1	MGG_09847.8	862	homologue to medA (a developmental regulator of <i>A. nidulans</i> conidiation); involved in conidiophore architecture and pathogenicity.	unknown domain	(Lau and Hamer, 1998; Nishimura et al., 2000)

*:available transcriptome and/or proteome data

REFERENCES (A2.1 Table)

- Nishimura M, Fukada J, Moriwaki A, Fujikawa T, Ohashi M, et al. (2009)** Mst1, an APSES Transcription Factor, Is Required for Appressorium-Mediated Infection in *Magnaporthe grisea*. *Bioscience Biotechnology and Biochemistry* 73: 1779-1786.
- Qi ZQ, Wang Q, Dou XY, Wang W, Zhao Q, et al. (2012)** MoSwi6, an APSES family transcription factor, interacts with MoMps1 and is required for hyphal and conidial morphogenesis, appressorial function and pathogenicity of *Magnaporthe oryzae*. *Molecular Plant Pathology* 13: 677-689.
- Dufresne M, Osbourn AE (2001)** Definition of tissue-specific and general requirements for plant infection in a phytopathogenic fungus. *Molecular Plant-Microbe Interactions* 14: 300-307.
- Tucker SL, Besi MI, Galhano R, Franceschetti M, Goetz S, et al. (2010)** Common Genetic Pathways Regulate Organ-Specific Infection-Related Development in the Rice Blast Fungus. *Plant Cell* 22: 953-972.
- Froeliger EH, Carpenter BE (1996)** *NUT1*, a major nitrogen regulatory gene in *Magnaporthe grisea*, is dispensable for pathogenicity. *Mol Gen Genet* 251: 647-656.
- Lee K, Singh P, Chung WC, Ash J, Kim TS, et al. (2006)** Light regulation of asexual development in the rice blast fungus, *Magnaporthe oryzae*. *Fungal Genetics and Biology* 43: 694-706.
- Zhou X, Liu W, Wang C, Xu Q, Wang Y, et al. (2011)** A MADS-box transcription factor MoMcm1 is required for male fertility, microconidium production and virulence in *Magnaporthe oryzae*. *Molecular Microbiology* 80: 33-53.
- Mehrabi R, Ding S, Xu JR (2008)** MADS-box transcription factor Mig1 is required for infectious growth in *Magnaporthe grisea*. *Eukaryotic Cell* 7: 791-799.
- Zhou ZZ, Li GH, Lin CH, He CZ (2009)** Conidiophore Stalk-less1 Encodes a Putative Zinc-Finger Protein Involved in the Early Stage of Conidiation and Mycelial Infection in *Magnaporthe oryzae*. *Molecular Plant-Microbe Interactions* 22: 402-410.
- Choi J, Kim Y, Kim S, Park J, Lee YH (2009)** *MoCRZ1*, a gene encoding a calcineurin-responsive transcription factor, regulates fungal growth and pathogenicity of *Magnaporthe oryzae*. *Fungal Genetics and Biology* 46: 243-254.
- Zhang H, Zhao Q, Liu K, Zhang Z, Wang Y, et al. (2009)** MgCRZ1, a transcription factor of *Magnaporthe grisea*, controls growth, development and is involved in full virulence. *FEMS Microbiology Letters* 293: 160-169.
- Kim S, Hu JN, Oh Y, Park J, Choi J, et al. (2010)** Combining ChIP-chip and Expression Profiling to Model the MoCRZ1 Mediated Circuit for Ca²⁺/Calcineurin Signaling in the Rice Blast Fungus. *PLoS Pathogens* 6: e1000909.
- Odenbach D, Breth B, Thines E, Weber RW, Anke H, et al. (2007)** The transcription factor Con7p is a central regulator of infection-related

- morphogenesis in the rice blast fungus *Magnaporthe grisea*. *Molecular Microbiology* 64: 293-307.
- Breth B, Odenbach D, Yemelin A, Schlinck N, Schroder M, et al. (2013)** The role of the Tra1p transcription factor of *Magnaporthe oryzae* in spore adhesion and pathogenic development. *Fungal Genetics and Biology* 57: 11-22.
- Zhang H, Zhao Q, Guo X, Guo M, Qi Z, et al. (2014)** Pleiotropic function of the putative zinc-finger protein MoMsn2 in *Magnaporthe oryzae*. *Mol Plant Microbe Interact* 27: 446-460.
- Yan X, Li Y, Yue X, Wang C, Que Y, et al. (2011)** Two Novel Transcriptional Regulators Are Essential for Infection-related Morphogenesis and Pathogenicity of the Rice Blast Fungus *Magnaporthe oryzae*. *PLoS Pathog* 7: e1002385.
- Tsuji G, Kenmochi Y, Takano Y, Sweigard J, Farrall L, et al. (2000)** Novel fungal transcriptional activators, Cmr1p of *Colletotrichum lagenarium* and Pig1p of *Magnaporthe grisea*, contain Cys₂His₂ zinc finger and Zn(II)₂Cys₆ binuclear cluster DNA-binding motifs and regulate transcription of melanin biosynthesis genes in a developmentally specific manner. *Molecular Microbiology* 38: 940-954.
- bin Yusof MT, Kershaw MJ, Soanes DM, Talbot NJ (2014)** FAR1 and FAR2 regulate the expression of genes associated with lipid metabolism in the rice blast fungus *Magnaporthe oryzae*. *PLoS One* 9: e99760.
- Lu J, Cao H, Zhang L, Huang P, Lin F (2014)** Systematic analysis of Zn₂Cys₆ transcription factors required for development and pathogenicity by high-throughput gene knockout in the rice blast fungus. *PLoS Pathog* 10: e1004432.
- Chung H, Choi J, Park SY, Jeon J, Lee YH (2013)** Two conidiation-related Zn(II)₂Cys₆ transcription factor genes in the rice blast fungus. *Fungal Genet Biol* 61: 133-141.
- Battaglia E, Klaubauf S, Vallet J, Ribot C, Lebrun MH, et al. (2013)** Xlr1 is involved in the transcriptional control of the pentose catabolic pathway, but not hemi-cellulolytic enzymes in *Magnaporthe oryzae*. *Fungal Genetics and Biology* 57: 76-84.
- Dong Y, Zhao Q, Liu X, Zhang X, Qi Z, et al. (2015)** MoMyb1 is required for asexual development and tissue-specific infection in the rice blast fungus *Magnaporthe oryzae*. *BMC Microbiol* 15: 37.
- Kim S, Park SY, Kim KS, Rho HS, Chi MH, et al. (2009)** Homeobox Transcription Factors Are Required for Conidiation and Appressorium Development in the Rice Blast Fungus *Magnaporthe oryzae*. *PLoS Genetics* 5: e1000757.
- Liu WD, Xie SY, Zhao XH, Chen X, Zheng WH, et al. (2010)** A Homeobox Gene Is Essential for Conidiogenesis of the Rice Blast Fungus *Magnaporthe oryzae*. *Molecular Plant-Microbe Interactions* 23: 366-375.
- Park G, Bruno KS, Staiger CJ, Talbot NJ, Xu J-R (2004)** Independent genetic mechanisms mediate turgor generation and penetration peg formation during plant infection in the rice blast fungus. *Molecular Microbiology* 53: 1695-1707.

- Park G, Xue C, Zheng L, Lam S, Xu JR (2002)** MST12 Regulates Infectious Growth but not Appressorium Formation in the Rice Blast Fungus *Magnaporthe grisea*. *Molecular Plant-Microbe Interactions* 15: 183-192.
- Swiegard JA, Carroll AM, Farrall L, Chumley FG, Valent B (1998)** *Magnaporthe grisea* Pathogenicity Genes Obtained Through Insertional Mutagenesis. *Molecular Plant-Microbe Interactions* 11: 404-412.
- Kong S, Park SY, Lee YH (2015)** Systematic characterization of the bZIP transcription factor gene family in the rice blast fungus, *Magnaporthe oryzae*. *Environ Microbiol* 17: 1425-1443.
- Guo M, Guo W, Chen Y, Dong SM, Zhang X, et al. (2010)** The Basic Leucine Zipper Transcription Factor Moatf1 Mediates Oxidative Stress Responses and Is Necessary for Full Virulence of the Rice Blast Fungus *Magnaporthe oryzae*. *Molecular Plant-Microbe Interactions* 23: 1053-1068.
- Guo M, Chen Y, Du Y, Dong YH, Guo W, et al. (2011)** The bZIP Transcription Factor MoAP1 Mediates the Oxidative Stress Response and Is Critical for Pathogenicity of the Rice Blast Fungus *Magnaporthe oryzae*. *PLoS Pathogens* 7: e1001302.
- Tang W, Ru Y, Hong L, Zhu Q, Zuo R, et al. (2015)** System-wide characterization of bZIP transcription factor proteins involved in infection-related morphogenesis of *Magnaporthe oryzae*. *Environmental Microbiology* 17: 1377-1396.
- Li GT, Zhou XY, Kong LG, Wang YL, Zhang H, et al. (2011)** MoSfl1 Is Important for Virulence and Heat Tolerance in *Magnaporthe oryzae*. *Plos One* 6: e19951.
- Li Y, Liang S, Yan X, Wang H, Li D, et al. (2010)** Characterization of MoLDB1 required for vegetative growth, infection-related morphogenesis, and pathogenicity in the rice blast fungus *Magnaporthe oryzae*. *Mol Plant Microbe Interact* 23: 1260-1274.
- Chen Y, Zhai S, Sun Y, Li M, Dong Y, et al. (2015)** MoTup1 is required for growth, conidiogenesis and pathogenicity of *Magnaporthe oryzae*. *Mol Plant Pathol*.
- Lu JP, Feng XX, Liu XH, Lu Q, Wang HK, et al. (2007)** Mnh6, a nonhistone protein, is required for fungal development and pathogenicity of *Magnaporthe grisea*. *Fungal Genetics and Biology* 44: 819-829.
- Yang J, Zhao XY, Sun J, Kang ZS, Ding SL, et al. (2010)** A Novel Protein Com1 Is Required for Normal Conidium Morphology and Full Virulence in *Magnaporthe oryzae*. *Molecular Plant-Microbe Interactions* 23: 112-123.
- Shin J-H, Han J-H, Kim K (2014)** Genome-wide analyses of DNA-binding proteins harboring AT-hook motifs and their functional roles in the rice blast pathogen, *Magnaporthe oryzae*. *Genes & Genomics* 36: 871-881.
- Chen Y, Zhai S, Zhang H, Zuo R, Wang J, et al. (2013)** Shared and distinct functions of two Gti1/Pac2 family proteins in growth, morphogenesis and pathogenicity of *Magnaporthe oryzae*. *Environ Microbiol* 16: 788-801.
- Wilson RA, Gibson RP, Quispe CF, Littlechild JA, Talbot NJ (2010)** An NADPH-dependent genetic switch regulates plant infection by the rice blast

fungus. Proceedings of the National Academy of Sciences of the United States of America 107: 21902-21907.

Fernandez J, Wright JD, Hartline D, Quispe CF, Madayiputhiya N, et al. (2012) Principles of Carbon Catabolite Repression in the Rice Blast Fungus: Tps1, Nmr1-3, and a MATE-Family Pump Regulate Glucose Metabolism during Infection. PLoS Genetics 8: ARTN e1002673.

Lau GW, Hamer JE (1998) Acropetal: a genetic locus required for conidiophore architecture and pathogenicity in the rice blast fungus. Fungal Genetics and Biology 24: 228-239.

Nishimura M, Hayashi N, Jwa NS, Lau GW, Hamer JE, et al. (2000) Insertion of the LINE Retrotransposon MGL Causes a Conidiophore Pattern Mutation in *Magnaporthe grisea*. Molecular Plant-Microbe Interactions 13: 892-894.

Annex Table A2.2. Complete list of Zn(II)₂Cys₆ binuclear family of transcriptional regulators present in *M. oryzae* genome.

Gene locus				Gene locus			
1	MGG_00021	1	376	59	MGG_06659	1	486
2	MGG_00032	2	552	60	MGG_06683	1	973
3	MGG_00049	2	708	61	MGG_06724	2	713
4	MGG_00096	1	590	62	MGG_06829	2	656
5	MGG_00318	1	863	63	MGG_06845	1	622
6	MGG_00320	1	518	64	MGG_06891	1	585
7	MGG_00329	2	708	65	MGG_06939	2	725
8	MGG_00417	2	464	66	MGG_06243	2	1,114
9	MGG_00494	0	676	67	MGG_06279	1	969
10	MGG_00672	1	740	68	MGG_06312	1	813
11	MGG_00934	1	995	69	MGG_06355	2	879
12 (Tpc1)	MGG_01285	1	839	70	MGG_06416	2	658
13	MGG_01414	2	995	71	MGG_06465	1	731
14	MGG_01486	2	1,074	72	MGG_06482	2	776
15	MGG_01518	2	988	73	MGG_06550	1	640
16	MGG_01624	2	1,023	74	MGG_06626	1	580
17	MGG_01734	1	698	75	MGG_06778	2	724
18	MGG_01777	2	960	76	MGG_06832	1	682
19	MGG_01779	1	637	77	MGG_06854	1	670
20	MGG_01833	2	781	78	MGG_07063	1	640
21	MGG_01836	1	941	79	MGG_07131	1	842
22	MGG_01870	1	504	80	MGG_07149	0	727
23	MGG_01887	2	1,017	81	MGG_07215	1	973
24	MGG_01946	1	522	82	MGG_07218	1	450
25	MGG_02089	1	518	83	MGG_07368	1	630
26	MGG_02226	2	849	84	MGG_07386	1	709
27	MGG_02269	1	475	85	MGG_07450	1	873
28	MGG_02377	2	883	86	MGG_07458	1	875
29	MGG_02408	2	928	87	MGG_07534	1	584

*: 0, Zn(II)₂Cys₆ domain not annotated in INTERPRO; 1, Zn(II)₂Cys₆ domain; 2, presence of additional domains other than Zn(II)₂Cys₆.

