UNIVERSIDAD POLITÉCNICA DE MADRID

**ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA
AGRONÓMICA, ALIMENTARIA Y DE
BIOSISTEMAS**

**Understanding pathogenicity-related mechanisms in
the rice blast fungus *Magnaporthe oryzae***

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EL PRESIDENTE

LOS VOCALES

EL SECRETARIO

**“Quien se para a llorar, quien se lamenta contra la piedra
hostil del desaliento, quien se pone a otra cosa que no sea el
combate, no será un vencedor, será un vencido lento”**

Miguel Hernández

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LIST OF ABBREVIATIONS

%	Percentage
µg	Micrograms
µL	Microliters
µM	Micromolar
3AT	3-Amino-1,2,4-triazole
APA	Alternative polyadenylation
AS	Alternative splicing
ATMT	<i>Agrobacterium tumefaciens</i> -mediated transformation
AVR	Avirulence
BIC	Biotrophic interaction complex
Bp	Base pair
BP	Branch point
cAMP	Cyclic 3',5' adenosine monophosphate
cDNA	Complementary DNA
CDS	Coding sequence
CFI	Cleavage Factor I
cm	Centimetre
CM	Complete medium
DCM	Defined complex medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate

DOB	Dropout base
dpi	Days post-inoculation
eIF	Eukaryotic initiation factor
EIHM	Extra-invasive hyphal membrane
ER	Endoplasmic reticulum
f2dU	5-fluoro-2'-deoxyuridine
g	Grams
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
h	Hour
hpi	Hours post-inoculation
HYG	Hygromycin
IH	Invasive hyphae
Kb	Kilobase
KO	Knock-out
MAPK	Mitogen-activated protein kinase
mg	Milligram
min	Minutes
miRNA	MicroRNA
ml	Millilitre
MM	Minimal media
mM	Millimolar

MM-C	Minimal media without carbon source
MM-N	Minimal media without nitrogen source
MM ^T	Minimal media with ammonium tartrate as unique nitrogen source
mRNA	Messenger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate
ncRNA	Non-coding RNA
ng	Nanograms
NMD	Nonsense-mediated decay
nt	Nucleotide
°C	Degree Celsius
ORF	Open reading frame
PAS	Polyadenylation site
PCR	Polymerase chain reaction
PHIL-PS	Polystyrene hydrophilic surfaces
PKA	Protein kinase A
poly(A)	Poly adenines
qRT-PCR	Quantitative reverse-transcription polymerase chain reaction
RBP35	RNA-binding protein 35
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
rpm	Revolutions per minute
RRM	RNA recognition motif

SD	Synthetic defined
ss	Splicing site
SUR	Sulfonylurea
T-DNA	Transfer DNA
TK	Thymidine kinase
TOR	Target of Rapamycin
uORF	Upstream open reading frame
UTR	Untranslated region
WA	Water agar
WT	Wild-type
YPDA	Yeast peptone dextrose adenine

RESUMEN

Magnaporthe oryzae es un hongo ascomiceto que causa la piriculariosis, una de las enfermedades fúngicas más graves del cultivo de arroz (*Oryza sativa* L.). La identificación de proteínas de unión a ARN, específicas de hongos, está ayudando a desentrañar las redes postranscripcionales y los procesos celulares que confieren identidad al reino fúngico. Rbp35 es una proteína de unión a ARN y un componente exclusivo de la maquinaria de poliadenilación de hongos filamentosos. Rbp35 no es esencial para la viabilidad fúngica, pero regula la longitud de los 3' UTRs de transcritos que tienen funciones asociadas al desarrollo y la virulencia. La falta de ortólogos claros de Rbp35 en levaduras, plantas y animales indica que Rbp35 podría usarse como una diana fúngica para desarrollar compuestos antifúngicos.

Aquí, analizamos la regulación ejercida por el intrón localizado en el 5' UTR de *RBP35* y el marco de lectura abierto aguas arriba (uORF1), e investigamos cómo esta regulación modula la función Rbp35 en la célula. También mostramos la influencia de la poliadenilación alternativa en la patogenicidad de *M. oryzae* al observar las diferencias mostradas por las isoformas de ARNm de *14-3-3B* durante la infección, las cuales son reguladas por Rbp35. Finalmente, estudiamos la regulación del Hrp1 de *M. oryzae*, otro componente del Factor de escisión I de la maquinaria de poliadenilación específica de hongos.

La región 5' no traducida (5' UTR) del gen *RBP35* tiene 733 nucleótidos (nt) de largo, inusualmente grande en comparación con el tamaño medio de los 5' UTRs en *M. oryzae*. El intrón localizado en el 5' UTR de *RBP35* incluye dentro de su secuencia un marco de lectura abierto aguas arriba (uORF1) y parte de un segundo uORF (uORF2) capaz de generar péptidos de 36 y 72 aminoácidos, respectivamente. Esta característica no se encuentra en ningún otro gen de *M. oryzae*. Mostramos que la presencia del intrón 5' UTR inhibe la traducción de la secuencia codificante de la proteína *RBP35*. Además, los enfoques bioquímicos y genéticos revelan que el intrón del 5' UTR bloquea la traducción de la secuencia principal de codificación aguas abajo, es decir, *RBP35*. Los perfiles polisomales confirman la capacidad del 5' UTR para desequilibrar la cantidad de ARNm de *RBP35* que se encuentra en las fracciones polisomales. Además, encontramos que el péptido uORF1

puede desempeñar un papel independiente en la célula fúngica, una característica inusual para los uORF.

Seleccionamos el gen *14-3-3B*, ya que su pre-ARNm está poliadenilado por Rbp35, para estudiar la influencia de la poliadenilación alternativa en la biología de *M. oryzae*. Las proteínas 14-3-3 son proteínas de armazón que participan en las cascadas de señalización, incluido en la vía de señalización de la ruta TOR. El mutante $\Delta 14-3-3b$ muestra defectos significativos en la respuesta fúngica al estrés osmótico y la patogenicidad del hongo. La reintroducción de construcciones genéticas de *14-3-3B* en el mutante $\Delta 14-3-3b$ que contienen un 3' UTR corto y largo revela que el desarrollo de hongos o la respuesta al estrés osmótico no se ven afectados por la selección del sitio de poliadenilación. Sin embargo, la selección del sitio de poliadenilación distal controla los niveles de proteína y ARNm de *14-3-3B*, y es crucial para el proceso de infección de *M. oryzae*.

Hrp1 es una proteína de unión a ARN del segundo componente CFI presente en hongos filamentosos. La levadura contiene solo un CFI formado por Hrp1, y carece del segundo CFI que se encuentra exclusivamente en hongos filamentosos, que está compuesto por Rbp35 y CFI25. Mientras que Hrp1 es un gen esencial en la levadura, la eliminación de *MoHRP1* en *M. oryzae* genera un mutante que todavía es viable, aunque muestra graves deficiencias de desarrollo. El Hrp1 de levaduras puede recuperar parcialmente los defectos del mutante *mohrp1*. Sin embargo, Hrp1 de *M. oryzae* no es funcional en la levadura. Estos resultados sugieren que las funciones de estas proteínas difieren entre estos dos organismos. Ensayos de doble híbrido en levadura demuestran la interacción entre MoHrp1 y Rbp35, apoyando la divergencia funcional encontrada entre estos dos organismos.

En general, este trabajo de investigación ha contribuido a aumentar nuestro conocimiento sobre la relevancia de los elementos reguladores localizados en regiones no codificantes de la proteína de unión a ARN Rbp35, y el papel significativo que juegan los componentes de la maquinaria de poliadenilación fúngica y la poliadenilación alternativa durante la colonización de *M. oryzae* en el arroz.

SUMMARY

Magnaporthe oryzae is an ascomycetous fungus that causes the rice blast, one of the most serious fungal diseases on rice (*Oryza sativa* L.). Identification of fungal-specific RNA-binding proteins is helping to unravel post-transcriptional networks and cellular processes that confer identity to the fungal kingdom. Rbp35 is an RNA-binding protein exclusive to the polyadenylation machinery of filamentous fungi. *M. oryzae* Rbp35 is not essential for fungal viability but regulates the length of 3'UTRs of transcripts with development- and virulence-associated functions. The lack of clear Rbp35 orthologues in yeast, plants and animals indicates that Rbp35 could be used as a fungal target to develop anti-fungal drugs.

Here, we analyse the regulation exerted by the *RBP35* 5'UTR intron and upstream open reading frame (uORF1), and investigate how this regulation modulates Rbp35 function in the cell. We also show the influence of alternative polyadenylation in *M. oryzae* pathogenicity by looking at the differences displayed by Rbp35-dependent mRNA isoforms of *14-3-3B* during rice blast infection. Finally, we study the regulation of *M. oryzae* Hrp1, another component of the Cleavage Factor I of the fungal-specific polyadenylation machinery.