	Gene locus	Motifs*	aa
88	MGG_07549	1	570
89	MGG_07636	2	616
90	MGG_07681	2	819
91	MGG_07777	2	742
92	MGG_07800	1	741
93	MGG_07830	1	473
94	MGG_07845	1	564
95	MGG_08058	0	337
96	MGG_08093	1	719
97	MGG_08094	1	645
98	MGG_08130	3	702
99	MGG_08168	1	717
100	MGG_08185	1	564
101	MGG_08199	2	1009
102	MGG_08314	2	894
103	MGG_08361	1	528
104	MGG_08618	2	616
105	MGG_08753	1	316
106	MGG_08777	2	823
107	MGG_08784	3	971
108	MGG_08917	1	648
109	MGG_08974	2	704
110	MGG_09002	1	215
111	MGG_09027	1	770
112	MGG_09118	1	747
113	MGG_09263	1	1099
114	MGG_09273	1	820
115	MGG_09276	1	971
116	MGG_09312	1	457

	Gene locus	Motifs	aa
117	MGG_09676	1	704
118	MGG_09780	1	539
119	MGG_09829	1	787
120	MGG_09950	1	957
121	MGG_10197	1	713
122	MGG_10212	2	661
123	MGG_10246	1	468
124	MGG_10307	1	751
125	MGG_10422	0	703
126	MGG_10528	2	1,002
127	MGG_10529	1	666
128	MGG_10594	1	755
129	MGG_10806	1	524
130	MGG_11116	2	809
131	MGG_11119	2	1,024
132	MGG_11160	1	657
133	MGG_11192	1	1,103
134	MGG_11247	0	613
135	MGG_11724	1	362
136	MGG_11764	1	582
137	MGG_12037	1	760
138	MGG_12097	1	793
139	MGG_12339	1	729
140	MGG_12349	1	659
141	MGG_12424	0	608
142	MGG_12776	2	729
143	MGG_13350	2	471
144	MGG_13360	1	597
145	MGG_13385	1	811

	Gene locus	Motifs	aa
146	MGG_13629	2	867
147	MGG_13894	1	610
148	MGG_13919	2	776
149	MGG_13927	1	498
150	MGG_13994	1	680
151	MGG_14175	2	886
152	MGG_14728	1	533
153	MGG_14816	1	664
154	MGG_14852	1	721
155	MGG_15021	1	485
156	MGG_15023	1	951
157	MGG_15085	2	880
158	MGG_15093	1	609
159	MGG_15139	1	1,167
160	MGG_15759	1	335
161	MGG_15915	2	675
162	MGG_16161	2	871
163	MGG_16444	2	900
164	MGG_16477	2	770
165	MGG_16756	2	688
166	MGG_16835	1	531
167	MGG_17012	1	766
168	MGG_17060	1	821
169	MGG_17264	1	419
170	MGG_17623	2	656
171	MGG_17669	2	925
172	MGG_17821	1	664
173	MGG_17841	1	555
174	MGG_18016	1	130
175	MGG_18117	0	202

*: 0, Zn(II)₂Cys₆ domain not annotated in INTERPRO; 1, Zn(II)₂Cys₆ domain; 2, presence of additional domains other than Zn(II)₂Cys₆

Annex Table A2.3. List of differentially expressed genes in *Δtpc1* identified by transcriptome analysis.

Fold change ¹	GENE ID	PROTEIN DESCRIPTION / ORTHOLOGUES ²	GO term name and number	REFERENCES ³
Signaling (3 down-regulated; 10 up-regulated)	-10.7 MGG_06800	Pepp1: Phosphatidylethanolamine-binding protein PEBP		this study
	-6.6 MGG_14045	Pepp2: Phosphatidylethanolamine-binding protein PEBP		
	-5.9 MGG_04787	Leucine carboxyl methyltransferase. Its activity may affect the heteromultimeric composition of protein phosphatase 2A	methyltransferase activity GO:0008168; methylation GO:0032259; transferase activity GO:0016740	
	7.0 MGG_01779	Zn-C ₂ fungal-type transcriptional regulator; not required for conidiation, growth and virulence but affected in stress response	Nucleus GO:0005634; zinc ion binding GO:0008270; regulation of transcription, DNA-templated GO:0006355; sequence-specific DNA binding transcription factor activity GO:0003700; sequence-specific DNA binding RNA polymerase II transcription factor activity GO:0000981	(Lu et al., 2014)
4.3 MGG_14489		Serine/threonine protein kinase; overexpressed after the transition from darkness to light (Kim, 2011 #2284); some similarity with Iine2 (e-3) and Rim11 (3,2e-3) of <i>S. cerevisiae</i> , kinases that regulate activation of meiosis in yeast	ATP binding GO:0005524; kinase activity GO:0016501; phosphorylation GO:0016310; protein kinase activity GO:0004672; protein serine/threonine kinase activity GO:0004674; protein phosphorylation GO:0006468; transferase activity, transferring phosphorus-containing groups GO:0016772	
3.6 MGG_00070		Zn-C ₂ fungal-type DNA-binding domain; pathogenicity, appressorium formation, germination and conidiation affected in the mutant; not listed in Lu et al. paper (2014)	Binding GO:0005488; nucleus GO:0005634; zinc ion binding GO:0008270; regulation of transcription, DNA-templated GO:0006355; sequence-specific DNA binding transcription factor activity GO:0003700; sequence-specific DNA binding RNA polymerase II transcription factor activity GO:0000981	(Jeon et al., 2007)
3.3 MGG_00080		C ₂ H ₂ Zinc finger DNA-binding domain	nucleic acid binding GO:0003676; zinc ion binding GO:0008270; intracellular GO:0005622; metal ion binding GO:0046872	
2.3 MGG_09138		glutathione S-transferase; participate in the detoxification of reactive electrophilic compounds by catalysing their conjugation with glutathione	cytoplasmic sequestering of protein GO:0057220; negative regulation of nucleic acid-templated transcription GO:1903507; phosphoprotein binding GO:0051219; response to aluminum ion GO:0010044; cytoplasmic sequestering of transcription factor GO:0042994; glutathione peroxidase activity GO:0004602; soluble fraction GO:0005625; transcription corepressor activity GO:0003714; cytosol GO:0005829; protein urmylation GO:0032447;	
2.2 MGG_06120		Homeodomain-like domain	DNA binding GO:0003677	
2.2 MGG_01475		NTD80 domain, similar to yeast NTD80 (e-10), and <i>N. crassa</i> Fsd-1 (e-100), which is involved in female sexual development and ascospore maturation	DNA binding GO:0003677; regulation of transcription, DNA-templated GO:0006355; sequence-specific DNA binding transcription factor activity GO:0003700	
2.2 MGG_01624		FZC3, Zn-C ₂ fungal-type transcriptional regulator; mutant had affected in conidiation, growth, virulence or stress response	metal ion binding GO:0046872; nucleus GO:0005634; DNA binding GO:0003677; zinc ion binding GO:0008270; transcription, DNA-templated GO:0006351; regulation of transcription, DNA-templated GO:0006355; sequence-specific DNA binding transcription factor activity GO:0003700	(Lu et al., 2014)
2.2 MGG_02055		APSES and ZnF C ₂ H ₂ domains; transcriptional regulator	DNA binding GO:0003677; nucleic acid binding GO:0003676; zinc ion binding GO:0008270; regulation of transcription, DNA-templated GO:0006355; intracellular GO:0005622; sequence-specific DNA binding transcription factor activity GO:0003700; metal ion binding GO:0046872	

2,0 MGG_02061	CAMK protein kinase; mutant not affected in pathogenicity	ATP binding GO:0005524; kinase activity GO:0016301; phosphorylation GO:0016310; protein kinase activity GO:0004672; protein serine/threonine kinase activity GO:0004674; protein phosphorylation GO:0006468; transferase activity, transferring phosphorus-containing groups GO:0016772	(Villalba et al., 2008)
Metabolism (68 down-regulated; 61 up-regulated)			
-9,8 MGG_15388	Lactose permease; TM	Membrane GO:0015020; integral component of membrane GO:0016021; transport GO:0006810; transmembrane transport GO:0055085; transporter activity GO:0005215; transmembrane transporter activity GO:0022857; substrate-specific transmembrane transporter activity GO:0022891	
-8,9 MGG_08359	Flavonol synthase; Oxoglutarate/iron-dependent oxygenase	oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114; iron ion binding GO:0005506; oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, 2-oxoglutarate as one donor, and incorporation of one atom each of oxygen into both donors GO:0016706	
-8,7 MGG_07863	Short-chain dehydrogenase-reductase; NAD(P)-binding domain	nucleotide binding GO:0000166; metabolic process GO:0008152; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114	
-8,0 MGG_08363	Alcohol dehydrogenase GroES-like/NAD(P)-binding domain; ApdC (A. nidulans; e-64), member of the aspyridone (apd) gene cluster (Andersen, 2013 #2654)	nucleotide binding GO:0000166; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114; zinc ion binding GO:0003270	
-8,0 MGG_15100	Polyketide synthase; NAD(P)-binding domain; synB	nucleotide binding GO:0000166; transferase activity GO:0016740; catalytic activity GO:0003824; metabolic process GO:0008152; binding GO:0005488; biosynthetic process GO:0009038; ACP phosphopantetheine attachment site binding involved in fatty acid biosynthetic process GO:0000036; phosphopantetheine binding GO:0031177	
-6,8 MGG_06950	L-serine dehydratase; Chai1p (S. cerevisiae; e-53)	catalytic activity GO:0003824; metabolic process GO:0008152; pyridoxal phosphate binding GO:0030170; cellular amino acid metabolic process GO:0006520	
-6,9 MGG_05010	P-loop containing nucleoside triphosphate hydrolase	nucleotide binding GO:0000166	
-6,1 MGG_03036	NmrA-like/NAD(P)-binding domain	glutathione biosynthetic process GO:0006750; gamma-glutamylcystidyltransferase activity GO:0003839	
-5,9 MGG_06340	Bulirosin biosynthesis, BirG-like domain	metal ion binding GO:0046872; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114; heme binding GO:0020037; iron ion binding GO:0005506; monooxygenase activity GO:0004487; electron carrier activity GO:0009035; oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen GO:0016705	
-4,9 MGG_08498	Cytochrome P450; oxidoreductase activity; TM		
-4,7 MGG_13895	Fructose-bisphosphate aldolase	catalytic activity GO:0003824; zinc ion binding GO:0008270; carbohydrate metabolic process GO:0005975; aldehyde-lyase activity GO:0016832	

-4,5 MGG_10893	Nicotinic acid transporter Tna1 (S. cerevisiae; e-54); TM	Integral component of membrane GO:0016021; transmembrane transport GO:005085	
-4,2 MGG_07419	Tyrosinase; SignalP-noTM	metabolic process GO:0008152; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0051114; metal ion binding GO:0046872	
-4,2 MGG_00097	Putative NAD(P) ⁺ coupled glycerol dehydrogenase; Ccy1 (N. crassa; e-143); it can bind mRNA	oxidoreductase activity GO:0016491; oxidation-reduction process GO:0051114; mycelium development GO:0043581	
-4,1 MGG_08486	Beta-lactamase-related; SignalP-noTM		
-4,1 MGG_05479	GH43 domain; xylosidase/arabinoxidase-Glycoside hydrolase	hydrolase activity, hydrolyzing O-glycosyl compounds GO:0004553; carbohydrate metabolic process GO:0005975	(Nguyen et al., 2011)
-4,0 MGG_08342	Phosphorylcholine phosphatase-like domain; SignalP-noTM		
-3,9 MGG_02530	Sugar/inositol transporter; TM; Hxt11 (S. cerevisiae; e-39)	Membrane GO:0016020; integral component of membrane GO:0016021; transport GO:0006810; transmembrane transport GO:005085; transporter activity GO:0005215; transmembrane transporter activity GO:0022857; substrate-specific transmembrane transporter activity GO:0022891	
-3,7 MGG_10533	Agmatinase 1; Ureohydrolase; SignalP-TM	metal ion binding GO:0046872; hydrolase activity GO:0016787; hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in linear amides GO:0013813	
-3,6 MGG_09307	Hexose transporter; TM ; SH12 (S. cerevisiae; e-31)	Membrane GO:0016020; integral component of membrane GO:0016021; transport GO:0006810; transmembrane transport GO:005085; transporter activity GO:0005215; transmembrane transporter activity GO:0022857; substrate-specific transmembrane transporter activity GO:0022891	
-3,5 MGG_08291	Cupredoxin; SignalP-noTM; paralogue of MGG_06653	interaction with host via protein secreted by type II secretion system GO:0052051	
-3,3 MGG_04997	Oligopeptide transporter OPT; TM; Opt1 (S. cerevisiae; e-116)	transmembrane transport GO:0055085; nucleus GO:0005634; cytosol GO:0005829	
-3,4 MGG_06653	Cupredoxin, multicopper oxidase; paralogue of MGG_08291	mycelium development GO:0043581	
-3,4 MGG_07247	Cytochrome P450; SignalP-TM	metal ion binding GO:0046872; oxidation-reduction process GO:0051114; heme binding GO:0020037; iron ion binding GO:0005506; monooxygenase activity GO:0004497; electron carrier activity GO:0009055; oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen GO:0016706	
-3,3 MGG_08474	Fatty acid desaturase; TM	lipid metabolic process GO:0006629	
-3,3 MGG_10860	Steroid 24-C-methyltransferase; ERGosterol biosynthesis methyltransferase; Erg6 (S. cerevisiae; e-99)	transferase activity GO:0016740; metabolic process GO:0008152; methyltransferase activity GO:0006166; methylation GO:0022393; steroid biosynthetic process GO:0006694; mitochondrial outer membrane GO:0005741; steroid 24-C-methyltransferase activity GO:0008383; lipid particle GO:0005811	
-3,2 MGG_03283	Aldehyde/methylidene dehydrogenase; Alc3/5 (S. cerevisiae; e-72)	metabolic process GO:0008152; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0051114; oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor GO:0016520	

-3,2 IMG_17097	Major facilitator superfamily; TM ; previously MGG_03409; Tna1 (S. cerevisiae; e-42)	Integral component of membrane GO:0016021; transmembrane transport GO:0055085	
-3,1 MGG_08937	Sugar-inositol transporter; TM ; Hxt1/4 (S. cerevisiae; e-52)	Membrane GO:0016020; integral component of membrane GO:0016021; transport GO:0006310; transmembrane transport GO:0055085; transporter activity GO:0005215; transmembrane transporter activity GO:0022857; substrate-specific transmembrane transporter activity GO:0022891	
-3,1 IMG_07431 -3,0 MGG_02817	Glutamine amidtransferase type 1 Glutamate decarboxylase; Gac1 (S. cerevisiae; e-122); T-DNA mutant is not affected in virulence	catalytic activity GO:0003824; lyase activity GO:0016829; pyridoxal phosphate binding GO:0030170; glutamate decarboxylase activity GO:0004351; glutamate metabolic process GO:0006535; carboxyl-lyase activity GO:0016831; carboxylic acid metabolic process GO:0019752; cytoplasm GO:0005737; cellular response to oxidative stress GO:0034599; glutamate catabolic process GO:0006538; calmodulin binding GO:0005516	(Jeon et al., 2007)
-2,7 IMG_03648	Xy1; NAD(P)H-dependent D-xylose reductase; similar to yeast Gre3	oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114; glucose 1-dehydrogenase (NADP+) activity GO:0047935; galactose catabolic process GO:0019333; D-xylose NADP reductase activity GO:0032866; cellular response to osmotic stress GO:0071470; arabinose catabolic process GO:0019568; aldolase activity GO:0003728	(Klaubauf et al., 2013; Battaglia et al., 2013)
-2,7 MGG_03038 -2,6 MGG_00973	P-loop_NTPase FAD binding domain-containing protein; SignalP-noTM	ADP binding GO:0043531; mycelium development GO:0043581 oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114; flavin adenine dinucleotide binding GO:0050660; UDP-N-acetylmuramate dehydrogenase activity GO:0008762; oxidoreductase activity; acting on CH-OH group of donors GO:0016614; catalytic activity GO:0003324; oxidoreductase activity GO:0016491	
-2,6 MGG_08095	Cysteine dioxygenase	oxidation-reduction process GO:0055114; iron ion binding GO:0005506; oxidoreductase activity; acting on single donors with incorporation of molecular oxygen, incorporation of two atoms of oxygen GO:0016702; cysteine dioxygenase activity GO:0017172; L-cysteine metabolic process GO:0046439	
-2,6 MGG_04345	Cytochrome P450 17A1; 1 TM	metal ion binding GO:0045872; oxidoreductase activity GO:0015491; oxidation-reduction process GO:0055114; heme binding GO:0020037; iron ion binding GO:0005508; monooxygenase activity GO:0004497; electron carrier activity GO:0009055; oxidoreductase activity; acting on paired donors, with incorporation or reduction of molecular oxygen GO:0016705	
-2,6 MGG_11327	Amino-acid permease; TM; similar to yeast Gap1 (e-124)	Membrane GO:0016020; integral component of membrane GO:0016021; transport GO:0006310; transmembrane transport GO:0055085; amino acid transmembrane transport GO:0003333; amino acid transport GO:0008865; amino acid transmembrane transporter activity GO:0015171	

-2.5 MGG_13764	cupredoxin	oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114; copper ion binding GO:0005507
-2.5 MGG_10005	glycerol kinase; there are two in <i>M. cryzae</i> (MGG_01281); similar to yeast Gut1 (e-67)	kinase activity GO:0016301; phosphorylation GO:0016310; transferase activity GO:0016740; carbohydrate metabolic process GO:0005975; phosphotransferase activity, alcohol group as acceptor GO:0016773; glycerol kinase activity GO:0004370; glycerol-3-phosphate metabolic process GO:0006072
-2.5 MGG_10408	FAD binding domain-containing protein; SignalP - noTM	catalytic activity GO:0003824; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114; flavin adenine dinucleotide binding GO:0050660; UDP-N-acetylmuramate dehydrogenase activity GO:0003762; oxidoreductase activity, acting on CH-OH group of donors GO:0016614
-2.5 MGG_00892	ATP synthase subunit 9; 2 TM ; similar to yeast Oll1	membrane GO:0016020; integral component of membrane GO:0016021; transport GO:0006810; ion transport GO:0006811; hydrogen ion transport transport activity GO:0015078; ATP hydrolysis coupled proton transport GO:0015991; proton-transporting two-sector ATPase complex, proton-transporting domain GO:0033177; ATP synthesis coupled proton transport GO:0015985; proton transport GO:0015992; lipid binding GO:0008289; proton-transporting ATP synthase complex, coupling factor F1o GO:0045283
-2.5 MGG_10859	cytochrome P450	oxidation-reduction process GO:0055114; peroxidase activity GO:0004601; response to oxidative stress GO:0006979; heme binding GO:0020037; iron ion binding GO:0005506; electron carrier activity GO:0009055; oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen GO:0016705
-2.4 MGG_09757	Neutral alpha-glucosidase; GH31	catalytic activity GO:0003824; hydrolase activity, hydrolyzing O-glycosyl compounds GO:0004553; carbohydrate metabolic process GO:0005975; carbohydrate binding GO:0030246; carbohydrate metabolic process GO:0005975; catalytic activity GO:0003824
-2.4 MGG_00743	Twin-arginine translocation pathway signal	metabolic process GO:0008152; catalytic activity GO:0003824; epoxide hydrolase activity GO:0004301; soluble fraction GO:0005625
-2.4 MGG_04340	transferase	transferase activity, transferring acyl groups other than amino-acyl groups GO:0016747; mycelium development GO:0043581
-2.4 MGG_10106	NmrA domain; NAD(P)-binding	nucleotide binding GO:0000166
-2.3 MGG_09852	Sugar transporter; similar to yeast Sit1 (e-123); TM	Membrane GO:0016020; integral component of membrane GO:0016021; transport GO:0006810; transmembrane transport GO:0055085; carbohydrate transport GO:0008643; transporter activity GO:0005215; transmembrane transporter activity GO:0022837; substrate-specific transmembrane transporter activity GO:0022891
-2.3 MGG_10751	Peroxisomal copper amine oxidase	metal ion binding GO:0048672; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114; copper ion binding GO:0005507; quinone binding GO:0048038; primary amine oxidase activity GO:0008131; amine metabolic process GO:0009308

-2,2 MGG_12175	Grammidin S synthetase 1, AMP-dependent synthetase/Ligase; DUF4009		catalytic activity GO:0003824; metabolic process GO:0008152; ligase activity GO:0016874; cofactor binding GO:0048037; ACP phosphopantetheine attachment site binding involved in fatty acid biosynthetic process GO:0006603; phosphopantetheine binding GO:0031177
-2,2 MGG_10334	Mannitol 2-dehydrogenase		coenzyme binding GO:0050652; metabolic process GO:0008152; catalytic activity GO:0003824; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114; oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor GO:0016615
-2,2 MGG_07962	Cytochrome P450 monooxygenase		metal ion binding GO:0046872; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114; heme binding GO:0020037; iron ion binding GO:0005506; monooxygenase activity GO:004497; electron carrier activity GO:0009055
-2,2 MGG_03793	2,3-dihydroxybenzoic acid decarboxylase		catalytic activity GO:0003824; metabolic process GO:0008152
-2,2 MGG_10583	12-oxophytenoate reductase 1		catalytic activity GO:0003824; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114; FMN binding GO:0010181
-2,2 MGG_06888	Glutamine synthetase		nucleotide binding GO:0000186; ATP binding GO:0005524; catalytic activity GO:0003824; ligase activity GO:0016874; nitrogen compound metabolic process GO:0006807; glutamate-ammonia ligase activity GO:0004356; glutamine biosynthetic process GO:0006542; cytoplasm GO:0005737
-2,2 MGG_08173	NADP-dependent malic enzyme		nucleotide binding GO:0000186; metal ion binding GO:0046872; oxidoreductase activity GO:0016491; oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor GO:0016616; oxidation-reduction process GO:0055114; NAD binding GO:0051237; malate metabolic process GO:0006108; malic enzyme activity GO:0004470
-2,1 MGG_06644	Tartrate transporter		integral component of membrane GO:0016021; transmembrane transport GO:0050585
-2,1 MGG_10561	tyrosinase; SignalP Ψ 10TM		metal ion binding GO:0046872; metabolic process GO:0008152; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114
-2,1 MGG_07200	Plasma membrane ATPase; TM, similar to yeast Pma1		nucleotide binding GO:0000186; ATP binding GO:0005524; metal ion binding GO:0046872; catalytic activity GO:0003824; metabolic process GO:0008152; membrane GO:0016020; integral component of membrane GO:0016021; hydrolase activity GO:0016787; obsolete ATP catalytic process GO:0005200; ATPase activity GO:0016887; cation transport GO:0006612; ATP biosynthetic process GO:0006754; ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism GO:0015692;

-2.1 MGG_08281	Polyketide synthase	transferase activity GO:0016740; catalytic activity GO:0003824; metabolic process GO:0008152; hydrolase activity, acting on ester bonds GO:0016788; binding GO:0005488; biosynthetic process GO:0009058; cofactor binding GO:0048337; ACP phosphopantetheine attachment site binding involved in fatty acid biosynthetic process GO:0000036; phosphopantetheine binding GO:0031177
-2.1 MGG_11916	CAP20; transaldolase	mycelium development GO:0043581
-2.1 MGG_14886	sphingolipid C3-methyltransferase ; TM	transferase activity GO:0016740; lipid biosynthetic process GO:0008610; methyltransferase activity GO:0008168; methylation GO:0032259; lipid biosynthetic process GO:0008610
-2.1 MGG_08468	Fatty acyl-CoA hydrolase; SignalP - noTM	hydrolase activity GO:0016787
-2.0 MGG_04225	MFS quinone transporter; 12 TM	Membrane GO:0016020; integral component of membrane GO:0016021; transport GO:0006870; transmembrane transport GO:0055085; transporter activity GO:0005215; transmembrane transporter activity GO:0022837; substrate-specific transmembrane transporter activity GO:0022891
-2.0 MGG_07793	Dehydrogenase/reductase SDR family member 13	nucleotide binding GO:0000168; metabolic process GO:0008152; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114
-2.0 MGG_07457	cyclase	tryptophan catabolic process to kynurenine GO:0019441; arylamidase activity GO:0004061
-2.0 MGG_01114	NADP-dependent mannitol dehydrogenase; SignalP-noTM	nucleotide binding GO:0000168; metal ion binding GO:0046872; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114; zinc ion binding GO:0008270
55.1 MGG_09601	Glycoside hydrolase, GH31; Alpha-xylosidase; carbohydrate metabolism; Rd2 (<i>S. cerevisiae</i> , e-25)	catalytic activity GO:0003824; hydrolase activity; hydrolyzing O-glycosyl compounds GO:0004553; carbohydrate metabolic process GO:0005975; carbohydrate binding GO:0030246
12.3 MGG_09602	Copper amine oxidase/Cooper amine oxidase; SignalP-noTM; co-immunoprecipitates with the histone deacetylase complex Tg1 [Ding, 2010 #2384]	oxidation-reduction process GO:0016740; copper ion binding GO:0005507; quinone binding GO:0048038; primary amine oxidase activity GO:0008131; amine metabolic process GO:0009338
10.3 MGG_09608	Glycoside hydrolase GH-28; Pectin lyase fold	metabolic process GO:0008152; hydrolase activity GO:0016787; carbohydrate metabolic process GO:0005975; hydrolase activity, acting on glycosyl bonds GO:0016798; polygalacturonase activity GO:0004650
5.4 MGG_05045	adenine phosphoribosyltransferase; catalyzes the formation of AMP from adenine and 5-phosphoribosylpyrophosphate. It also contains a higher eukaryotic phosphomevalonate kinase/FAD linked oxidase; overexpressed after the transition from darkness to light. ; Km, 2011 #2284; APT1 (e-11)	transferase activity GO:0016740; catalytic activity GO:0003824; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114; cytoplasm GO:0005737; flavin adenine dinucleotide binding GO:0005080; UDP-N-acetylmuramate dehydrogenase activity GO:0008762; oxidoreductase activity, acting on CH-OH group of donors GO:0016614; nucleoside metabolic process GO:0009116; transferase activity, transferring glycosyl groups GO:0016757; phosphomevalonate kinase activity GO:0004631; cholesterol biosynthetic process GO:0006895; cellular nitrogen compound biosynthetic process GO:0044271; cellular nitrogen compound biosynthetic process GO:0044271