The 5' untranslated region (5' UTR) of the *RBP35* gene is 733 nucleotides (nt) long, unusually large for a standard fungal 5' UTR. The *RBP35* 5'UTR intron includes within its sequence a full upstream open reading (uORF1) and part of a second uORF (uORF2) capable of generating 36- and 72-aminoacid long peptides, respectively. This feature is not found in any other *M. oryzae* gene. We show that the presence of the 5' UTR intron inhibits the translation of the *RBP35* protein-coding sequence. Furthermore, biochemical and genetic approaches reveal that uORF1 blocks the translation of the main downstream coding sequence, i.e. *RBP35*. Polysomal profiles confirm the ability of the 5' UTR to unbalance the amount of *RBP35* mRNAs found in polysomal fractions. In addition to the inhibitory role of uORF1 over the main downstream protein-coding sequence, we found that uORF1 peptide can play an independent role in the fungal cell, an unusual feature for uORFs.

We select the *14-3-3B* gene since its pre-mRNA is polyadenylated by Rbp35 to study the influence of alternative polyadenylation in *M. oryzae* biology. The 14-3-3 proteins are scaffold proteins that participate in signaling cascades, including the target of rapamycin

(TOR) signaling pathway. The $\Delta 14\text{-}3\text{-}3B$ mutant shows significant defects in the fungal response to osmotic stress and fungal pathogenicity. The reintroduction of genetic constructs of *14-3-3B* in the $\Delta 14\text{-}3\text{-}3B$ mutant that contain a short and a long 3'UTR reveals that fungal development or the osmotic stress response are not affected by the polyadenylation site selection. However, the selection of distal polyadenylation site controls *14-3-3B* mRNA and protein levels, and is crucial for fungal infection.

Hrp1 is a RNA-binding protein component of the second CFI present in filamentous fungi. Yeast contains only one CFI formed by Hrp1, and lacks the second CFI found exclusively in filamentous fungi, which is composed by Rbp35 and CFI25. Whereas Hrp1 is an essential gene in yeast, the deletion of *MoHRP1* in *M. oryzae* is still viable although the mutant displays severe development deficiencies. Yeast Hrp1 is able to recover partially the defects of *M. oryzae mohrp1* mutant. However, the *M. oryzae* Hrp1 is not functional in yeast. These results suggest that the roles of these proteins differ between these two organisms. Yeast two hybrid assays demonstrate the interaction between MoHrp1 and Rbp35, supporting the functional divergence found.

Overall, this research work has contributed to increase our knowledge on the relevance of the regulatory elements localized in non-coding regions of the RNA-binding protein Rbp35, and the significant role that components of the fungal polyadenylation machinery and alternative polyadenylation play during *M. oryzae* rice colonisation.

1-GENERAL INTRODUCTION

This section is a modified version of the review published in. In Encyclopedia of Life Sciences (eLS), John Wiley & Sons, Ltd (Ed.). doi:10.1002/9780470015902.a0021311.pub2. (2018)

Magnaporthe and Its Relatives

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1.1. *Magnaporthe oryzae* relatives

The *Magnaporthaceae* family named by PF Cannon is a complex phylogenetic group (Cannon, 1994), and includes fungal genera like *Buergeriella*, *Ceratosphaeria*, *Gaeumannomyces*, *Magnaporthe*, *Mycoleptodiscus*, *Ophioceras*, *Pseudohalonectria*, and *Pyricularia*. The *Gaeumannomyces* and *Magnaporthe* genera are studied in more detail due to their ability to infect cereals and grasses of economic significance. The similarities found on teleomorph morphology and host range in fungal species of these genera have justified their inclusion within the same family (Cannon, 1994), and further phylogeny studies support it (Hibbett *et al.*, 2007).

Figure 1.1. *G. graminis* and *M. oryzae* penetrate plant roots by means of hyphopodia. A) Light microscope image and (b) scanning electron micrograph of lobed hyphopodia on Mylar films developed by *G. graminis* strains. Reproduced with permission from (Money *et al.*, 1998). © Elsevier. C) Confocal image of *M. oryzae* hyphopodia on rice roots. D) Scanning electron micrograph of *M. oryzae* hyphopodia on hydrophilic polystyrene. C,D) Reproduced with permission from (Illana *et al.*, 2013). © Springer Nature.

The *Magnaporthaceae* family is included within the ascomycetous *Magnaporthales* order. This order was proposed based on the nuclear 18S and 28S ribosomal DNA (rDNA) analysis, and the morphological similarities found in fungal species of the *Magnaporthaceae* family and *Diaporthales* / *Ophiostomatales* orders (Thongkantha *et al.*, 2009). However, there is not a well-defined consensus about these phylogenetic similarities. Currently, more

than two hundred species have been described as *Magnaporthales*, including a significant number of wood saprophytic and plant pathogens (Thongkantha *et al.*, 2009). Additional phylogenetic analyses have proposed the division of *Magnaporthales* in three different lineages that correspond to the families of *Ophioceraceae*, *Magnaporthaceae* and *Pyriculariaceae* (Klaubauf *et al.*, 2014; Luo *et al.*, 2015). The *Ophioceraceae* differs clearly from the other clades and is composed of terrestrial and aquatic species that maintain a saprophytic lifestyle. The other two clades contain fungal plant pathogens. The *Magnaporthaceae* clade is associated with root-infecting fungi that use a penetration structure named hyphopodia to infect underground parts of the plant. The *Pyriculariaceae* clade includes fungi that colonise above ground plant tissues and make use of the appressorium to penetrate leaves.

1.1.1. *Gaeumannomyces graminis* and *G. tritici*

The *Gaeumannomyces* genus, established in 1952 by Arx and Olivier (Arx & Olivier, 1952) is formed by seven species. *G. caricis*, *G. medullaris*, *G. incrustrans*, *G. amomi* and *G. wongoonoo* were originally isolated from distinct grass species whereas *G. cylindrosporus* and *G. graminis* were found in cereals (Freeman & Ward, 2004). By far, the most damaging of all is the take-all fungus *G. graminis*, a homothallic fungus frequently found in the soil that infects mainly underground plant tissues. Depending on host range, infection capability and fungal structures, four different varieties are found within *G. graminis*, i.e. *graminis*, *maydis*, *avenae* and *tritici*. Many similarities exist between *G. graminis* var. *tritici* and *G. graminis* var. *avenae*, suggesting that they are closely related compared to *G. graminis* var. *graminis* or *G. graminis* var. *maydis* (Bryan *et al.*, 1995). A recent phylogenetic analysis determined that *G. graminis* var. *tritici* and *G. graminis* var. *avenae* represent different fungal species, and their proposed names are now *G. tritici* and *G. avenae*, respectively (Hernandez-Restrepo *et al.*, 2016).

G. graminis var. *graminis* is currently the only described *Gaeumannomyces* that forms lobed brown hyphopodia when it colonizes the plant (Figure 1.1A-1.1B). Other fungi of this family form simple hyphopodia during root colonization (Figure 1.1C-1.1D). *G. graminis* var. *graminis* is considered a weak pathogen of different cereals. It causes the sheath

blast in rice (*Oryza sativa* L.) (Ou, 1985; Walker, 1972) and the dieback in Bermuda grass (*Cynodon dactylon* L.) (Elliott, 1991).

G. tritici is the fungal species of this genus that causes most serious crop damages. It is the causal agent of the devastating take-all disease of wheat. The main host of *G. tritici* is wheat but is frequently found in barley (*Hordeum vulgare* L.) and rye (*Secale cereale* L.) (Walker, 1972). *G. tritici* is normally very aggressive and induces white heads in wheat crops due to the collapse of the root vascular system. Once the plant has been infected by the fungus (Figure 1.2A), the root becomes dark brown with severe necrotic lesions in more affected areas, reaching the necrosis to the base of the stem (Figure 1.2B). This damage leads to a disruption of the root architecture precluding the correct nutrient transport to the aerial tissues of the plant. The lack of nutrients impairs plant growth and blocks the maturation of the grain, which frequently is not formed, producing white-coloured empty heads. When the infection spreads to neighbouring plants, this is seen as white patches in the field (Figure 1.2C).

Figure 1.2. Disease symptoms of take-all on wheat and summer patch disease on turfgrass. A) Pigmented runner hyphae of *G. tritici* on the surface of a wheat root. Reproduced with permission from (Cook, 2003). © Elsevier. B) Adult wheat plants with severe take-all rot symptoms on the roots and the stem base. Courtesy of AHDB (Agriculture and Horticulture Development Board, UK)/BASF. C) Circular patches of wheat plants in a field showing typical take-all disease symptoms with visible white heads and stunted growth. Courtesy of Kansas State University. D) Necrotic root lesions caused by *M. poae*. E) Typical circular patches of yellow-brown colour on turfgrass caused by *M. poae*. D, E) Courtesy of Dr. Lane Tredway, North Carolina State University.

To avoid complete yield losses, several possible actions have been studied. The biological control of take-all is relatively efficient and is based on the use of suppressive soils

(Cook, 2003). Cultural methods such as crop rotation using legumes or other non-host crops of *G. tritici* instead of wheat also help to control the disease (Yeates, 1986), and they must be undertaken within the first two years depending on the climate area. A break in wheat cultivation promotes take-all decline, which can be enhanced by growing some weed grasses, e.g. ryegrass or barren brome (Home-Grown Cereals Authority Report, 2006). To our knowledge, resistance genes against *G. tritici* have not been found yet, which hampers the generation of more resistant wheat cultivars by traditional breeding (Freeman & Ward, 2004).