6,0 MGG_03348	phosphate-repressible phosphate permease; N crassa Pho4 (NCU0564) and yeast: PHO89 (e-130); Pho4 is a sodium-phosphate symporter	membrane GO:0016020; inorganic phosphate transmembrane transporter activity GO:0005315; phosphate ion transport GO:000817; sodium inorganic phosphate symporter activity GO:0015319; phosphate ion transmembrane transport GO:0035435; sodium ion transmembrane transport GO:0035725; transmembrane transport GO:0055085; plasma membrane GO:0005886; identical protein binding GO:0042802
5,7 MGG_05045	GTP cyclohydrolase I; catalyses the biosynthesis of formic acid and dihydroneopterin triphosphate from GTP; Nitrile oxidoreductase; Fd2 (e-42)	Cytoplasm GO:0005737; hydrolase activity GO:0016787; GTP cyclohydrolase I activity GO:0003834; tetrahydrofolate biosynthetic process GO:0046554
5,4 MGG_17476	Dienolactone hydrolase; play a crucial role in chlorocatechol degradation via the modified ortho cleavage pathway; <i>S. cerevisiae</i> AIM2 (e-23)	hydrolase activity GO:0016787
5,0 MGG_08890	uncharacterized protein	cellular nitrogen compound biosynthetic process GO:004271
4,8 MGG_00402	polyketide synthase; short-chain dehydrogenase/reductase SDR/Glucose ribitol dehydrogenase/NAD(P)-binding domain	nucleotide binding GO:0001666; metabolic process GO:0008152; oxidoreductase activity GO:0016481; oxidant-reduction process GO:0035114
4,7 MGG_11496	RTA-like protein; TM; <i>S. cerevisiae</i> RRS1 (e-16) putative integral membrane transporter or flippase that may transport long chain bases (LCBs) from the cytoplasmic side toward the extracytoplasmic side of the membrane	integral component of membrane GO:0016021; response to stress GO:0006950
4,7 MGG_08725	dehydrogenase, NAD-binding/NAD(P)-binding domain; SER3 (e-26), 3-phosphoglycerate dehydrogenase; catalyzes the first step in serine and glycine biosynthesis	nucleotide binding GO:0001666; metabolic process GO:0008152; oxidoreductase activity GO:0016481; oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor GO:0016616; oxidation-reduction process GO:0055114; cofactor binding GO:0046037; NAD binding GO:0051287; mycelium development GO:0046381
4,6 MGG_04126	CYS14; TM; similar to <i>S. cerevisiae</i> Sul1 (e-139) and Sul2 (e-139); high affinity sulfate permease of the SulP anion transporter family	Membrane GO:0016020; integral component of membrane GO:0016021; transport GO:0006910; transmembrane transport GO:0055085; transporter activity GO:0005215; secondary active sulfate transmembrane transporter activity GO:0008271; sulfate transport GO:0008272; sulfate transmembrane transporter activity GO:0015116
4,1 MGG_01511	MF5 transporter; ABC transporter Flr1 (<i>S. cerevisiae</i> ; e-81); TM	integral component of membrane GO:0016021; transmembrane transport GO:0055085; fluconazole transport GO:0015903; drug transmembrane transport GO:0006855; fluconazole transporter activity GO:0015244; plasma membrane GO:0005886
3,7 MGG_03476	Protein-L-isoaspartate O-methyltransferase	transferase activity GO:0016740; methyltransferase activity GO:0008168; methylation GO:0032259; cellular protein modification process GO:0006464; protein-L-isoaspartate (D-aspartate) O-methyltransferase activity GO:0004719; cytoplasm GO:0005737; nucleus GO:0005634; cytosol GO:0005329
3,5 MGG_11021	ATPase, AAA+ type; it is overexpressed after the transition from darkness to light; (Kim, 2011 #2284);	nucleotide binding GO:0001666; ATP binding GO:0005524; nucleoside-triphosphatase activity GO:0017111; mycelium development GO:0043581
3,4 MGG_05398	uncharacterized protein	cellular nitrogen compound biosynthetic process GO:004271

3.4 MGG_09607	Maltose permease; TM : MA_31 (e-92) member of the 12 transmembrane domain superfamily of sugar transporters	membrane GO:0016020; integral component of membrane GO:0016021; transport GO:0006810; transmembrane transport GO:0055085; transporter activity GO:0005215; transmembrane transporter activity GO:0022857; substrate-specific transmembrane transporter activity GO:0022891
3.3 MGG_17602	Aminotransferase, class V; Cysteine desulfurase; NFS1 (e-4); in yeast involved in iron-sulfur cluster (Fe/S) biogenesis and in thio-modification of mitochondrial and cytoplasmic tRNAs; essential protein located predominantly in mitochondria	catalytic activity GO:0003824; metabolic process GO:0008152; pyridoxal phosphate binding GO:0030170; nucleus GO:0005634; cytosol GO:0005829; mycelium development GO:0043581; ergothioneine biosynthetic process GO:0052689; mercaptopyruvate sulfoxide lyase activity (ergothioneine-forming) GO:1990411; selenoneine biosynthetic process GO:1903257
3.3 MGG_00020	SUMF1; required for post-translational sulfatase modification; Sulfatase-modifying factor enzyme	binding GO:0005486
3.3 MGG_00632	Cytochrome P450; TM: ERG5 (e-6) C-22 sterol desaturase; a cytochrome P450 enzyme involved in ergosterol biosynthesis	metal ion binding GO:0046872; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114; heme binding GO:0020037; iron ion binding GO:0005506; monooxygenase activity GO:0004497; electron carrier activity GO:0009055; oxidoreductase activity acting on paired donors, with incorporation or reduction of molecular oxygen GO:0016705; mycelium development GO:0043581; membrane GO:0016020
3.2 MGG_06373	Aromatic ring hydroxylase-like-metabolic process; oxidoreductase activity	metabolic process GO:0008152; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114
3.1 MGG_10274	Short-chain dehydrogenase reductase SDR/Glucose-ribitol dehydrogenase/NAD(P)-binding domain	nucleotide binding GO:0000166; metabolic process GO:0008152; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114; mycelium development GO:0043581
3.1 MGG_04304	Short-chain dehydrogenase reductase SDR/Glucose-ribitol dehydrogenase/NAD(P)-binding domain; YMR28C (e-16) is a NAD(P)-dependent serine dehydrogenase and carbonyl reductase; acts on serine, L-allo-threonine, and other 3-hydroxy acids	nucleotide binding GO:0000166; metabolic process GO:0008152; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114
3.1 MGG_05554	Alcohol dehydrogenase superfamily, zinc-type/GroES-like/NAD(P)-binding domain; ADH3 (e-7) Mitochondrial alcohol dehydrogenase involved in the shuttling of mitochondrial NADH to the cytosol under anaerobic conditions and ethanol production	nucleotide binding GO:0000166; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114; zinc ion binding GO:0008270
3.0 MGG_09359	Alcohol dehydrogenase superfamily, zinc-type/GroES-like/NAD(P)-binding domain; Xyl2 (e-7) xylitol dehydrogenase; converts xylitol to D-xylulose; expression induced by xylose, even though this pentose sugar is not well utilized by <i>S. cerevisiae</i>	nucleotide binding GO:0000166; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114; zinc ion binding GO:0008270
3.0 MGG_16375	Aldo-keto reductase/NADP-dependent oxidoreductase domain	oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114
3.0 MGG_02210	type 2 phosphatidic acid phosphatase (PAP), enzymatic activity with a central role in the synthesis of phospholipids and triacylglycerol through its product diacylglycerol, and it also generates and/or degrades lipid-signalling molecules.	catalytic activity GO:0003824; oxidation-reduction process GO:0055114; membrane GO:0016020; peroxidase activity GO:0004601

2,8 MGG_06326	Vacuolar ATP synthase V0 subunit; TM	Membrane GO:0016020; integral component of membrane GO:0016021; transport GO:0006810; ion transport GO:0006811; hydrogen ion transmembrane transporter activity GO:0015078; ATP hydrolysis coupled proton transport GO:0015991; proton-transporting two-sector ATPase complex, proton-transporting domain GO:0033177; proton-transporting V-type ATPase, V0 domain GO:0033179; proton transport GO:0015992
2,7 MGG_05555	monooxygenase	oxidation-reduction process GO:0055114; monooxygenase activity GO:0004497; oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen GO:0016705
2,7 MGG_10710	Oxidoreductase	metabolic process GO:0008152; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114
2,7 MGG_08074	NADP-specific glutamate dehydrogenase	nucleotide binding GO:0000166; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114; cellular amino acid metabolic process GO:0006520; oxidoreductase activity, acting on the CH-NH2 group of donors, NAD or NADP as acceptor GO:0016639; cytosol GO:0005829; mitochondrion GO:0005739; nucleus GO:0005634
2,6 MGG_18853	Putative uncharacterized protein ; previously MGG_05055	oxidation-reduction process GO:0055114; flavin adenine dinucleotide binding GO:0050660; oxidoreductase activity, acting on CH-OH group of donors GO:0016614; alcohol metabolic process GO:0006086; choline dehydrogenase activity GO:0008812
2,6 MGG_07219	Conidial yellow pigment biosynthesis polyketide synthase	transferase activity GO:0016740; catalytic activity GO:0003824; metabolic process GO:0008152; hydrolase activity, acting on ester bonds GO:0016788; binding GO:0005488; biosynthetic process GO:0009058; cofactor binding GO:0048037; ACP phosphopantetheine attachment site binding involved in fatty acid biosynthetic process GO:0000036; phosphopantetheine binding GO:0031177
2,6 MGG_08297	NADH:flavin oxidoreductase/NADH oxidase	catalytic activity GO:0003824; oxidoreductase activity GO:0015491; oxidation-reduction process GO:0055114; FMN binding GO:0010181
2,6 MGG_09376	FAD binding contain-containing protein; SignalP - notM	catalytic activity GO:0003824; oxidoreductase activity GO:0015491; oxidation-reduction process GO:0055114; flavin adenine dinucleotide binding GO:0050660; UDP-N-acetylmuramate dehydrogenase activity GO:0008762; oxidoreductase activity, acting on CH-OH group of donors GO:0016614
2,5 MGG_03823	NADH oxidase	catalytic activity GO:0003824; oxidoreductase activity GO:0015491; oxidation-reduction process GO:0055114; FMN binding GO:0010181; mycelium development GO:0043581
2,5 MGG_18812	FAD linked oxidase	catalytic activity GO:0003824; oxidoreductase activity GO:0015491; oxidation-reduction process GO:0055114; flavin adenine dinucleotide binding GO:0050660; UDP-N-acetylmuramate dehydrogenase activity GO:0008762; oxidoreductase activity, acting on CH-OH group of donors GO:0016614
2,4 MGG_09333	Nucleoside-diphosphate-sugar epimerase; SignalP - notM	nucleotide binding GO:0000166

2.4	MGG_03662	Phosphoadenylyl-sulfate reductase		catalytic activity GO:0003824; metabolic process GO:0008152; oxidation-reduction process GO:0055114; phosphoadenylyl-sulfate reductase (thioredoxin) activity GO:0004604; cysteine biosynthetic process GO:0019344; sulfate assimilation, phosphoadenylyl sulfate reduction by phosphoadenylyl-sulfate reductase (thioredoxin) GO:0019379; nucleus GO:0005634; cytosol GO:0005829	
2.4	MGG_09007	Alcohol dehydrogenase; GroES-like chaperone domain		nucleotide binding GO:0000166; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114; zinc ion binding GO:0008270; endoplasmic reticulum GO:0005783; Golgi apparatus GO:0005794; cytoplasm GO:0005737	
2.5	MGG_00156	Nmr1; lack of nmr1 has no effect in disease symptoms production; targeted deletion of NMR1 partially restores Δlps1 pathogenicity defects		nucleotide binding GO:0000166; negative regulation of nitrogen compound metabolic process GO:0051172	(Wilson et al., 2010)
2.4	MGG_05059	SDH1; scylalone dehydratase, enzyming involved in melanin synthesis		melanin metabolic process GO:0005552; scylalone dehydratase activity GO:0030411	(Chumley and Valent, 1990)
2.4	MGG_08943	ATPase		ATP binding GO:0005524	
2.3	MGG_05940	Short-chain dehydrogenase/reductase, SDR		nucleotide binding GO:0000166; metabolic process GO:0008152; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114	
2.3	MGG_09189	Cellobiose dehydrogenase; SignalP - no TM		oxidation-reduction process GO:0055114; flavin adenine dinucleotide binding GO:0050680; oxidoreductase activity, acting on CH-OH group of donors GO:0016614	
2.3	MGG_08656	multidrug resistance MdrG protein domain, TM		integral component of membrane GO:0016021; plasma membrane GO:0005886; transmembrane transport GO:0055085; transporter activity GO:005215	
2.3	MGG_07216	Persicollin reductase; homologue of Bnf1, involved in melanin synthesis		nucleotide binding GO:0000166; metabolic process GO:0008152; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114	
2.3	MGG_12983	Short-chain dehydrogenase/reductase SDR		nucleotide binding GO:0000166; metabolic process GO:0008152; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114	
2.3	MGG_04736	AD(P)-binding domain		nucleotide binding GO:0000166; mycellium development GO:0043581	
2.2	MGG_12228	Alcohol dehydrogenase		nucleotide binding GO:0000166; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114; zinc ion binding GO:0008270	
2.2	MGG_05331	Protein-S-isoprenylcysteine O-methyltransferase		transferase activity GO:0016740; integral component of membrane GO:0016021; methyltransferase activity GO:0008168; methylation GO:0032259; protein C-terminal S-isoprenylcysteine carboxyl O-methyltransferase activity GO:0004671; C-terminal protein methylation GO:0006481; cellular protein localization GO:0034813; endoplasmic reticulum GO:0005783	
2.2	MGG_08989	Short-chain dehydrogenase/reductase SDR		nucleotide binding GO:0000166; metabolic process GO:0008152; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114	

2.2 MGG_13464	Laccase; SignalP - noTM	metal ion binding GO:0046872; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114
2.2 MGG_17812	Alcohol dehydrogenase; GroES-like chaperone domain; renamed from MGG_09328	nucleotide binding GO:000156; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114; zinc ion binding GO:0008270; mycelium development GO:0043581
2.1 MGG_17542	Putative uncharacterized protein	oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114
2.1 MGG_02095	NmrA-like domain	
2.1 MGG_10861	FAD linked oxidase; SignalP - noTM	catalytic activity GO:0003824; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114; flavin adenine dinucleotide binding GO:005060; UDP-N-acetylmuramate dehydrogenase activity GO:0008762; oxidoreductase activity, acting on C-H-OH group of donors GO:0016614
2.1 MGG_01368	Short-chain dehydrogenase/reductase SDR	nucleotide binding GO:000156; metabolic process GO:0008152; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114
2.1 MGG_10316	MFS (major facilitator superfamily) domain; TM	integral component of membrane GO:0016021; transmembrane transport GO:0055085
2.0 MGG_11463	NmrA-like domain	nucleotide binding GO:000156
2.0 MGG_06740	Purine transporter, homology with <i>A. nidulans</i> AzgA (e-152)	Membrane GO:0016020; Transport GO:0006810; transmembrane transport GO:0055085; transporter activity GO:005215
Secreted/cell wall-related (100 down-regulated; 40 up-regulated)		
-41.8 MGG_08355	uncharacterised protein; SignalP-noTM	
-21.5 MGG_08844	uncharacterised protein; SignalP-noTM	
-19.1 MGG_05805	uncharacterised protein; SignalP-noTM	
-17.3 MGG_07356	uncharacterised protein; SignalP-noTM	
-13.9 MGG_09019	secreted phospholipase A2; SignalP-noTM; paralogue of MGG_01367, also down in <i>Δlpc1</i>	MoPDA is MGG_14014; paper Lyso-Phosphatidylcholine is a Signal in the Arbuscular Mycorrhizal Symbiosis (Liu et al., 2011)
-13.8 MGG_09321	uncharacterised protein; SignalP-noTM	
-12.1 MGG_05406	uncharacterised protein; SignalP-TM	
-10.9 MGG_01941	FAD binding domain; FAD linked oxidase; SignalP-noTM. The UDP-N-acetylmuramate dehydrogenase participates in amino sugars metabolism. One of the most common amino sugars is N-Acetyl-D-glucosamine, the main component of chitin.	catalytic activity GO:0003824; oxidoreductase activity GO:0016491; oxidation-reduction process GO:0055114; flavin adenine dinucleotide binding GO:005060; UDP-N-acetylmuramate dehydrogenase activity GO:0008762; oxidoreductase activity, acting on C-H-OH group of donors GO:0016614
-8.3 MGG_12068	uncharacterised protein; SignalP-noTM	
-8.0 MGG_07630	uncharacterised protein; SignalP-noTM	
-8.0 MGG_10394	uncharacterised protein; SignalP-noTM	
-7.9 MGG_02987	carboxylesterase, para-nitrobenzyl esterase; SignalP-noTM; paralogue of MGG_12798 and MGG_00593, also down in <i>Δlpc1</i>	
-7.2 MGG_03593	uncharacterised protein; SignalP-noTM	mycelium development GO:0043581

-6.7 MGG_07246	uncharacterised protein; SignalP-noTM	interaction with host via protein secreted by type II secretion system GO:0052051	(Xue et al., 2002)
-6.2 MGG_00703	DUF3129; SignalP-noTM; paralogue of Mas3/Gas1	mycelium development GO:0043561	
-5.5 MGG_08501	Fringe-like domain (a beta-1,3-N-acetylglucosaminyltransferase enzyme); SignalP-noTM	Membrane GO:0016020; transferase activity, transferring glycosyl groups GO:0016757	
-5.4 MGG_07868	Glycosyl hydrolase family 10; SignalP-noTM. Closely related to MGG_14243; xylanase GH10; specifically expressed in the compatible interaction; PH-base number:2209	catalytic activity GO:0003824; metabolic process GO:0008152; hydrolase activity GO:0016787; hydrolase activity hydrolyzing O-glycosyl compounds GO:0004553; carbohydrate metabolic process GO:0003973; hydrolase activity, acting on glycosyl bonds GO:0016798; cation binding GO:0043169; endo-1,4-beta-xylanase activity GO:0031176; polysaccharide catabolic process GO:0000272	(Nguyen et al., 2011; Kim et al., 2015)
-5.4 MGG_09232	uncharacterised protein; SignalP-noTM	calcium ion binding GO:0005509	
-5.2 MGG_03966	Calcium-binding EF-hand-like domain; SignalP-noTM		
-5.1 MGG_15553	ECM (extracellular matrix)-Hyaluronan synthase domain; nucleotide diphospho sugar transferase domain; domain with a Rossmann like fold and can be found in diverse glycosyltransferases		
-5.0 MGG_07793	Ccp2; chitin-binding protein; SignalP-noTM; not expressed in <i>Δcon7</i> {Odenbach, 2007 #1285}	chitin-binding GO:0008031	
-4.9 MGG_04547	GH61; SignalP-noTM; Endoglucanase II		
-4.8 MGG_02384	NoFLP1; FAS1 cell adhesion domain; SignalP-noTM		(Liu et al., 2008)
-4.7 MGG_12558	Carboxylesterase, type BLipase GDXX; SignalP-noTM; paralogue of MGG_02987 and MGG_00593 also down in <i>Δlpc1</i>	metabolic process GO:0008152; hydrolase activity GO:0016787	
-4.7 MGG_03886	ECM (extracellular matrix)-Cell wall galactorhamnoprotein; highly up-regulated in appressorium {Oh, 2008 #2483}; mannosylated protein		
-4.9 MGG_07625	uncharacterised protein; SignalP-noTM		
-4.5 MGG_04258	uncharacterised protein; SignalP-noTM		
-4.5 MGG_08373	uncharacterised protein; SignalP-noTM		
-4.4 MGG_09875	DUF3129; CAS1 domain; SignalP-noTM; paralogue of Mas3/Gas1		(Xue et al., 2002)
-4.4 MGG_13953	uncharacterised protein; SignalP-noTM		
-4.4 MGG_13971	Glycosyltransferase AER61; Catalyzes the transfer of a single N-acetylglucosamine from UDP-GlcNAc to a serine or threonine residue in extracellular proteins resulting in their modification with a beta-linked N-acetylglucosamine (O-GlcNAc)	transferase activity, transferring glycosyl groups GO:0016757	
-4.2 MGG_07631	GH61; Fungal cellulose binding domain-containing protein; signalP-noTM		
-4.2 MGG_00283	uncharacterised protein; SignalP-noTM		
-4.2 MGG_09817	Serine-type endopeptidase; SignalP-noTM	hydrolase activity GO:0016787; proteolysis GO:0006508; peptidase activity GO:0006233; serine-type peptidase activity GO:0006236; serine-type endopeptidase activity GO:0004252	
-4.0 MGG_02265	NAD(P)-binding domain; SignalP-noTM	nucleotide binding GO:0000166	

-4.0 MGG_15751	uncharacterised protein; SignalP-TM				
-4.0 MGG_09998	uncharacterised protein; SignalP-TM			interaction with host via protein secreted by type II secretion system GO:0052051	
-4.0 MGG_08830	uncharacterised protein; SignalP-noTM				
-4.0 MGG_04757	uncharacterised protein; SignalP-noTM				
-3.8 MGG_09352	Minor extracellular protease vpr; serine-type endopeptidase activity Peptidase S8/S53; DUF1034; SignalP-noTM			Membrane GO:0016020; hydrolase activity GO:0016787; proteolysis GO:0005008; peptidase activity GO:0006233; serine-type peptidase activity GO:0008236; serine-type endopeptidase activity GO:0004252; cell wall GO:0005618	
-3.7 MGG_05785	Bas113; GH32 Glycosyl hydrolases family 32; SignalP-noTM			metabolic process GO:0008152; hydrolase activity GO:0016787; hydrolase activity, hydrolyzing O-glycosyl compounds GO:0004553; carbohydrate metabolic process GO:0005975; hydrolase activity, acting on glycosyl bonds GO:0016788; cation binding GO:0043169; chitinase activity GO:0004568; chitin catabolic process GO:0006032	(Giraldo et al., 2013)
-3.6 MGG_16585	uncharacterised protein; SignalP-TM				
-3.6 MGG_08497	uncharacterised protein; SignalP-TM				(Mosquera et al., 2009)
-3.6 MGG_07969	similar to Bas2(e-29); SignalP-noTM				
-3.6 MGG_04732	GH16; chitinase-Glycoside hydrolase, family 18; SignalP-noTM			catalytic activity GO:0003824; metabolic process GO:0008152; hydrolase activity GO:0016787; hydrolase activity, hydrolyzing O-glycosyl compounds GO:0004553; carbohydrate metabolic process GO:0005975; hydrolase activity, acting on glycosyl bonds GO:0016788; cation binding GO:0043169; chitinase activity GO:0004568; chitin catabolic process GO:0006032	this study
-3.4 MGG_00593	Carboxylesterase, type B; SignalP-noTM; paralogue of MGG_02967 and MGG_12798			hydrolase activity GO:0016787	
-3.3 MGG_09956	SignalP-TM; similar to cell fusion protein Ham6 (<i>M. crassa</i> ; e-75)(Fu, 2014 #2658), and NoxD/Fro41 (Lacaze, 2014 #2657; Slegmund, 2015 #2658)				this study
-3.1 MGG_07311	uncharacterised protein; SignalP-noTM				
-3.1 MGG_00282	endopeptidase; subtilisin-related; DUF1034; no predicted SignalP-no TM			Membrane GO:0016020; proteolysis GO:0006508; serine-type endopeptidase activity GO:0004252; cell wall GO:0005618	
-3.0 MGG_02531	Minor extracellular protease vpr; peptidase, subtilisin-related; SignalP-noTM this gene is down regulated in Mosom1 mutant(Yan, 2011 #1900); gene knockout shows no detectable changes in conidial germination and appressorium formation but reduced disease symptoms			hydrolase activity GO:0016787; proteolysis GO:0006508; peptidase activity GO:0008233; serine-type peptidase activity GO:0008236; serine-type endopeptidase activity GO:0004252	(Guo et al., 2011)
-3.0 MGG_05608	uncharacterised protein; SignalP-noTM				
-2.9 MGG_07624	uncharacterised protein; SignalP -noTM				
-2.9 MGG_02309	carboxypeptidase S1; similar to CPY (e-34); a broad-specificity C-terminal exopeptidase involved in non-specific protein degradation in the vacuole; SignalP-noTM				
-2.9 MGG_06234	uncharacterized protein; SignalP -noTM			interaction with host via protein secreted by type II secretion system; GO:0052051	

-2.9 MGG_03439	acid phosphatase; SurE-like nucleotidase; SignalP - noTM	hydrolase activity GO:0016787	
-2.9 MGG_12337	DUF329; SignalP-noTM; paralogue of Mss3/Gas1		(Xue et al., 2002)
-2.9 MGG_03995	carboxypeptidase S1*, similar to yeast CPY (e=40), a broad-specificity C-terminal exopeptidase; SignalP-noTM	proteolysis GO:0006508; carboxypeptidase activity GO:0004180; serine-type carboxypeptidase activity GO:0004185	
-2.9 MGG_00992	uncharacterized protein; SignalP - noTM		
-2.8 MGG_04603	uncharacterized protein; SignalP - noTM		
-2.8 MGG_00245	uncharacterized protein; SignalP-noTM	viral capsid GO:0019028	
-2.8 MGG_09818	peptidase; SignalP - TM	Proteolysis GO:0006508; aspartic-type endopeptidase activity GO:0004190	
-2.8 MGG_10330	glutamate carboxypeptidase 2; SignalP - noTM	Proteolysis GO:0006508; peptidase activity GO:0006233; carboxypeptidase activity GO:0004180; mycelium development GO:0043581	
-2.7 MGG_08415	Serin endopeptidase; SignalP - noTM	Membrane GO:0016020; Proteolysis GO:0006508; serine-type endopeptidase activity GO:0004252; cell wall GO:0005618; proteolysis GO:0006503	
-2.7 MGG_01532	uncharacterized protein; SignalP-noTM		
-2.7 MGG_16033	zinc metalloprotease; previously MGG_02107; fibronectin type3 and M28 peptidase domain; SignalP - noTM		
-2.6 MGG_02339	Carboxypeptidase S1; SignalP - noTM	Proteolysis GO:0006508; carboxypeptidase activity GO:0004180; serine-type carboxypeptidase activity GO:0004185	
-2.6 MGG_09641	CW_beta-glucan_synthetis; SignalP - noTM	mycelium development GO:0043581	
-2.6 MGG_00859	Glucan 1,3-beta-glucosidase; peptidyl-lyase fold; SignalP - TM		
-2.6 MGG_05092	uncharacterized protein; SignalP - noTM		
-2.5 MGG_05539	Carboxypeptidase 2; SignalP-noTM	Proteolysis GO:0006508; zinc ion binding GO:0006270; carboxypeptidase activity GO:0004180; metalocarboxypeptidase activity GO:0004181	
-2.5 MGG_09468	AB_hydrolase; SignalP - noTM		
-2.5 MGG_08480	Alpha/beta hydrolase; SignalP - noTM		
-2.5 MGG_02234	uncharacterized protein; SignalP - noTM	interaction with host via protein secreted by type II secretion system GO:0052051	
-2.4 MGG_01149	Extracellular CFEM domain; SignalP-noTM		
-2.4 MGG_07646	Alpha-glucuronidase; SignalP - noTM	catalytic activity GO:0003824; extracellular region GO:0005578; carbohydrate metabolic process GO:0005975; cation binding GO:0043169; xylan catabolic process GO:0045493; alpha-glucuronidase activity GO:0046569;	
-2.4 MGG_06224	uncharacterized protein; SignalP - noTM		
-2.3 MGG_09844	uncharacterized protein; SignalP - TM		
-2.3 MGG_08748	peptidase M6-like; SignalP - noTM	Proteolysis GO:0006508; peptidase activity GO:0006233	

-2.2 MGG_17532	DUF1348; SignalP - no TM; previously MGG_00114		metabolic process GO:0008152 hydrolase activity GO:0016787; hydrolase activity, hydrolyzing O-glycosyl compounds GO:0044553; carbohydrate metabolic process GO:0005975; hydrolase activity, acting on glycosyl bonds GO:0016798
-2.2 MGG_09314	Lipolytic enzyme; esterase; SignalP - TM		
-2.2 MGG_01863	Aminopeptidase Y; SignalP-noTM		Membrane GO:0016020; calcium ion binding GO:0005559; proteolysis GO:0006508; peptidase activity GO:0008233; aminopeptidase activity GO:0004177; mannosyl-oligosaccharide 1,2-alpha-mannosidase activity GO:0004571
-2.2 MGG_02756	uncharacterized protein; SignalP - noTM		
-2.2 MGG_08971	uncharacterized protein; SignalP - noTM		
-2.2 MGG_04078	DUF1596; SignalP - noTM		
-2.2 MGG_01892	Alpha carbonic anhydrase; SignalP-noTM		
-2.1 MGG_02368	galactose oxidase; Kelch and DUF1929 domains; SignalP - no TM		mycelium development GO:0043581
-2.1 MGG_09428	uncharacterized protein; SignalP - TM		
-2.1 MGG_10318	uncharacterized protein; SignalP - noTM		
-2.1 MGG_02582	galactose oxidase/kelch, beta-propeller; SignalP - 1TM		
-2.1 MGG_07748	uncharacterized protein; SignalP - noTM		
-2.1 MGG_06303	AB-hydrolase; SignalP - TM		
-2.1 MGG_09639	Alpha-1,3-glucan synthase AgsB(A. nidulans; e0.0); SignalP - TM		metabolic process GO:0008152 hydrolase activity GO:0016787; endocytosis GO:0006908; cytoplasm GO:0005737
-2.1 MGG_03671	uncharacterized protein; SignalP - noTM		metabolic process GO:0008152 hydrolase activity GO:0016787; hydrolase activity, hydrolyzing O-glycosyl compounds GO:0044553; carbohydrate metabolic process GO:0005975; biosynthetic process GO:0009058; cation binding GO:0043169
-2.1 MGG_01001	SignalP-noTM; GH81; similar to A. nidulans engA (e0.0); a 1,3-beta-glucosidase with a role in carbon starvation-induced autolytic cell wall degradation (Schlag, 2010 #2653)		cell wall macromolecule catabolic process GO:0016998; endo-1,3(4)-beta-glucanase activity GO:0033903
-2.1 MGG_01367	Secreted phospholipase A2; SignalP-1 TM; paralogue of MGG_09019 that is also down in $\Delta bpc1$		
-2.1 MGG_10440	Endonuclease L-PSP; SignalP - noTM		cellular nitrogen compound biosynthetic process GO:0044271; deaminase activity GO:0019239
-2.0 MGG_09272	Beta-galactosidase 1; SignalP - noTM		metabolic process GO:0008152 hydrolase activity GO:0016787; hydrolase activity, hydrolyzing O-glycosyl compounds GO:0044553; carbohydrate metabolic process GO:0005975; hydrolase activity, acting on glycosyl bonds GO:0016798
-2.0 MGG_16250	AB hydrolase; SignalP - noTM		mycelium development GO:0043581
-2.0 MGG_12712	uncharacterized protein; SignalP - noTM		
-2.0 MGG_12468	uncharacterized protein; SignalP - TM		mycelium development GO:0043581
9.1 MGG_08253	uncharacterised protein; SignalP-noTM		