Despite the relevance of the take-all disease, the molecular mechanisms that regulate *Gaeumannomyces* infection have not been studied in detail. *G. tritici* and *G. graminis* are genetically intractable and very inefficient for gene disruption methods. The pH signalling pathway seems to operate very similar to the one described in other fungi (Daval *et al.*, 2013). The only reported gene successfully disrupted is the *avecinase* gene in *G. avenae* (previously *Gga*). The *avecinase* gene encodes an enzyme that detoxifies the saponin *avenacin*. This metabolite is produced by oats and confers resistance against several pathogens, like *G. tritici* (Osborn *et al.*, 1994). The lack of *avecinase* in *G. avenae* avoids the colonization of its oat host, suggesting a central role for this enzyme in fungal host range (Bowyer *et al.*, 1995).

1.2. The blast fungus *Magnaporthe oryzae* (syn. *Pyricularia oryzae*)

Magnaporthe oryzae has become a leading organism for the study of fungal pathogenesis in crops due to its genetic feasibility and the economic impact of the blast disease. The fungus can cause complete yield losses under favourable conditions (Hossain, 2004; Illana *et al.*, 2013). *M. oryzae* infects multiple grasses and cereals (Ou, 1980), and has been renamed several times since its first description. The previous name *M. grisea* (Hebert) Barr (anamorph *Pyricularia grisea* Sacc) was found to be a species-complex (Couch & Kohn, 2002). Phylogenetic analyses inferred the presence of two distinct clades within the *M. grisea* species-complex (Couch & Kohn, 2002). Currently, *M. grisea* is used for the clade infecting *Digitaria* and related grasses. The other clade, formed by fungi that infect rice and wheat received a new name species, *Magnaporthe oryzae*. It is important to highlight that no differential morphological features are observed between isolates belonging to these two clades.

In the field, the main control strategy of the rice blast disease is the use of rice resistant cultivars, although the fungus can overcome newly introduced resistance genes in 2-3 years due in part to its genome instability (Nicholas J. Talbot, 2003; Zhou *et al.*, 2007). Blast has also become a relevant disease on wheat during the last decades (Cruz & Valent, 2017), especially in South America where it seems that the wheat blast disease has originated, and more recently in Asia (Islam *et al.*, 2016). Under these conditions, other wheat-producing regions like Europe or North America are at risk.

To understand the complex genetic structure and evolutionary history of *M. oryzae*, a phylogenomic study has been carried out using 12 *M. oryzae* isolates of grass and cereal genera (Gladieux *et al.*, 2018). This study suggests the existence of multiple divergent lineages within *M. oryzae*, each preferentially associated with one host genus. It also suggests that gene flow has contributed to the emergence of multiple lineages within *M. oryzae* and contradicts the proposal of the new species name *Pyricularia graminis-tritici* for the wheat-infecting blast isolates. Additional phylogenetic analyses using *M. oryzae* wheat isolates supported that this strain cannot be considered a new specie. In addition, this study alerts that the recombination of a wheat-infecting strain with sexually fertile rice isolates could produce a new strain more virulent to rice and wheat (Valent *et al.*, 2019).

In contrast to wheat isolates, the rice-infecting strains of *M. oryzae* are present worldwide. Rice blast symptoms are observed in all plant tissues including leaves, stems, nodes, collar, panicles and grains (Figure 1.3A-E). It can also infect roots under laboratory conditions (Figure 1.3F) (Dufresne & Osbourn, 2001; Marcel *et al.*, 2010; Ane Sesma & Osbourn, 2004; Tucker *et al.*, 2010), which suggests that underground infection can be a source of primary inoculum in the field (Figure 1.4) (Ane Sesma & Osbourn, 2004). On leaves, symptoms appear like diamond shaped spots. These spots are usually gray in the middle surrounded by a brown-coloured region. The panicle can fall over if it is seriously affected, causing the neck blast. Neck and panicle blast produce the most severe yield losses in this crop (Ou, 1985).

1.2.1. *M. oryzae* root infection

Research on *M. oryzae* root infection pathways is helping to expand our knowledge in the underground infection mechanisms used by other close relatives within the *Magnaporthales*

such as *Gaeumannomyces* species, which are genetically intractable (Besi *et al.*, 2009; Luo *et al.*, 2015).

M. oryzae leaf and root penetration mechanisms differ (Ane Sesma & Osbourn, 2004). During leaf penetration, the fungus develops an appressorium, which is a melanised and highly specialized structure (Figure 1.3G). The fungus generates within the appressorium the high turgor pressure required to cross the tough leaf cell wall by mechanical means (Howard *et al.*, 1991). The protein kinase A (PKA) regulates this process by driving the mobilization of lipid and glycogen (Thines *et al.*, 2000). The melanisation of appressoria is essential to maintain the high turgor pressure within the appressorium (Chumley & Valent, 1990). *M. oryzae* root colonisation is initiated using simple hyphopodia that allow the fungus to penetrate directly into the root tissue (Figure 1.1C-1.1D) (Ane Sesma & Osbourn, 2004), similar to those observed in *G. graminis*. The $\Delta cpka$ and melanin-deficient mutants produce

Figure 1.3. Disease symptoms and fungal structures produced by the rice blast fungus *M. oryzae*. **A)** Panicle blast symptoms in a rice field. **B)** Non-infected (left) and infected (right) rice panicle with necrotic lesions on neck (a), leaf (b) and collar (c). A,B) Courtesy of Dr. Yulin Jia, USDA-ARS, Dale Bumpers National Rice Research Center. **C)** Fallen panicle showing necrosis in the neck. **D-E)** Typical diamond-shaped lesions with brown margins on 3-week-old rice leaves. **F)** Necrotic symptoms on rice roots. **G)** Scanning electron micrograph of *M. oryzae* conidia (CO) producing appressoria (AP) on barley leaves. **H)** Confocal image of *M. oryzae* conidia on hydrophilic polystyrene membranes producing simple hyphopodia-like structures and pre-invasive hyphae. The image was taken at 24 h using tetramethylrhodamine isothiocyanate (TRITC)-labelled wheat germ agglutinin (anti-chitin lectin), which is shown in red. The pre-invasive hyphae (pre-IH) shows reduced levels of chitin compared to the cell wall of the hyphopodium (HY).

hyphopodia and can penetrate roots, which indicates that turgor generation is not required for hyphopodia-mediated root penetration.

M. oryzae also penetrates roots by means of specialized hyphae, named pre-invasive hyphae (pre-IH, Figure 1.3H) (Tucker *et al.*, 2010). Pre-IH is escorted by changes in the fungal cell wall structure, which can be induced by polystyrene hydrophilic surfaces (PHIL-PS) (Tucker *et al.*, 2010). The use of PHIL-PS allowed to further dissect *M. oryzae* pre-IH development at molecular level. The formation of pre-IH relies on the mitogen-activated protein kinase (MAPK) Pmk1 (J. R. Xu & Hamer, 1996), its downstream transcriptional regulator Mst12 (Park *et al.*, 2002), and the upstream effector Cbp1 (Kamakura *et al.*, 2002). In leaves, these proteins regulate appressorium development and function, and fungal invasive growth, providing evidence of the existence of common requirements for aerial and underground plant infection. Interestingly, pre-IH have a reduced content of chitin and β -glucans in the fungal cell wall (Tucker *et al.*, 2010), possibly to avoid or to reduce the plant immune response (Boller & Felix, 2009; Zipfel & Oldroyd, 2017).

In an attempt to identify more genetic determinants regulating *M. oryzae* structures associated with root infection, a T-DNA insertional screening of nearly 3,000 fungal transformants was carried out on PHIL-PS (Tucker *et al.*, 2010). Twenty T-DNA fungal lines showed altered growth on PHIL-PS and defects in pathogenicity. The majority of them displayed appressorium penetration defects. Six of them showed no relevant growth defects on complete medium, and only one, M1373 presented strong defects specifically towards roots. These results supported a strong developmental link between hyphopodium, pre-IH and appressorium structures (Tucker *et al.*, 2010).

The mutant M1373 has the T-DNA inserted in the coding sequence of a gene encoding for exportin-5 (Exp5). The generation of the deletion mutant $\Delta exp5$ confirmed the phenotype displayed by M1373. Exp5 is the karyopherin/ β -importin orthologue of yeast Msn5 and human exportin-5. Karyopherins are involved in nucleocytoplasmic transport in eukaryotic cells (Aramburu & Lemke, 2017; Sloan *et al.*, 2015). The human Exp5 can translocate non-coding RNAs and proteins between the nucleus and the cytoplasm, and the yeast Msn5 is involved in cell cycle, stress response, glucose, and nitrogen and phosphate metabolism. The $\Delta exp5$ mutant is unable to produce necrotic symptoms on roots although retains the capability to penetrate leaves (Tucker *et al.*, 2010). In addition, the blast lesions

produced by the $\Delta exp5$ mutant on leaves are smaller, an indication of invasive growth defects (Tucker *et al.*, 2010), which strengthens the association between leaf invasive growth and root penetration.