8,7 MGG_14057	lipase; AB hydrolase; SignalP - noTM	lipid catabolic process GO:0016042; triglyceride lipase activity GO:0004806	
7,5 MGG_00311	Peptidase G1/Concanavalin A-like lectin/gucanase; SignalP-noTM; regulated by PdeH; mutant has no virulence defects	Proteolysis GO:0005508; aspartic-type endopeptidase activity GO:0004190; interaction with host via protein secreted by type II secretion system GO:0052051	(Zhang et al., 2011)
6,6 MGG_14053	uncharacterised protein; SignalP-noTM		
4,2 MGG_D3685	Avr-Pf54 allele; SignalP-noTM		(Devanra et al., 2014)
4,0 MGG_D6647	Peptidase A1; SignalP-noTM	Proteolysis GO:0005508; aspartic-type endopeptidase activity GO:0004190	
3,8 MGG_D2139	uncharacterised protein; SignalP-noTM		
3,7 MGG_D7558	uncharacterised protein; SignalP-noTM		
3,5 MGG_D9106	uncharacterised protein; SignalP-noTM		
3,5 MGG_D1944	uncharacterised protein; SignalP-noTM		
3,5 MGG_D7643	uncharacterised protein; SignalP-noTM		
3,4 MGG_D6045	uncharacterized protein; SignalP-noTM		
3,3 MGG_12582	uncharacterized protein; SignalP-noTM		
3,2 MGG_D7644	uncharacterized protein; SignalP-noTM		
3,2 MGG_D9849	uncharacterized protein; SignalP-noTM		
3,0 MGG_D6771	Chitin-binding domain; SignalP-no TM	chitin binding GO:0008061	
2,9 MGG_D7571	LysM domain-containing protein; mutant shows no defects; SignalP-noTM	cell wall macromolecule catabolic process GO:0016938	(Zhang et al., 2011)
2,7 MGG_18035	uncharacterized protein; SignalP - TM		
2,7 MGG_D3495	uncharacterized protein; SignalP - noTM		
2,6 MGG_15902	uncharacterized protein; SignalP - TM	mycelium development GO:0343581	
2,6 MGG_15427	uncharacterized protein; SignalP - noTM		
2,6 MGG_D8774	Chitin deacetylase; SignalP - no TM	catalytic activity GO:0003824; carbohydrate metabolic process GO:0005975; hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds GO:0016810	
2,5 MGG_D8451	uncharacterized protein; SignalP - noTM		
2,5 MGG_D9669	uncharacterized protein; SignalP - noTM		
2,5 MGG_D2754	Carbohydrate-binding WSC domain; SignalP - noTM		
2,5 MGG_D5671	Plh11; CFEM protein; G-protein couple receptor; required for appressorium formation and virulence; SignalP - TM; regulated by Tra1 transcription factor [Breth, 2013 #2557]	cellular nitrogen compound biosynthetic process GO:0044271	(DeZwaan et al., 1999)
2,4 MGG_12551	uncharacterized protein; SignalP - TM		
2,4 MGG_17624	uncharacterized protein; SignalP - noTM		

2.3 MGG_07411	uncharacterized protein; SignalP - noTM		
2.3 MGG_02989	uncharacterized protein; SignalP - noTM		
2.3 MGG_14712	uncharacterized protein; SignalP - noTM; reannotated from MGG_00209		
2.3 MGG_01255	Cellobiose dehydrogenase, extracellular flavoxychrome that degrades both cellulose and lignin; SignalP - TM	catalytic activity GO:0003824; carbohydrate binding GO:0030246; carbohydrate catabolic process GO:0015052	
2.3 MGG_05982	uncharacterized protein; SignalP - noTM	cellular nitrogen compound biosynthetic process GO:0044271	
2.2 MGG_10467	uncharacterized protein; SignalP - noTM		
2.2 MGG_03347	uncharacterized protein; SignalP - noTM		
2.2 MGG_10001	uncharacterized protein; SignalP - noTM		
2.2 MGG_00815	uncharacterized protein; SignalP - noTM		
2.1 MGG_07766	uncharacterized protein; SignalP - noTM		
2.1 MGG_02097	uncharacterized protein; SignalP - noTM		
2.0 MGG_01764	Polysaccharide lyase; SignalP - noTM		
Others (19 down-regulated; 35 up-regulated)			
-5.8 MGG_08360	Serine hydrolase FSH		
-5.4 MGG_07615	Methyltransferase	methyltransferase activity GO:0008168; methylation GO:0032259	
-5.1 MGG_00099	HAD-superfamily hydrolase; Cut1 (<i>N. crassa</i> ; e0.0)	metabolic process GO:0008152; hydrolase activity GO:0016787; mycelium development GO:0043581	
-4.6 MGG_14657	Sadenosyl-L-methionine-dependent methyltransferase-like		
-3.3 MGG_02942	Ribonuclease Inhibitor (CATH Superfamily 3.80.10.10)		
-3.3 MGG_06751	Uncharacterised protein family UPF0761; TM		
-3.3 MGG_02246	Conidiation-specific protein 6; Con6 (<i>N. crassa</i>)	ribonuclease activity GO:0004540 Nucleus GO:0005634; cytosol GO:0005829	this study; (White and Yandofsky, 1993)
-2.9 MGG_05855	peptidase; 1 TM	peptidase activity GO:0008233	
-2.7 MGG_13765	peptidase S28	Proteolysis GO:0006508; serine-type peptidase activity GO:0008236	
-2.7 MGG_10103	Het domain (heterokaryon incompatibility)		
-2.4 MGG_05505	Vacuole effluxer Atg22 like domain; 1 TM; autophagy protein		
-2.4 MGG_10657	acyl transferase; TM	transferase activity, transferring acyl groups other than amino-acyl groups GO:0016747	
-2.3 MGG_09681	6-blade_b-propeller_TolB-like		
-2.2 MGG_08019	F-box domain-containing protein	mycelium development GO:0043581	

-2.1 MGG_04582	Glycoside hydrolase; similar to yeast SCW11; T-DNA mutant shows reduced virulence	catalytic activity GO:0003824; carbohydrate metabolic process GO:0005975; cation binding GO:0043189; mycelium development GO:0043531; carbohydrate metabolic process GO:0005975; pathogenesis GO:0008405; spore germination GO:0009847; cytokinesis, completion of separation GO:0007109; fungal-type cell wall GO:0009277	(Jeon et al., 2007)
-2.1 MGG_08592	RNA-binding protein; survival motor neuron (SMN); domain involved in the assembly of spliceosomal small nuclear ribonucleoproteins (snRNPs)	Nucleus GO:0005634; mRNA processing GO:0006397; cytoplasm GO:0005737; RNA binding GO:0003723	
-2.1 MGG_16393	aminoacyl-tRNA hydrolase	aminoacyl-tRNA hydrolase activity GO:0004045; mitochondrial translation GO:00032543	
-2.1 MGG_08034	Rhodanese-like protein		
-2.0 MGG_11237	dsRNA-binding-like domain		
12.9 MGG_03212	uncharacterised protein, orthologue of <i>N. crassa</i> annotated as a methyltransferase (metabolism of beta-lactams)	mycelium development GO:0043581	
12.1 MGG_05719	Heat shock protein Hsp20HSP20-like chaperone	response to stress GO:0006950	
7.2 MGG_09227	uncharacterised protein, TM; <i>N. crassa</i> orthologue NCU03873 localises in developing septa [Lai, 2010 #2539]		
6.3 MGG_06790	Cir5 domain: This domain is found at the N terminus of the Cir5 protein, which is involved in silencing in fission yeast.		
4.2 MGG_15049	OTT_1508-like nucleic acid/nucleotide deaminase		
4.2 MGG_03329	HSP20-like chaperone; mutant defective in pathogenicity, appressorium formation, and conical morphology	response to stress GO:0006950; mycelium development GO:0043581	(Jeon et al., 2007)
4.1 MGG_09600	Heterokaryon incompatibility region	lipid catabolic process GO:0016042; triglyceride lipase activity GO:0004806	
3.3 MGG_11445	Cir5 domain: This domain is found at the N terminus of the Cir5 protein, which is involved in silencing in fission yeast.		
3.2 MGG_07250	DEAD/DEAH box helicase; promote translational arrest, polysome disassembly and decapping; Mtr4 (e-19), Skt2 (e-19)	ATP binding GO:0005524; hydrolase activity GO:0016787; nucleic acid binding GO:0003676; helicase activity GO:0004386; ATP-dependent helicase activity GO:0008026; cytosol GO:0005829	
3.1 MGG_11084	chromosomal proteins modulating gene activities and/or chromatin structure -SET1-domain, SET5 (e-3) SET domain protein of unknown function: deletion heterozygote is sensitive to compounds that target ergosterol biosynthesis	Binding GO:0005488	
2.7 MGG_04358	HSP20-like chaperone		
2.7 MGG_02648	dynamitin GTPase involved in membrane transport between compartments through the scission of a wide range of vesicles and organelles. The dynamitin family are also microtubule-associated force-producing proteins which are involved in the production of microtubule bundles.	nucleotide binding GO:0000166; GTPase activity GO:0003924; GTP binding GO:0005525; obsolete GTP catabolic process GO:0006184	
2.7 MGG_06739	SAM-dependent methyltransferase domain		
2.6 MGG_07560	peptidase A1	Proteolysis GO:0006508; aspartic-type endopeptidase activity GO:0004190	

2.5 MGG_00768	cyclin-like F-box domain		
2.5 MGG_000_8	Integral membrane protein, Major Facilitator Superfamily	transmembrane transport GO:005085; integral component of membrane GO:0016021; mycelium development GO:0043581	
2.5 MGG_00836	NUDIX hydrolase domain	hydrolase activity GO:0016787	
2.5 MGG_17265	Metalloprotein domain	metal ion binding GO:0046872	
2.4 MGG_01294	PAZ and PIWI domains found in Argonaute protein family; DUF1785	nucleic acid binding GO:0003676	
2.4 MGG_08113	Marvel (MAL) and related proteins for vesicle trafficking and membrane link domain; TM	mycelium development GO:0043581; membrane GO:0016020	
2.4 MGG_17222	glutathione S-transferase; participates in the detoxification of reactive electrophilic compounds by catalysing their conjugation to glutathione; reannulated form MGG_10108		
2.4 MGG_07159	Stc1 domain; in <i>S. pombe</i> , proteins containing this domain act as protein linkers, which link the chromatin modifying CLR complex to RNAi by tethering it to the RITS complex.		
2.3 MGG_07227	Cys-rich domain; TM		
2.3 MGG_07594	methyltransferase type 11	methylation GO:0032259	
2.2 MGG_09400	NUDIX hydrolase domain	metabolic process GO:0008152; methyltransferase activity GO:0008168;	
2.1 MGG_00675	cyclin-like F-box domain		
2.1 MGG_09384	Chloramphenicol acetyltransferase-like domain		
2.1 MGG_05825	Transferase	transferase activity GO:0016740; transferase activity, transferring acyl groups other than aminoacyl groups GO:0016747	
2.1 MGG_08052	glycosyl transferase; TM	transferase activity, transferring glycosyl groups GO:0016757	
2.0 MGG_03639	Arginine-tRNA ligase	nucleotide binding GO:0000166; ATP binding GO:0005524; Cytoplasm GO:0005737; arginine-tRNA ligase activity GO:0004814; aglycyl-tRNA aminoacylation GO:0003420	
2.0 MGG_02400	prin family, highly conserved nuclear proteins that may function as transcriptional regulators with a role in apoptosis; TM		
2.0 MGG_12911	RNA 2'-phosphotransferase 1	transferase activity GO:0016740; transferase activity, transferring phosphorus-containing groups GO:0016772; tRNA splicing, via endonucleolytic cleavage and ligation GO:0006388	
2.0 MGG_07796	Anaphase-promoting complex, subunit Cdc26	anaphase-promoting complex GO:0005680; regulation of mitotic metaphase/anaphase transition GO:0030071; anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolic process GO:0031145	
2.0 MGG_02114	cyclin GTPase involved in membrane transport between compartments through the scission of a wide range of vesicles and organelles. The cyclin family are also microtubule-associated force-producing proteins which are involved in the production of microtubule bundles.	nucleotide binding GO:0000166; GTPase activity GO:0003924; GTP binding GO:0005525; obsolete: GTP catabolic process GO:0006184	

2.0	MGG_02065	Kinesin light chain, variant	calalytic activity GO:0003524; binding GO:0005488; nucleoside metabolic process GO:0009116; ADP binding GO:0043531
unknown (27 down-regulated; 39 up-regulated)			
-8.5	MGG_10245	DUF3328; TM	
-5.3	MGG_15150	uncharacterised protein	
-5.0	MGG_03403	uncharacterised protein	
-4.9	MGG_03383	uncharacterised protein	
-4.4	MGG_15129	uncharacterised protein	
-4.1	MGG_08207	DUF3176; TM	
-3.7	MGG_11809	DUF3405; TM	
-3.6	MGG_14523	uncharacterised protein	
-3.3	MGG_11255	uncharacterised protein	
-3.2	MGG_06081	uncharacterised protein	
-3.0	MGG_16612	uncharacterized protein	
-2.8	MGG_10006	uncharacterized protein	
-2.6	MGG_13716	uncharacterized protein; TM	
-2.6	MGG_03504	DUF3129	
-2.6	MGG_04120	uncharacterized protein	
-2.6	MGG_00947	uncharacterized protein	
-2.5	MGG_05747	uncharacterized protein	
-2.5	MGG_00513	uncharacterized protein	
-2.5	MGG_01986	DUF1986; TM	
-2.3	MGG_04339	uncharacterized protein; 7 TM	
-2.3	MGG_09571	uncharacterized protein	
-2.3	MGG_09469	WD40 repeat domain	
-2.2	MGG_01940	uncharacterized protein; TM	
-2.1	MGG_08936	uncharacterized protein	
-2.1	MGG_16497	uncharacterized protein	
-2.1	MGG_10257	uncharacterized protein; TM	
-2.0	MGG_04181	uncharacterized protein	
6.2	MGG_15337	Zygot-specific protein; TM	
5.8	MGG_09612	uncharacterized protein; TM : fumarase folding)	
3.9	MGG_17073	uncharacterized protein	mycelium development GO:0043581

3.7 MGG_02956	uncharacterized protein		
3.7 MGG_19055	uncharacterized protein		
3.6 MGG_02206	uncharacterized protein		
3.5 MGG_01177	uncharacterized protein; TM	integral component of membrane GO:0016021	
3.3 MGG_15965	uncharacterized protein; TM		
3.2 MGG_03629	uncharacterized protein		
3.0 MGG_14358	uncharacterized protein	zinc ion binding GO:0008270; intracellular GO:0005622	
2.9 MGG_05131	uncharacterized protein		
2.9 MGG_03864	uncharacterized protein; TM		
2.8 MGG_15099	uncharacterized protein	nucleotide binding GO:0000166	
2.7 MGG_11694	uncharacterized protein; TM		
2.7 MGG_13028	uncharacterized protein		
2.7 MGG_07798	uncharacterized protein		
2.6 MGG_10921	uncharacterized protein		
2.6 MGG_17107	uncharacterized protein		
2.6 MGG_09604	uncharacterized protein; TM		
2.5 MGG_04595	uncharacterized protein		
2.5 MGG_05106	uncharacterized protein; TM		
2.4 MGG_00071	uncharacterized protein		
2.4 MGG_02208	uncharacterized protein; TM		
2.3 MGG_00287	uncharacterized protein		
2.3 MGG_13772	uncharacterized protein		
2.3 MGG_10788	uncharacterized protein		
2.3 MGG_03279	uncharacterized protein		
2.2 MGG_10480	uncharacterized protein; TM		
2.2 MGG_09916	uncharacterized protein		
2.2 MGG_09752	uncharacterized protein		
2.2 MGG_03358	uncharacterized protein; TM		
2.2 MGG_08026	uncharacterized protein; TM		
2.1 MGG_05143	uncharacterized protein		
2.1 MGG_02105	uncharacterized protein		
2.1 MGG_09412	uncharacterized protein		
2.1 MGG_17400	uncharacterized protein; previously MGG_11531		
2.0 MGG_00433	uncharacterized protein		
2.0 MGG_04910	uncharacterized protein;		
2.0 MGG_04729	uncharacterized protein		

REFERENCES (Table A2.3)

- Lu J, Cao H, Zhang L, Huang P, Lin F (2014)** Systematic analysis of Zn2Cys6 transcription factors required for development and pathogenicity by high-throughput gene knockout in the rice blast fungus. *PLoS Pathog* 10: e1004432.
- Kim S, Singh P, Park J, Park S, Friedman A, et al. (2011)** Genetic and molecular characterization of a blue light photoreceptor MGWC-1 in *Magnaporthe oryzae*. *Fungal Genetics and Biology* 48: 400-407.
- Jeon J, Park SY, Chi MH, Choi J, Park J, et al. (2007)** Genome-wide functional analysis of pathogenicity genes in the rice blast fungus. *Nature Genetics* 39: 561-565.
- Villalba F, Collemare J, Landraud P, Lambou K, Brozek V, et al. (2008)** Improved gene targeting in *Magnaporthe grisea* by inactivation of MgKU80 required for non-homologous end joining. *Fungal Genetics and Biology* 45: 68-75.
- Andersen MR, Nielsen JB, Klitgaard A, Petersen LM, Zachariassen M, et al. (2013)** Accurate prediction of secondary metabolite gene clusters in filamentous fungi. *Proceedings of the National Academy of Sciences of the United States of America* 110: E99-E107.
- Nguyen QB, Itoh K, Van Vu B, Tosa Y, Nakayashiki H (2011)** Simultaneous silencing of endo-beta-1,4 xylanase genes reveals their roles in the virulence of *Magnaporthe oryzae*. *Molecular Microbiology* 81: 1008-1019.
- Battaglia E, Klaubauf S, Vallet J, Ribot C, Lebrun MH, et al. (2013)** Xlr1 is involved in the transcriptional control of the pentose catabolic pathway, but not hemi-cellulolytic enzymes in *Magnaporthe oryzae*. *Fungal Genetics and Biology* 57: 76-84.
- Klaubauf S, Ribot C, Melayah D, Lagorce A, Lebrun MH, et al. (2013)** The pentose catabolic pathway of the rice blast fungus *Magnaporthe oryzae* involves a novel pentose reductase restricted to few fungal species. *FEBS Lett* 587: 1346-1352.
- Ding SL, Liu WD, Iliuk A, Ribot C, Vallet J, et al. (2010)** The Tlg1 Histone Deacetylase Complex Regulates Infectious Growth in the Rice Blast Fungus *Magnaporthe oryzae*. *Plant Cell* 22: 2495-2508.
- Wilson RA, Gibson RP, Quispe CF, Littlechild JA, Talbot NJ (2010)** An NADPH-dependent genetic switch regulates plant infection by the rice blast fungus. *Proceedings of the National Academy of Sciences of the United States of America* 107: 21902-21907.
- Chumley FG, Valent B (1990)** Genetic analysis of melanin deficient, nonpathogenic mutants of *Magnaporthe grisea*. *Molecular Plant-Microbe Interactions* 3: 135-143.
- Liu XH, Zhuang FL, Lu JP, Lin FC (2011)** Identification and molecular cloning MoplaA gene, a homologue of Homo sapiens PLAA, in *Magnaporthe oryzae*. *Microbiological Research* 167: 8-13.

- Xue C, Park G, Choi W, Zheng L, Dean RA, et al. (2002)** Two Novel Fungal Virulence Genes Specifically Expressed in Appressoria of the Rice Blast Fungus. *Plant Cell* 14: 2107-2119.
- Kim SG, Wang Y, Lee KH, Park Z-Y, Park J, et al. (2013)** In-depth insight into in vivo apoplastic secretome of rice-*Magnaporthe oryzae* interaction. *Journal of proteomics* 78: 58-71.
- Odenbach D, Breth B, Thines E, Weber RW, Anke H, et al. (2007)** The transcription factor Con7p is a central regulator of infection-related morphogenesis in the rice blast fungus *Magnaporthe grisea*. *Molecular Microbiology* 64: 293-307.
- Liu TB, Chen GQ, Min H, Lin FC (2009)** MoFLP1, encoding a novel fungal fasciclin-like protein, is involved in conidiation and pathogenicity in *Magnaporthe oryzae*. *Journal of Zhejiang University-Science B* 10: 434-444.
- Oh Y, Donofrio N, Pan HQ, Coughlan S, Brown DE, et al. (2008)** Transcriptome analysis reveals new insight into appressorium formation and function in the rice blast fungus *Magnaporthe oryzae*. *Genome Biology* 9: R85.
- Giraldo MC, Dagdas YF, Gupta YK, Mentlak TA, Yi M, et al. (2013)** Two distinct secretion systems facilitate tissue invasion by the rice blast fungus *Magnaporthe oryzae*. *Nature Communications* 4: 1996.
- Fu C, Ao J, Dettmann A, Seiler S, Free SJ (2014)** Characterization of the *Neurospora crassa* Cell Fusion Proteins, HAM-6, HAM-7, HAM-8, HAM-9, HAM-10, AMPH-1 and WHI-2. *Plos One* 9: e107773.
- Lacaze I, Lalucque H, Siegmund U, Silar P, Brun S (2014)** Identification of NoxD/Pro41 as the homologue of the p22(phox) NADPH oxidase subunit in fungi. *Molecular Microbiology* 95: 1006-1024.
- Siegmund U, Marschall R, Tudzynski P (2015)** BcNoxD, a putative ER protein, is a new component of the NADPH oxidase complex in *Botrytis cinerea*. *Molecular Microbiology* 95: 988-1005.
- Yan X, Li Y, Yue X, Wang C, Que Y, et al. (2011)** Two Novel Transcriptional Regulators Are Essential for Infection-related Morphogenesis and Pathogenicity of the Rice Blast Fungus *Magnaporthe oryzae*. *PLoS Pathog* 7: e1002385.
- Guo M, Chen Y, Du Y, Dong YH, Guo W, et al. (2011)** The bZIP Transcription Factor MoAP1 Mediates the Oxidative Stress Response and Is Critical for Pathogenicity of the Rice Blast Fungus *Magnaporthe oryzae*. *PLoS Pathogens* 7: e1001302.
- Szilagyi M, Kwon NJ, Dorogi C, Poci I, Yu JH, et al. (2010)** The extracellular beta-1,3-endoglucanase EngA is involved in autolysis of *Aspergillus nidulans*. *Journal of Applied Microbiology* 109: 1498-1508.
- Zhang HF, Liu KY, Zhang X, Tang W, Wang JS, et al. (2011)** Two Phosphodiesterase Genes, PDEL and PDEH, Regulate Development and Pathogenicity by Modulating Intracellular Cyclic AMP Levels in *Magnaporthe oryzae*. *Plos One* 6: e17241.
- Devanna NB, Vijayan J, Sharma TR (2014)** The Blast Resistance Gene Pi54

of Cloned from *Oryza officinalis* Interacts with Avr-Pi54 through Its Novel Non-LRR Domains. Plos One 9.

Breth B, Odenbach D, Yemelin A, Schlinck N, Schroder M, et al. (2013) The role of the Tra1p transcription factor of *Magnaporthe oryzae* in spore adhesion and pathogenic development. Fungal Genetics and Biology 57: 11-22.

DeZwaan TM, Carroll AM, Valent B, Sweigard JA (1999) *Magnaporthe grisea* Pth11p Is a Novel Plasma Membrane Protein That Mediates Appressorium Differentiation in Response to Inductive Substrate Cues. Plant Cell 11: 2013-2030.

White BT, Yanofsky C (1993) Structural Characterization and Expression Analysis of the *Neurospora* Conidiation Gene Con-6. Developmental Biology 160: 254-264.

Lai J, Ng SK, Liu FF, Patkar RN, Lu Y, et al. (2010) Marker fusion tagging, a new method for production of chromosomally encoded fusion proteins. Eukaryot Cell 9: 827-830.

Annex Table A2.4. List of primers used in this study.