M. oryzae Nut1 is the orthologue of *Aspergillus nidulans* AreA and acts as a central regulator of nitrogen assimilation (Froeliger & Carpenter, 1996). *NUT1* is essential for the growth of the fungus in underground tissues, and therefore the development of blast symptoms on roots (Dufresne & Osbourn, 2001; Froeliger & Carpenter, 1996). Interestingly, the $\Delta nut1$ mutant induces normal disease symptoms on leaves, which supports the existence of specific metabolic requirements for fungal colonization in different plant tissues. The *M. oryzae* *FOW1* also confirms the existence of specific fungal metabolic needs during invasion of different plant tissues. The *M. oryzae* $\Delta fow1$ mutant can penetrate the cortical cells of rice roots but cannot reach the vascular system, in contrast to the wild-type strain (Ane Sesma & Osbourn, 2004). This gene codifies for a mitochondrial carrier protein orthologue to the *Fusarium oxysporum* Fow1 protein involved for vascular invasion of tomato plants (Inoue *et al.*, 2002). These findings support that cellular respiration can be playing a key role during *M. oryzae* and *F. oxysporum* invasion of roots, as it has been shown for other root-colonizing fungi (Tamasloukht *et al.*, 2003).

1.2.2. *M. oryzae* leaf infection

The *M. oryzae* foliar infection begins when a three celled conidium, usually dispersed by wind or water splash, lands on rice leaf surface. Then, the spore apex secretes a mucilage, sticking it onto the hydrophobic cuticle (Hamer *et al.*, 1988). Once attached, a polarize germ tube emerges normally from the most apical cell. Within few hours, the tip of the germ tube tip swells and a specialized penetration structure, called appressorium, is developed. This structure is highly melanised and its formation requires high humidity and a hard hydrophobic surface. But other compounds, like plant cutin monomers, can also induce germ tube differentiation (Gilbert *et al.*, 1996). *M. oryzae* can break the tough leaf cuticle due to the high turgor pressure produced within the appressorium structure, which accumulates high concentrations of glycerol (De Jong *et al.*, 1997). This high content in glycerol derives from the degradation of lipids and glycogen storages of the spore (Thines *et al.*, 2000). The appressorium contains a melanin layer between a chitin-enriched cell wall and the cell

membrane. This semi-permeable layer acts as an efflux barrier for glycerol (Howard *et al.*, 1991). This physical force combined with cell wall-degrading enzymes permits the emergence of a penetration peg at the base of the appressorium, which breaks the leaf cuticle to begin fungal cell invasion.

Subsequently, *M. oryzae* colonizes plant epidermal cells by means of ramified hyphae. Six days after fungal penetration, reproduction structures, namely conidiophores, emerge at the leaf surface where lesions can be visualized. In this step, thousands of conidia during several days within each lesion and dispersed by wind or water splash, starting again the fungal cycle of infection (Figure 1.4).

Figure 1.4. Rice blast disease infection cycle. Right panel: *M. oryzae* leaf cycle. *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 the fungal growth on leaf 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 during weeks to the environment. Reproduced with permission from (Ribot *et al.*, 2008). © Elsevier. 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. Reproduced with permission from (Illana *et al.*, 2013). © Springer Nature.

1.2.3. *M. oryzae* autophagy

Outside the rice plant, the fungus survives in a poor nutrient environment. During germination, the fungus can grow due to the storage of trehalose, glycogen, polyols and lipid bodies within the spore until it penetrates the plant cell, where a high nutrient environment is then available (Andrew J. Foster *et al.*, 2016; Thines *et al.*, 2000). One of the most important pathways involved in cell adaptation to a nutrient-changing environment is autophagy (Kroemer *et al.*, 2010). The process of autophagy degrades proteins, lipids or entire organelles by surrounding them with double-membrane vesicles, named autophagosomes. This highly conserved mechanism fuses autophagosomes to lysosomes, where the cytoplasmic compounds are hydrolysed (Galluzzi *et al.*, 2014).

During *M. oryzae* germination, only the serine/threonine protein kinase *MoATG1* seems to contribute to this process but autophagy is not essential for conidia germination (X. H. Liu *et al.*, 2007). However, autophagy plays a central role during appressorium maturation. The fungus needs to degrade spore compounds to obtain enough energy and to develop high turgor pressure within the appressorium. This autophagic mechanism leads into a conidial cell collapse. The energy provided from autophagic events are also necessary for asexual differentiation. Some genes like *MoATG8*, *MoATG4*, *MoATG1* and recently *MoATG14* have been described as essential genes for a correct conidium programmed cell death and turgor pressure accumulation in the appressorium (Kershaw & Talbot, 2009; X. H. Liu *et al.*, 2017). *MoATG8* and the cysteine protease named *MoATG4* interact *in vivo* (T. B. Liu *et al.*, 2010; Veneault-Fourrey *et al.*, 2006). The degradation of lipid deposits also provides the metabolic energy required for appressoria formation, and where vacuoles are implicated in this mechanism (Weber *et al.*, 2001). The *MoSNF1* complex permits a free nutrient environment adaptation through peroxisomal-dependent lipid metabolism (Zeng *et al.*, 2014). *MoSga1* has been described as a vacuolar glucoamylase that acts close to autophagy through glycogen catabolism developing a correct asexual maturation (Deng & Naqvi, 2010).

1.2.4. *M. oryzae* cell cycle

Mitotic control is necessary for fungal infection and a round of mitosis is completed before the appressorium is fully developed (Veneault-Fourrey *et al.*, 2006). When the spore

germinates on the rice leaf surface, the spore nucleus passes quickly from G1 to S phase during germination. After that, an entry in mitosis is necessary to start appressorium differentiation. Finally the progression of mitosis does not affect appressorium maturation but it is crucial for repolarization and infection progression in the plant (Saunders *et al.*, 2010a; Saunders *et al.*, 2010b). Recently, it has been proposed that the cell nucleus is arrested in a G1 phase in the early appressorium and that turgor generation is necessary to entry into the S phase and for cytoskeleton remodelling. This remodelling is controlled by the NADPH oxidase Nox2 complex that acts on septins GTPases, which form the appressorium pore required to initiate plant penetration (Oses-Ruiz *et al.*, 2017).

1.2.5. *M. oryzae* septins

Septins are a family of GTPases conserved in all eukaryotes, except in plants (Nishihama *et al.*, 2011). These proteins are involved in several fungal processes like asymmetric cell development, cell differentiation or cytoskeleton organization (Khan *et al.*, 2015). These processes have a clear link with fungal virulence (Vargas-Muniz *et al.*, 2016), being cell shape determination crucial during invasive growth (Momany & Talbot, 2017). In *M. oryzae* there are five septin genes, four of which are core septins (Dagdas *et al.*, 2012). These proteins are crucial for fungal pathogenicity; all the mutants on these genes are defective in infection. Septins play a key role in the assembly of the toroidal F-actin network in appressorium pore. In this appressorium region cell wall thickness is reduced and septins contribute to maintain cell wall rigidity (Dagdas *et al.*, 2012). A direct link between septins and reactive oxygen (ROS) synthesis has been found in *M. oryzae*. NADPH oxidases are involved in the reduction of oxygen molecules to superoxide (Lambeth, 2004), and three of these oxidases have been described to be relevant during *M. oryzae* infection (Egan *et al.*, 2007; Galhano *et al.*, 2017; Ryder *et al.*, 2013). Nox2 is involved in the assembly of the toroidal F-actin network which permits the correct pore formation. But cell polarization is also important once the hypha has penetrated. During infection Nox1 is crucial for polarized growth maintenance. Nox1 promotes invasive hypha elongation during plant cell penetration (Ryder *et al.*, 2013). NoxD was recently found to be the missing subunit component of fungal NADPH complexes (Galhano *et al.*, 2017).

Finally, the appressorium remodelling is coupled with exocyst complex, which helps sustaining the hyphal tip. Both processes are needed for the morphogenetic changes followed during infection (Gupta *et al.*, 2015). The exocyst complex contributes to the secretory system, which plays a key role during infection. *Magnaporthe* secretes several proteins into host cells to avoid the plant immune response. This complex is recruited in the appressorium pore by septins and NADPH oxidases to carry out a successful penetration (Gupta *et al.*, 2015).

1.2.6. *M. oryzae* secretion systems and protein effectors

After appressorium formation and the rice cell cuticle breaking *M. oryzae* invades the cells through invasive hyphae (IH). The fungus lives inside as a hemibiotrophic pathogen and biotrophic IH are surrounded by a plant membrane named extra-invasive hyphal membrane (EIHM) (Kankanala *et al.*, 2007). *Magnaporthe* secretes several proteins towards rice cell with the aim to suppress the host immunity. These effector proteins can be secreted by apoplastic- or by exocyst-mediated systems (Giraldo *et al.*, 2013).

Apoplastic effectors are secreted from the endoplasmic reticulum (ER) to Golgi pathway. These effectors have a peptide signal and are not in contact with the plant cell cytoplasm. Instead, they accumulate extracellularly in the EIHM (Mosquera *et al.*, 2009). A correct function of actin, microtubules, ER and Golgi genes are necessary for apoplastic effectors secretion (S. Zhang & Xu, 2014). By contrast, exocyst-dependent routes secrete cytoplasmic effectors that are delivered into the plant cell (Giraldo *et al.*, 2013). Exo70 and Sec5 components of the exocyst complex are necessary for an efficient protein translocation. These cytoplasmic effectors are accumulated in a membrane-rich structure formed by the plant known as the biotrophic interfacial complex (BIC). In *M. oryzae* several hundreds of candidate effector genes are found but determining its direct role during infection is not trivial due to their redundancy (Giraldo & Valent, 2013). These effectors are normally less than 200 amino acids in length, have no conserved sequences or domains and are expressed only during plant infection. The analyses of the three unique effectors, AVR1-CO39, AVR-Pita and AvrPiz-t, which have been structurally characterized in *M. oryzae* has defined the presence of a core six β -sandwich structure common to all of them (de Guillen *et al.*, 2015). These three effectors are stabilized by hydrophobic residues and disulfides bonds in the

beginning of the 1 β and 5 β sheets. This structure also possesses a hydrophobic region that it seems relevant for interaction with other proteins. All these features have allowed to cluster these effectors in a new family known as MAX effectors (for Magnaporthe Avrs and ToXB-like) (de Guillen *et al.*, 2015). These results helped to establish a new method for effector identification in *M. oryzae*.

1.2.7. *M. oryzae* kidnaps rice hormone pathways

The modulation of several hormone pathways like jasmonic acid (JA), salicylic acid and ethylene increases plant protection against pathogens (Ning *et al.*, 2017; Pieterse *et al.*, 2012). However, some pathogens have developed strategies to neutralize these hormone-associated immunity pathways in order to obtain nutrients and grow within its host plant (Chanclud & Morel, 2016). An example of this strategy is the *M. oryzae* antibiotic biosynthesis monooxygenase (*ABM*) gene, which is involved in rice immunity repression (Patkar *et al.*, 2015). This fungal gene encodes an enzyme that is responsible for the conversion of the active form of JA into the inactive form by hydroxylation (12OH-JA), and its secretion during *M. oryzae* host penetration helps to decrease rice immune responses. Strikingly, *ABM* closest orthologues are only found in rhizosphere bacteria like *Burkholderia* and *Ralstonia* species (Patkar *et al.*, 2015), which suggests that *M. oryzae* could share a common soil habitat with some root bacterial pathogens. This would imply the *M. oryzae* *ABM* gene has been acquired by horizontal gene transfer.

M. oryzae has also developed other strategies for hijacking plant metabolism by manipulating cytokine (CK) pathways. *M. oryzae* produces identical or similar chemical compounds to plant CKs, and modulates its levels during plant cell invasion. *M. oryzae* *CKS1* gene is required for fungal CK biosynthesis and the $\Delta cks1$ mutant is defective in pathogenicity (Chanclud *et al.*, 2016). CKs secreted by *M. oryzae* change the transcriptional expression of rice responsive CK genes, indicating that *M. oryzae* CKs are detected and able to modulate CK-mediated plant processes. *M. oryzae* CKs reduce the accumulation of soluble sugars involved in early response immunity pathways in infected tissues. *M. oryzae* is also able to use the plant vitamin B3 when the fungal nicotinamide adenine dinucleotide biosynthesis is disrupted (Wilson *et al.*, 2019).

1.3. Post-transcriptional regulation: controlling mRNA fate during fungal infection

Fungal pathogens grow under unfavourable conditions during infection since they trigger the host immune reaction that tries to prevent their invasion. Within this context, post-transcriptional regulation offers to the fungal invader an important mechanism for rapid adaptation to an adverse environment. In addition to the important role of *de novo* transcription in cellular adaptation, post-transcriptional mechanisms can regulate other significant stages of the mRNA life cycle, including pre-mRNA half-life and translation efficiency. The main advantage, compared to the transcriptional control, is a faster cellular response that can induce permanent changes in the physiology of the cell by controlling protein levels and localisation (A. Sesma, 2016). In the next sections, several post-transcriptional mechanisms and its relevance during fungal infection are detailed.

Figure 1.5. mRNA life cycle in eukaryotic cells. RNA-binding proteins play an essential role in the mRNA life cycle. They are required for several maturation steps of the pre-mRNA including 5' capping, splicing, polyadenylation, export, localisation, degradation and translation. Non-coding RNAs can also be polyadenylated and exported. Ribonucleoprotein (RNP) complexes, formed by RNA-binding proteins and RNAs, play a key role in establishing RNA networks.

1.3.1. Life cycle of the mRNA

Any pre-mRNA that encodes a functional protein undergoes different maturation steps (Figure 1.5). Most of these steps occur co-transcriptionally and include capping, splicing,

polyadenylation, export, localisation, degradation and translation (Jens & Rajewsky, 2015; M. J. Moore & Proudfoot, 2009).

Post-transcriptional regulatory pathways are driven mainly by RNA-binding proteins and non-coding RNAs (ncRNAs) (Jens & Rajewsky, 2015). RNA-binding proteins play a key role in post-transcriptional regulation due to its ability to bind RNA and other RNA-binding proteins. The association of RNA-binding proteins and RNAs drives the formation of ribonucleoprotein complexes (RNPs), which can interact with proteins and *cis*-acting elements present in target RNAs such as primary sequences or secondary structures (Anderson & Kedersha, 2009). The functional diversity of these RNPs is determined by the nature of RNA-binding proteins and RNAs present in the complex. This protein/RNA composition controls the localisation and translation efficiency of the mRNA subpopulations (Hentze *et al.*, 2018).

In addition to transcriptional regulation, small RNAs (sRNAs) play an important role in post-transcriptional regulation, e.g. mRNA stability, localisation and translation efficiency (Afonso-Grunz & Muller, 2015; Bartel, 2009; Jens & Rajewsky, 2015). One of the most interesting features recently discovered is that they can be transferred among organisms belonging to different kingdoms. This crosstalk between evolutionary distant organisms supposes an important field of study. In fungal pathogens, the mobilisation of sRNAs into the host can interfere with host immune response pathways by silencing mRNA expression (Baulcombe, 2015; Knip *et al.*, 2014).

1.4. Alternative pre-mRNA 3' end processing in filamentous fungi

Splicing and 3' processing mechanisms have a remarkable functional similarity (Richard & Manley, 2009; Zhu *et al.*, 2018). Both mechanisms generate mRNA isoforms that have different regulatory properties or can synthesise different proteins from an unique gene (Tian & Manley, 2017).

Pre-mRNA 3' end processing is a critical feature in higher eukaryotes. In contrast to yeast, the average 3' untranslated region (UTR) length of human mRNAs shows a ten-fold increase. In addition, more mRNA isoforms with different 3' UTR lengths are generated in humans compared to *S. cerevisiae*, where the number of protein coding genes and produced mRNAs are similar (Mayr, 2017). Almost all metazoan pre-mRNAs are cleavage at their 3'

end and polyadenylated, with the exception of some histone metazoan genes, which do not contain a polyadenosine tail (Elkon *et al.*, 2013).

Alternative polyadenylation is the mechanism involved in the control of the 3' UTR length of pre-mRNAs (Sandberg *et al.*, 2008). For the correct processing of the 3' UTR, two consecutive steps are required. First, a specific endonucleolytic cleavage occurs in the pre-mRNA at the canonical or a non-canonical site. In higher eukaryotes, the cleavage is produced by CPSF73, or by Cft2/Hrp1 in yeast. During the second step, polyadenosine [poly(A)] polymerases add the adenosine tail (Elkon *et al.*, 2013; Sonenberg & Hinnebusch, 2009).

The poly(A) site selection is controlled by both cellular signals and the primary/secondary structure of RNAs. The *cis* elements present in the 3' UTR have the potential to regulate the mRNA fate. Therefore, the existence of mRNA populations with different 3' UTR length permits a regulation that is dependent on the presence of conserved *cis* elements (Pichon *et al.*, 2012). Some *cis* elements are miRNAs target sites or AU-rich elements, which are responsible for the mRNA stability and translation efficiency, respectively (Barreau *et al.*, 2005; Bartel, 2009). The 3' UTR can also contain *cis* elements recognized by RNA-binding proteins that transport the mRNA to specific locations within the cell where their translation occurs; this mechanism ensures a timely accurate localization of proteins in specific cellular locations (Martin & Ephrussi, 2009). In addition to the alternative polyadenylation, the number of adenosines of the poly(A) tail can also influence the translation, localisation and stability of the mRNA (Weill *et al.*, 2012).

In humans, more than 80 proteins are found to interact with the 3' UTR of pre-mRNAs. The RNA-binding proteins are involved in the recognition of the *cis* elements present in the 3' end region. The cleavage and regulation of the 3' UTR length is determined by the composition of RNA-binding proteins bound to this region, which in turn is controlled by environmental factors. Other features like the mRNA secondary structure can affect the recognition of 3' UTRs by RNA-binding proteins. These secondary structures are less well conserved, but the primary sequences of regulatory elements present in its 3' UTR are well maintained across species.