General primers	
M13F	5' CGCCAGGGTTTTCCAGTCACGAC 3'
M13R	5' AGCGGATAACAATTTACACAGGA 3'
HY	5' GGATGCCTCCGCTCGAAGTA 3'
YG	5' CGTTGCAAGACCTGCCTGAA 3'
PCR-amplified hygromycin probe for Southern	
M13F	5' CGCCAGGGTTTTCCAGTCACGAC 3'
M13R	5' AGCGGATAACAATTTACACAGGA 3'
PCR-amplified probes for Southern	
B1RN-MGG_14045	GGGGACTGCTTTTTTGTACAAACTTGTTGGAAACCTTTGGTGAA
PEBP2-F-probe	ATTACGGACACAATGCCGAC
5'-PEBP1-Probe	GCGCACATCATGCAAATCTTG
3'-PEBP1-Probe	TTAACCCATCAAGACCGATGC
TPC1-Fw-probe	AGAACACGGCCATCTTCAAC
TPC1-Rv-probe	ACAGGCAGCTTCTCCTTGAC
B4-TPC1	5' GGGGACAACTTTGTATAGAAAAGTTGCTGTGCATCCTGAAGCCTGGAAAC 3'
B1RNTPC1KO	5' GGGGACTGCTTTTTTGTACAAACTTGATGTTGCTGAAAGTCGGCTCC 3'
B4-MGG_09956	GGGGACAACTTTGTATAGAAAAGTTGACTCGCTCGCCTCGCTTGAT
B1RN-MGG_09956	GGGGACTGCTTTTTTGTACAAACTTGCTGTGCTGTTTTTTTTCTGTTGTT
B4-MGG_02246	GGGGACAACTTTGTATAGAAAAGTTGCACAGGTTCTCACCAGTGCC
B1RN-MGG_02246	GGGGACTGCTTTTTTGTACAAACTTGATTGATGGTTGGGGTGTT
B4-MGG_04732	GGGGACAACTTTGTATAGAAAAGTTGGCTTCAGCATTACCCTGTA
B1RN-MGG_04732	GGGGACTGCTTTTTTGTACAAACTTGTTGCTGGAGTCCTTAATT
Primers for qPCR analysis	
PRO41-q-F	CCTGATGTTAGCGTGGGAGT
PRO41-q-R	TAGTAGACGGCAGCGTTGGT
PEBP2-q-F	ATGGTGGACCCAGACCTGAC
PEBP2-q-R	CCTGGTTGATGCTGACGTTG
PEBP1-q-F	GCCAGAGCACTGACAACCAA
PEBP1-q-R	ATGTAGGGCGCCAAGGTATG
CON6-q-F	CTCGAGGAGCAGTTTGATGG
3'-CON6-Probe	GCTCCTTGGCAGACTCCTTG
qTPC1 For	TTTCCAACACACGCCCATTTG
qTPC1 Rev	TCCTGCCAATTTGCAATGCC
GH18-q-F	GCTACTTTGCCGCTACCAC
GH18-q-R	GTCTCGGCAACGCGTACTT
Primers for PCR mutants analysis	
ext-5'-CON6	CATCCCGTGGCAGGTGCG
ext-3'-CON6	CGCAAGTCTTGGCACCAGTCC
5'-rev-PEBP1	GCCGTTTACGTCAAAGCTG
ext-5'-PEBP1	GAGATGCAGAGGAAAGTGCC
ext-5'-PEBP2	GTAGCGATCAAAATCCGAGG
ext-3'-PEBP2	CGCTTGGACGACGACCCG
ext-5'-PRO41	GACGGCTGTATGCGACTAG
ext-3'-PRO41	GGCTGGGCTTCGTTCAGGATG
CT74-1	CACGCCATGTAGTATTGACC
AndyF_T3	AATTAACCCTCACTAAAGGGA
5-HYGR	GCCGATAGTGGAACCGACGC
HSVtk-Probe	CCGAGCCGATGACTTACTGG
SU	5' GCTCCTCGATCAGAGTCTGAGGC 3'
UR	5' ACAGTCGGTTACCCTGGCGGTGC 3'
ext-5'-GH18	GGTAAGCGCTCCCTCTCCCTC
CT74-3	GTAGAATAGGTAAGTCAGATTG
Primers for analysis of T-DNA insertion in M1422	
AT-RB	5' GATTGTCGTTTCCGCTTCAG 3'
AT-LB2	5' CCAGTACTAAAATCCAGATCCC 3'
Primers for generating deletion mutants	
B4-TPC1	5' GGGGACAACTTTGTATAGAAAAGTTGCTGTGCATCCTGAAGCCTGGAAAC 3'
B1RNTPC1KO	5' GGGGACTGCTTTTTTGTACAAACTTGATGTTGCTGAAAGTCGGCTCC 3'
B2RCRTPC1KO	5' GGGGACAGCTTTCTGTACAAAGTGCCCTTGCCTACAACTGACGCTTC 3'
B3-TPC1	5' GGGGACAACTTTGTATAAATAAGTTGAGCAGGTGTGAGGCGAGGTAGC 3'
B4-MGG_09956	5' GGGGACAACTTTGTATAGAAAAGTTGACTCGCTCGCCTCGCTTGAT 3'
B1RN-MGG_09956	5' GGGGACTGCTTTTTTGTACAAACTTGCTGTGCTGTTTTTTTTCTGTTGTT 3'

B2RC-MGG_09956	5' GGGGACAGCTTTCTTGACAAAAGTGGGCTATTGCTTCGCGCCCGCC 3'
B3-MGG_09956	5' GGGGACAACCTTTGTATAATAAAGTTGGTTCTGCAAATGCTTCTTGT 3'
B4-MGG_02246	5' GGGGACAACCTTTGTATAGAAAAGTTGCACAGGTTCTCACCGATGCC 3'
B1RN-MGG_02246	5' GGGGACTGCTTTTTGTACAAACTTGATTGATGGTTGGGGGTGGTT 3'
B2RC-MGG_02246	5' GGGGACAGCTTTCTTGACAAAAGTGAAGCAGACTACATTAGTCGC 3'
B3-MGG_02246	5' GGGGACAACCTTTGTATAATAAAGTTGCTCCCTACCTGCCTACCCCTT 3'
B4-MGG_06800	5' GGGGACAACCTTTGTATAGAAAAGTTGATCCTGAAGACGAGGACGAG 3'
B1RN-MGG_06800	5' GGGGACTGCTTTTTGTACAAACTTGGGCTAGTCTAGTCTAGTTTG 3'
B2RC-MGG_06800	5' GGGGACAGCTTTCTTGACAAAAGTGGGTTATTTTCGGGGCCACTCT 3'
B3-MGG_06800	5' GGGGACAACCTTTGTATAATAAAGTTGATCATTAGCCGCCATTGCGCC 3'
B4-MGG_14045	5' GGGGACAACCTTTGTATAGAAAAGTTGGAAGAGCACTACAATAGACA 3'
B1RN-MGG_14045	5' GGGGACTGCTTTTTGTACAAACTTGTGTTGAAACCTTTGGGTGAA 3'
B2RC-MGG_14045	5' GGGGACAGCTTTCTTGACAAAAGTGGTTTGTGAGTGATTATGGGTC 3'
B3-MGG_14045	5' GGGGACAACCTTTGTATAATAAAGTTGTGGAGGACTGCGCGACGAA 3'
B4-MGG_04732	GGGGACAACCTTTGTATAGAAAAGTTGGCTTCAGCATTACCCCTGTA
B1RN-MGG_04732	GGGGACTGCTTTTTGTACAAACTTGGTTTGCTGGAGTCCTTAATT
B3-MGG_04732	GGGGACAACCTTTGTATAATAAAGTTGTGTGAGCGATTAAACGAAATG
B2RC-MGG_04732	GGGGACAGCTTTCTTGACAAAAGTGGGCTTGGAATGTAGAGGTT
Primers for generation of GFP protein fusion constructs	
7.GFP_F	5' ATGTTGAGCAAGGGCGAGGAGCTG 3'
TPC1_GFP_F	5' CCTGCCAACACCAATCCAC 3'
TPC1_GFP_Fnest	5' GTCGTGAACCTTGATTGTGGGC 3'
TPC1_GFP_R	5' CAGCTCCTCGCCCTTGCTCACCATTGGCAGCAATGACCAACTTG 3'
GFP_4658_R	5' GTCATGCCGTGAGTGATCC 3'
B1RC-TPC1	5' GGGGACTGCTTTTTGTACAAACTTGGATTGGCACGAATGACCAACTTGG 3'
B4-TPC1	5' GGGGACAACCTTTGTATAGAAAAGTTGCTGTGCATCCTGAAGCCTGGAAAC 3'
B2RC-TPC1	5' GGGGACAGCTTTCTTGACAAAAGTGGGACACTTGCCGATTATCAGTGTC 3'
B3-TPC1	5' GGGGACAACCTTTGTATAATAAAGTTGAGCAGGTGTGAGCGAGGTAGC 3'
Primers for generation of mRFP protein fusion constructs	
B4-MGG_09956	5' GGGGACAACCTTTGTATAGAAAAGTTGCAACCTCGGTATCCGACGTTCTG 3'
B1RN-MGG_09956	5' GGGGACTGCTTTTTGTACAAACTTGTGACACGCGCACCAACGCC 3'
B2RC-MGG_09956	5' GGGGACAGCTTTCTTGACAAAAGTGGGCTATTGCTTCGCGCCCGCC 3'
B3-MGG_09956	5' GGGGACAACCTTTGTATAATAAAGTTGCCCTTCGTCACCCCGTCTATGC 3'
B1-PRO41	5' GGGGACAAGTTTGTACAAAAAGCAGGCTAACGAAAAAAGACGACACA 3'
B2	5' GGGGACCACTTTGTACAAGAAAGCTGGGT 3'
Primers for PCR-site directed mutagenesis constructs	
TPC1noNLS_F	5' GGACCTTGCGAATGACAGACTTGGTGGGCT 3'
TPC1noNLS_R	5' GTCTGTCAATCGCAAGGGTCTCTTCTGCC 3'
TPC1noKK_F	5' GACAGAAGTTGGCCGCCGCAAGGGT 3'
TPC1noKK_R	5' TTCTGTCTTGCCGTCCTTGCC 3'
TPC1noRR_F	5' GTCATTACGTCAACTGGCAAGGAC 3'
TPC1noRR_R	5' AATGACAGACTTGGTGGGCT 3'
TPC1noSUMO_F	5' GCCGTCTTGCCAGTTGAC 3'
TPC1noSUMO_R	5' GGACGGCGCGACAGAAAAGAAGG 3'
TPC1_T168G_F	5' GTGTTGGAACCCGTCCTCGA 3'
TPC1_T168G_R	5' CCAACACGCGCCCATTTAGT 3'
TPC1_T349G_F	5' CTCGGTCTGTTGATGGTGG 3'
TPC1_T349G_R	5' CAACAGACCGAGGCGATCAGCC 3'
1R.TrpC.EcoRI_nest	5' ACGAATTCAGTGTACCTGTGCATTCTGGG 3'
primers for generation of Y2H constructs and NoxD:GFP	
NoxD_EcoRI	5' GGAGGCCAGTGAATTCATGGGAAGACTTATCAAGAACCAC 3'
NoxD_SmaI	5' CGATGCCC ACCCGGGTTAGACACGCGCACCAACGCCCTCC 3'
Nox1_EcoRI	5' CATGGAGGCCGAATTCATGTCGTCGGAGAGTTCTTGGCT 3'
Nox1_BamHI	5' GCAGGTCGACGGATCCCTAGAAATGCTCCTTCCAGAAGCG 3'
Nox2_EcoRI	5' CATGGAGGCCGAATTCATGTCGATACGGCTACGGAGGA 3'
Nox2_BamHI	5' GCAGGTCGACGGATCCCTAGAAATCTCCTTGCCCATAC 3'
NoxR_EcoRI	5' CATGGAGGCCGAATTCATGTCGCTCAAGCAGGAGATAGAA 3'
NoxR_BamHI	5' GCAGGTCGACGGATCCCTCATGCATCCTGAATCCAAACCTC 3'
NoxD_GFPF	5' TAGAAGTAGTGGATCTATCGAATGAAGTGGTTGTTCCGAG 3'
NoxD_GFPR	5' GACACGCGCACCAACGCCCTCCACG 3'
GFPF	5' CGTGGTGCGCGTGTCTATGGTGTGAGCAAGGGCGAGGAGCTG 3'
TrpCR	5' CCGTATCGATAAGCTGTGGAGATGTGGAGTGGGCGCTTA 3'

Annexes

Annex Table A3.1. Exp5 is mainly involved in metabolism. List of Go Terms obtained from immunoprecipitation results. FungiFun 2.2 web tool was used for functional enrichment analysis of fungal protein obtained from immunoprecipitation experiment (Priebe et al., 2015). Standard parameters were used. ^a p-value adjust <0.05 (adjust to number of genes input).

FunCat description	Adjusted p-value ^a	genes/category	% genes/category	genes/input	% genes/input
Cell rescue, defense and virulence					
stress response	0.000065536	28 / 258	10.9	28 / 258	10.9
unfolded protein response (e.g. ER quality control)	0.012882	10 / 77	12.98	10 / 258	3.9
Cellular transport, transport facilitation and transport routes					
electron transport	0.00026168	28 / 288	9.7	28 / 258	10.9
protein transport	0.0081995	21 / 239	8.8	21 / 258	8.14
transport ATPases	0.0000082609	17 / 91	18.7	17 / 258	6.9
mitochondrial transport	0.00011391	17 / 114	14.9	17 / 258	6.9
ABC transporters	0.016878	10 / 81	12.3	10 / 258	3.9
Energy					
glycolysis and gluconeogenesis	0.00018172	14 / 84	16.7	14 / 258	5.4
electron transport and membrane-associated energy conservation	0.00066716	14 / 98	14.3	14 / 258	5.4
aerobic respiration	0.00094489	14 / 102	13.7	14 / 258	5.4
respiration	0.0031278	11 / 75	14.7	11 / 258	4.3
metabolism of energy reserves (e.g. glycogen, trehalose)	0.00018172	10 / 43	23.3	10 / 258	3.9
tricarboxylic-acid pathway (citrate cycle, Krebs cycle, TCA cycle)	0.00047279	10 / 49	20.4	10 / 258	3.9
energy generation (e.g. ATP synthase)	0.027868	5 / 25	20	5 / 258	1.9
Interaction with the environment					
homeostasis of protons	0.027868	8 / 61	13.1	8 / 258	3.1
Metabolism					
C-compound and carbohydrate metabolism	0.00094489	54 / 800	6.75	54 / 258	20.9
phosphate metabolism	0.015443	37 / 554	6.7	37 / 258	14.3
sugar, glucoside, polyol and carboxylate catabolism	0.015443	18 / 203	8.9	18 / 258	6.9
sugar, glucoside, polyol and carboxylate anabolism	0.00011391	13 / 69	18.8	13 / 258	5.04
purin nucleotide/nucleoside/nucleobase metabolism	0.040174	12 / 126	9.5	12 / 258	4.7
tetrahydrofolate-dependent C-1-transfer	0.02621	4 / 15	26.7	4 / 258	1.6
C-1 compound anabolism	0.030134	3 / 8	37.5	3 / 258	1.2
Protein fate (folding, modification, destination)					
protein targeting, sorting and translocation	0.030745	23 / 313	7.3	23 / 258	8.9
modification with sugar residues (e.g. glycosylation, deglycosylation)	0.010741	9 / 62	14.5	9 / 258	3.5
Protein synthesis					
translation	0.0000021682	25 / 172	14.5	25 / 258	9.6
aminoacyl-tRNA-synthetases	0.000037359	11 / 42	26.2	11 / 258	4.3
translation elongation	0.0074951	6 / 26	23.1	6 / 258	2.3
Protein with binding function or cofactor requirement (structural or analytic)					
protein binding	0.023353	79 / 1483	5.3	79 / 258	30.6
ATP binding	0.00000075672	54 / 590	9.2	54 / 258	20.9
nucleotide/nucleoside/nucleobase binding	0.0035602	32 / 414	7.7	32 / 258	12.4
GTP binding	0.00018217	18 / 135	13.3	18 / 258	6.9
structural protein binding	0.023084	8 / 58	13.8	8 / 258	3.1
thiamine pyrophosphate binding	0.023084	3 / 7	42.9	3 / 258	1.2
Regulation of metabolism and protein function					
regulation by binding / dissociation	0.00058737	15 / 109	13.8	15 / 258	5.8