Different mRNA isoforms with different exons can be generated by alternative polyadenylation. Moreover, alternative polyadenylation has also the potential to exert other

regulatory mechanisms. For example, the mRNA fragment cleaved off from de pre-mRNA during polyadenylation can act by itself as a long non-coding RNA (lncRNA) or these 3' UTRs cleaved fragments can modulate protein-protein interactions (Berkovits & Mayr, 2015; Chao & Vogel, 2016).

In humans, the polyadenylation machinery is formed by a multi-subunit protein complex, and ca. 18 proteins form the core of this complex. The main elements of polyadenylation machinery are the poly(A) polymerase, poly(A) binding proteins (PABPs), the large subunit of RNA polymerase II (RNAPII) and four multi-subunits complexes: the cleavage and polyadenylation specificity factor (CPSF), cleavage factor I (CFI_m), cleavage and stimulation factor (CstF) and cleavage factor II (CFII_m).

Figure 1.6. Schematic model of polyadenylation machinery in *M. oryzae*. Known proteins in *M. oryzae* and homologues to proteins of *S. cerevisiae* polyadenylation machinery are inside boxes. Conserved RNA sequences recognised by proteins/complexes are indicated. Rbp35 is a protein present exclusively in filamentous fungi. Hrp1 is conserved in both *M. oryzae* and *S. cerevisiae* but not in metazoans. CFI_m proteins (green box) are not conserved in yeast.

The polyadenylation proteins differ between mammals and yeast, but several yeast factors have a counterpart in mammals (Figure 1.6). Mammal CFI_m function is carried out by Hrp1. The same occurs with CstF, which in yeast its role is played by CFIA (complex formed by Clp1, Pcf11, RNA14 and RNA15). And the functional equivalent in yeast of the mammal CPSF is CFII (composed by Ydh1, Pta1, Yhh1 and Ysh1) (see Figure 1.6). In humans, the selection of the polyadenylation site is driven by CFI_m, whereas in yeast this function is carry out by Hrp1. The mammal CFI_m is composed by a small subunit CFI_m25 and a large subunit CFI_m68 and these factors are not only involved in polyadenylation, but they are tightly coupled to splicing due to its interaction with splicing factors and the spliceosome. In contrast, Hrp1 carries out the role of these two mammals' factor in yeast, where it is also necessary for mRNA export and non-mediated decay (NMD) (C. Gonzalez *et al.*, 2000).

NMD is involved in the pre-mRNA degradation with premature termination codons (Hug *et al.*, 2016).

CFI_m has been studied in more detail in humans, where they regulate alternative polyadenylation. CFI_m25 and CFI_m68 interact through their RRM domains. CFI_m25 binds to UGUA elements present in pre-mRNAs but it is not clear how this complex regulates alternative polyadenylation (APA).

1.5. Rbp35, a component of the polyadenylation machinery exclusive to filamentous fungi

Studies in alternative polyadenylation have been performed mainly in humans and yeast. The mechanism and the components of the pre-mRNA processing machinery involved in the polyadenylation of mRNAs are poorly known in filamentous fungi. A new component of the 3' end processing machinery in *M. oryzae* provided some insights into this area of study. The RNA-binding protein Rbp35 is exclusive to filamentous fungi and it is involved in alternative polyadenylation in *M. oryzae* (Franceschetti *et al.*, 2011). This component of the polyadenylation machinery is necessary for full virulence and development in *M. oryzae*. The unique presence of this protein in filamentous fungi and its pivotal role in *M. oryzae* virulence highlight the potential of Rbp35 to be considered as a target for rice blast disease control and the relevance of this study.

The Rbp35 protein is 424 aa long and contains several protein domains (Figure 1.7). An RNA recognition motif (RRM) is localised in its amino-end with the ability to recognise the UGUAAH motif by itself without the need of CFI25 (Rodríguez-Romero *et al.*, 2019). This is a singular feature, because as previously described, the human CFI_m59/68 requires of CFI_m25 to bind RNA (Dettwiler *et al.*, 2004). In Rbp35 middle region, six tripeptide repeats are found, the Arginine-Glycine-Glycine (RGG) motifs (Rodríguez-Romero *et al.*, 2015). The deletion of the RGG-containing region increases protein stability and nuclear delocalisation, suggesting that the RGGs are also controlling Rbp35 steady-state levels and nuclear import (Rodríguez-Romero *et al.*, 2015). The methionine-asparagine-glycine (Met-Asn-Gly) region is found in Rbp35 carboxy-terminal sequence and possesses two classical nuclear localisation signals (NLS), which suggests that Rbp35 has different nuclear import routes. A truncated variant of Rbp35 without the Met-Asn-Gly domain shows a 10-fold

increase in their cellular concentration without altering its degradation rate (Rodríguez-Romero *et al.*, 2015). This suggests that the Met-Asn-Gly region controls negatively Rbp35 translation and can act as an autoregulatory domain, potentially interacting with translational factors.

Figure 1.7. Rbp35 multilayer regulation in *M. oryzae*. Three polyadenylated transcripts have been detected by Northern in the *RBP35* locus. Two small and polyadenylated transcripts upregulated under carbon starvation conditions correspond to *uORF1* and *uORF2* found in the 5' UTR and the full length mRNA that contains *RBP35* coding sequence. The full length Rbp35 protein (Rbp35A) contains an RRM domain that binds to RNA sequence UGUAH, required for selection of distal polyadenylation sites in alternatively polyadenylated genes. The Arg-Gly-Gly tripeptides (RGG) control Rbp35 localisation, degradation and cleavage efficiency. Rbp35 C-terminal domain is enriched in Met, Asp and Gly, this region regulates negatively *RBP35* mRNA translation efficiency. The cleavage of Rbp35A C-terminal domain generates a shorter Rbp35 isoform (Rbp35B) that interacts with CFI25 and Rbp35A.

The different levels of regulation found in Rbp35 suggest the relevance of its role in *M. oryzae* cell biology. The expression of *RBP35* mRNA is relatively high compared to other RNA-binding proteins, and independent of the environmental changes. A naturally occurring carboxy-terminal processing of the protein results in two Rbp35 isoforms, Rbp35A and Rbp35B (Franceschetti *et al.*, 2011). This processing occurs after the RGG cluster, and arginine methylation of the RGGs seems to modulate the cleavage efficiency (Figure 1.7) (Rodríguez-Romero *et al.*, 2015). The full-length isoform Rbp35A has a size of 44 kDa and is expressed usually under rich media conditions. Rbp35A is not detected under nutritional

stresses such as carbon or nitrogen starvation, indicating either the relevance of the Rbp35 isoform ratios to overcome these environmental stresses or a lack of newly synthesised Rbp35A molecules (Rodríguez-Romero *et al.*, 2015). The shorter isoform Rbp35B is found in the cell under all the nutritional conditions studied. Rbp35A interacts *in vivo* with Rbp35B and CFI25, indicating that both isoforms are part of the *M. oryzae* CFI complex. The presence of both isoforms in the complex supports the idea that CFI can vary its composition depending on environmental factors, and consequently the pre-mRNAs that interact with (Rodríguez-Romero *et al.*, 2015).

Figure 1.8. *uORF1* regulates *M. oryzae* growth in the presence of rapamycin. Growth of *M. oryzae* wild-type (WT) strain is affected negatively by rapamycin. M35 is a *RBP35* T-DNA mutant that lacks *uORF1* in its genome. $\Delta rbp35$ has the *uORF1* but the full *RBP35* CDS has been deleted. Both mutants, $\Delta rbp35$ and M35 show higher tolerance to rapamycin, a growth inhibitor. *RBP35* UTRs construct contains the *uORF1*, whereas PrRP27+TerNOS and 5'UTR intronless constructs do not. The presence of *uORF1* either in the mutant genome or in the construct is necessary for WT phenotype recovery under rapamycin treatment (Rodríguez-Romero *et al.*, 2015).

Another level of regulation is provided by the identified elements in the 5' untranslated region (UTR) of *RBP35* mRNA (Figure 1.7). The 5' UTR is uncommonly large (733 bp) in the *RBP35* transcript (Rodríguez-Romero *et al.*, 2015). Its most remarkable feature is the presence of an intron that contains two potential upstream open reading frames (uORF), whereas no other introns with these characteristics have been observed in *M. oryzae* gene complement (Rodríguez-Romero *et al.*, 2015). The first uORF (uORF1) is fully included within the 5' UTR intron whereas the start of the second uORF (uORF2) is located

at the 3' end of the intron and expands beyond the length of the 5' UTR intron. The uORF2 stop codon is found inside 5' end of *RBP35* CDS. Northern blots reveal the presence of three polyadenylated transcripts in the *RBP35* locus, *uORF1*, *uORF2* and *RBP35*. Whereas both *uORF1* and *uORF2* mRNAs are overexpressed under carbon starvation, *RBP35* mRNA shows steady-state expression levels in CM and in carbon starvation (Rodríguez-Romero *et al.*, 2015).

Surprisingly, the uORF1 located in the 5' UTR seems to act *in trans* over Rbp35. When Target of rapamycin (TOR) pathway is inactivated by rapamycin, the growth of the *rbp35* mutants Δ *rbp35* and M35 is less affected. The individual presence of Rbp35 or uORF1 in the fungus is not enough for a correct response to rapamycin. Only when both elements are present in the cell, *M. oryzae* is able to recover a proper regulation by the TOR pathway. Additionally, the uORF1 acts over Rbp35 *in trans* because independently where the uORF1 is located – either in the background of the mutant genome or in the same construct – uORF1 is able to recover a wild-type response to rapamycin if Rbp35 is present (Figure 1.8) (Rodríguez-Romero *et al.*, 2015).

1.6. The importance of fungal introns and their splicing

Introns were discovered in eukaryotes in 1977 and for many years these elements were categorised as “junk DNA” (Jo & Choi, 2015). Introns are non-coding DNA sequences localised normally between protein-coding DNA sequences. During pre-mRNA maturation, introns are removed from the pre-mRNA by a mechanism named splicing. This maturation step of the pre-mRNA is carried out by a complex ribonucleoprotein machinery, the spliceosome (Dunn & Rader, 2014; Irimia & Roy, 2014). The spliceosome removes the introns of pre-mRNAs co-transcriptionally during transcription elongation wherever the RNA polymerase II recruits splicing factors through its carboxy terminal domain (CTD) (Peñate & Chávez, 2014).

Five small nuclear RNAs (snRNAs U1, U2, U4, U5 and U6) and more than 200 proteins are part of this complex (Baralle & Giudice, 2017; Irimia & Roy, 2014). Most of the spliceosomal introns possess well-conserved elements that need to be recognised by the spliceosome to begin the intron removal: the 5' splice site (5'ss; defined by a short nucleotide sequence, normally GT), the 3' splice site (3'ss, containing the dinucleotide AG) and the

branch point (BP, sequences defining this region are more variable and contain several nucleotides). In spite of the huge diversity of splicing machinery and gene architecture among organisms, the splicing chemical reactions are identical in all eukaryotes (Dunn & Rader, 2014).

Introns are usually located in the coding sequence of the gene, but they can be localised as well in the 5' or 3' UTRs of the pre-mRNA. In yeast, the introns localised in the 5' UTR are important for enhanced protein synthesis. Whereas this protein synthesis enhancement is due exclusively to a translational regulation, introns localised inside coding sequences are able to enhance their mRNA expression (Furger *et al.*, 2002; Hoshida *et al.*, 2017; Moabbi *et al.*, 2012).

A clear role described for introns is the intron-mediated enhancement (IME), which increases the accumulation of the mRNA (Gallegos & Rose, 2015). This function has also been found in plants. The presence of a 5' UTR intron in the *Arabidopsis thaliana* *GGT1* aminotransferase gene enhances its mRNA promoter activity (Laxa *et al.*, 2016). The mechanisms involved in this process are still unknown, but while expression of some genes is affected by the presence of the introns in their 5' UTRs, other genes do not show differences in expression with or without an intron (Emami *et al.*, 2013). The intron retention in the mRNA can affect the 5' UTR length, which subsequently is affecting its translation, stability or introducing new start codons that can trigger NMD (Gallegos & Rose, 2015).

In some genes the splicing plays a role in the expression of the mRNA (Akua *et al.*, 2010), while in other genes mRNA levels barely change (Rose & Beliakoff, 2000). The same occurs at a sequence level, some introns seems to regulate the mRNA levels through regions rich in T nucleotides (Clancy & Hannah, 2002), whereas in other introns the sequence are enriched in GC octamers (Donath *et al.*, 1995).

Fungal species are composed by genomes with abundant intron-containing genes, except for *Saccharomyces cerevisiae* (Neueglise *et al.*, 2011). Some examples of fungal genomes with a low percentage of intro-containing genes are *S. cerevisiae* (4%) and *Candida albicans* (6%). Other fungi such as *Cryptococcus neoformans* (98%), *Aspergillus fumigatus* (78%) and *M. oryzae* (76%) show genomes with a high percentage of introns (Mitrovich *et al.*, 2007; Schwartz *et al.*, 2008).

S. cerevisiae is a good model organism for the study of splicing because it has less introns than any other eukaryotic organism. The yeast's genome has only 295 introns distributed in 280 genes and they are usually shorter than 500 nt (Hartung *et al.*, 2002; Parenteau *et al.*, 2019). A study using a yeast whole mutant collection with introns deleted one by one in the 280 genes showed that their deletion increases mutant's resistance to starvation. This effect is independent of the function of the gene where the introns are located. Introns are able to recover the mutant phenotype by themselves, even when the gene is not synthesising a protein. To explain this, the authors of this work suggest that the introns are repressing the ribosomal protein genes (RPG), and therefore coupling the response of the TOR and PKA pathway. The presence of the introns by themselves can confer a better cellular growth, probably due to a reduction of nutrient consumption (Parenteau *et al.*, 2019). Additionally, excised introns with short distance between its branch point and 3'ss are not degraded and they have a biological role in the TOR growth-signalling network (Morgan *et al.*, 2019).

The major role described for introns is the alternative splicing (AS), which has been largely described in eukaryotes. The purpose of AS is the generation of several mRNA and proteins isoforms starting from a single gene (Irimia & Roy, 2014). The most common processing is produced due to the intron non-splicing but existence of other mechanisms such as exon exclusion and alternative cleavage of 5'ss or 3'ss can also generate truncated exons by AS, and therefore protein variants from the same gene (Irimia *et al.*, 2012). The production of different protein isoforms can help to promote differential biological processes and to fine-tune regulation of cellular processes, including localisation of mRNAs and proteins (Gabut *et al.*, 2011; Lopez, 1998).

Examples of fungal proteins required for pathogenicity and involved in AS or regulated by AS include the *Fusarium graminearum* FgPrp4 protein kinase (Wang *et al.*, 2011) and the *Ustilago maydis* GADPH glycolytic enzyme (Freitag *et al.*, 2012), respectively. Several epigenetic mechanisms, which are tightly coupled with splicing like chromatin structure, histone marks, DNA methylation or nucleosome position are required for fungal pathogenesis (A. Sesma, 2016). An important role in virulence is played by histone deacetylases in *Fusarium fujikuroi* (Studt *et al.*, 2013), and methyltransferases in *F. graminearum* (Connolly *et al.*, 2013) and *M. oryzae* (Pham *et al.*, 2015). More recently, a

link between autophagy and splicing has been shown in *M. oryzae* through the arginine methyltransferase MoHmt1 protein. *MoHMT1* is necessary for fungal growth and virulence, and it is required for the di-methylation of arginine residues in the *M. oryzae* U1 snRNP. The lack of MoHmt1 alters AS of 558 genes, including the autophagic gene *MoATG4*. MoHmt1 localises in the autophagosome during nitrogen starvation and light exposure and its lack produce defects in autophagy induction (Li *et al.*, 2020).

1.7. Translation initiation: The importance of regulatory elements in the 5' UTR

The existence of regulatory elements in the 5' UTR of main ORFs (mORFs) can affect its expression or translation efficiency. Upstream ORFs (uORFs) are important regulatory elements found in 5' UTRs that control gene expression in eukaryotes (Barbosa *et al.*, 2013). Currently, the only roles described for uORFs are regulating the expression of the downstream mORF, acting always *in cis* over it.

Typically, uORFs are affecting the ribosome scanning during the translation process using different mechanisms. *Leaky scanning*: if the uORF start codon (uATG) is in an unfavourable context, the ribosome scanning continues through the transcript until it reaches the start codon of the mORF, which has an optimum context to initiate translation. Then, the ribosome subunits are assembled (downstream of the uORF) and the protein synthesis start in the mORF. In this situation, the presence of the uORF is not affecting the translation of the mORF, being the most common situation of the uORFs in the genome (Figure 1.9A). *Preemptive initiation*: if the uATG has a favourable context, the ribosomes are able to translate the uORF. Once the uORF is translated, the ribosomes are disassembled from the mRNA. In this mechanism, the localisation of the uORF stop codon plays a key role. When the stop codon is close to the downstream mORF, the uATG and the ribosomes are not able to re-assemble again into the mRNA, reducing translation efficiency of the mORF (Figure 1.9B). Alternatively, if the uORF stop codon is far enough, the ribosomes can be assembled again and the mORF is not affected by the presence of the uORF. *Reinitiation*: In some of the uORFs, the presence of a sequence after its stop codon promotes ribosome reinitiation. This mechanism stimulates an efficient translation of the mORF (Figure 1.9C). *Ribosome stalling*: the presence of uORF in the 5' UTR can block the ribosomal scanning through the mRNA. This ribosome stalling avoids the downstream scanning and prevent the protein synthesis of

the mORF (Figure 1.9D). *mRNA degradation*: the NMD mechanism has the capacity of identifying upstream stop codons in the mRNA to trigger its degradation (Figure 1.9E). The main player of NMD in eukaryotes is the protein Upf1.

Figure 1.9. Regulatory roles of upstream open reading frames (uORFs) over the expression of downstream main ORF (mORF). The two ribosome subunits scan the mRNA molecule looking for an ATG with an appropriate context to initiate the protein synthesis. **A) Leaky scanning:** the upstream ATG (uATG) context is not favourable and the ribosomes bypass the uATG of the uORF. This leaky scanning increases the translation of the downstream mORF. **B) Preemptive initiation:** if uORF ATG has a favourable context, ribosomes translate a peptide and then disassemble from the mRNA, This avoids the mORF translation. **C) Reinitiation:** the ribosomes scan the mRNA and translate the uORF1. A certain distance between ORFs and the presence of a sequence promotes the ribosome assembly in the mORF, promoting its translation. **D) Ribosome stalling:** ribosome can translate or not the uORF. Ribosomes stall in the uORF region stopping the downstream scanning and avoiding the mORF translation. **E) mRNA degradation:** Upf1 plays a key role in the nonsense-mediated decay (NMD) of the mRNA molecule. Upf1 is able to detect stop codons upstream of the mORF. When Upf1 identifies these stop codons, it promotes the degradation of the entire mRNA molecule.

The study of uORFs as potential targets in biotechnology is a new area of research that is quickly expanding. In plants, the presence of uORF can be used to increase protein translation. In *Lactuca sativa*, GGP2 is an enzyme involved in the vitamin C biosynthesis pathway. The uORF edition of this gene promotes tolerance to oxidative stress and an accumulation of ascorbate (H. Zhang *et al.*, 2018). However, the potential of uORFs to synthesise small peptides implies that some of these uORFs could act *in trans*. The function

of the small peptides synthesised from these uORFs has been always altering either the translation efficiency or function of the downstream mORF.

1.8. Target of Rapamycin (TOR) pathway: environment sensing and growth in fungi

In living organisms, the sensing of nutrients in the environment is crucial since it determines their ability to grow and survive. In eukaryotes, the response to external nutrients availability is driven by the TOR signalling pathway. The activation of the TOR pathway represses autophagy and promotes lipid, protein and nucleotide synthesis (A. Gonzalez & Hall, 2017). One of the roles of the mammalian TOR cascade (mTOR) is to regulate translation. mTOR promotes translation of some mRNAs containing particular elements in their 5' UTRs such as the 5'-terminal oligopyrimidine tract (TOP), which is present in all ribosomal proteins, elongation factors and a few other translational regulators, and the 5'-pyrimidine-rich translational element (PRTE), which stimulates mRNAs-polysomes association. Additionally, mTOR activation promotes the shortening of 3' UTRs that enhances the translation of some mRNAs (Chang *et al.*, 2015).

Mammalian cells only have one TOR kinase that can form two different complexes with differential structure and function: the TOR complex 1 (TORC1) that is sensitive to rapamycin, and the TOR complex 2 (TORC2) that is insensitive (Loewith & Hall, 2011). In yeast, the essential components of the TOR pathway are the two TOR kinases (TOR1, TOR2), Kog1 and Lst8 (A. Gonzalez & Hall, 2017). Amino acids presence in the medium modulates the binding efficiency of Gtr1/2 RAG GTPases and TOR1 (Godard *et al.*, 2007; Hirose *et al.*, 1998). This has allowed to identify several upstream regulators of TOR1 in *S. cerevisiae* using amino acid sufficiency conditions. Yeast TOR1 is localised either in the vacuolar membrane or in localised perivacuolar sites independently of the nitrogen source. TOR1 responds better to glutamine than other amino acids (Hughes Hallett *et al.*, 2015; Kira *et al.*, 2014).

In filamentous fungi, there is only one essential TOR kinase and environmental nitrogen can modulate the TOR pathway and fungal virulence in *F. oxysporum* and *M. oryzae* (Lopez-Berges *et al.*, 2010). In *M. oryzae*, repression of the TOR pathway is vital for appressorium formation, and therefore for plant penetration (Marroquin-Guzman & Wilson, 2015). Appressorium formation occurs in a poor nutrient environment and requires activation

of autophagy for penetration. Asd4 is a GATA transcription factor that acts negatively in the expression of *GLN1*, a gene involved in nitrogen assimilation and distribution. Inappropriate levels of Gln1 in the cell promotes glutamine accumulation, which acts as a TOR antagonist. Therefore, *M. oryzae* Asd4 controls glutamine levels and modulates TOR inhibition, a necessary step for appressorium development (Marroquin-Guzman & Wilson, 2015).

Carbon metabolism also regulates appressorium development through the TOR pathway in *M. oryzae* (Marroquin-Guzman *et al.*, 2017). The *ABL1* gene involved in glucose signalling is a negative regulator of the TOR pathway in the absence of glucose or glucose-6-phosphate in the cell. This allows TOR to regulate mitosis in response to glucose levels in *M. oryzae* (Marroquin-Guzman *et al.*, 2017). Another gene, *IMP1* has been identified as a new component of *M. oryzae* TOR pathway. Imp1 is a vacuolar protein required for membrane trafficking, organelle acidification, V-ATPase assembly and autophagy induction. During infection, Imp1 is necessary for maintenance of *M. oryzae* biotrophic phase, which is required for subsequent fungal infection progression (G. Sun *et al.*, 2018).

Germ tube formation and appressorium development require an active cAMP/PKA signalling whereas the TOR pathway remains inactive. In turn, TOR inactivation arrests the cell division cycle at G2 checkpoint and triggers autophagy activation. Simultaneously, Abl1 activation mediated by cAMP promotes TOR inactivation, and explains the link between cAMP/PKA and TOR signalling pathways (Marroquin-Guzman *et al.*, 2017; Sun *et al.*, 2019). These evidences indicate that autophagy, TOR pathway and cell cycle are tightly coupled during fungal development and infection in *M. oryzae*.

More genes have been identified in *M. oryzae* TOR signalling pathway. *SNT2* is a TOR-dependent gene that encodes a protein which recognises histone H3 acetylation and regulates expression of autophagy-related genes *ATG6,15,16* and *22*. This gene is required for fungal pathogenicity linking virulence to an epigenetic mechanism (He *et al.*, 2018). Moreover, Ppe1 and Sap1 form a complex that regulates negatively cell wall integrity through TOR signalling (Qian *et al.*, 2018). During appressorium formation, cell wall integrity is essential for full virulence in the rice blast fungus.

As previously described, the Rbp35 protein is an RNA-binding protein involved in alternative polyadenylation. The expression of the TOR kinase gene is down-regulated in the $\Delta rbp35$ mutant, which is reflected by its nutrient-dependent behaviour, accelerated

autophagy and higher tolerance to rapamycin (Franceschetti *et al.*, 2011). The two *14-3-3* genes and *ASD4* gene known to be part of the TOR signalling cascade and aminoacid biosynthesis, respectively, have altered 3' UTRs in $\Delta rbp35$ (Franceschetti *et al.*, 2011). Possibly, the alternative polyadenylation of these genes is important for full virulence in *M. oryzae*.

1.9. Role of 14-3-3 proteins in cell biology

14-3-3 are a family of well conserved, acidic and dimeric proteins present in all studied eukaryotes. Their molecular weight is ca. 30 kDa and several isoforms can be found depending on the organism (van Heusden, 2009). Compared to mammals or *Arabidopsis thaliana* that have seven and thirteen isoforms, respectively, only two 14-3-3 isoforms (*BMH1* and *BMH2*) are found in yeasts (van Heusden *et al.*, 1995). In filamentous fungi such as *F. graminearum* (Brauer *et al.*, 2020) and *M. oryzae* (Rodríguez-Romero *et al.*, 2015; Rodríguez-Romero *et al.*, 2019) there are also two 14-3-3 proteins. Crystallization studies have identified their ability to dimerise with a U-shape structure. This shape facilitates the location of binding partners or peptides commonly phosphorylated by 14-3-3 (Xiao *et al.*, 1995; Yaffe, 2002). The 14-3-3 proteins are also regulated through phosphorylation of some of their serine residues (Bodenmiller *et al.*, 2008; Chi *et al.*, 2007; Morrison, 2009). 14-3-3 proteins can regulate cellular localisation and enzymatic activity of target proteins. They can also act as scaffold proteins in multi-protein complexes (Obsilova *et al.*, 2008).

In humans, 14-3-3 malfunction is associated to nervous system disorders and cancer (Berg *et al.*, 2003; Morrison, 2009). In *S. cerevisiae*, the lack of one of the isoforms has a minor effect on fungal viability. However, the double mutant is lethal (van Heusden *et al.*, 1995). 14-3-3 proteins are localised widely in the yeast cell with the exception of vacuoles, and they can partner with ca. 4% of yeast proteins (Kakiuchi *et al.*, 2007). 14-3-3 proteins can promote the cellular localisation of the transcription factor GLN3, thus controlling the nitrogen catabolite repression through the TOR pathway (Tate *et al.*, 2017). In addition, both 14-3-3 yeast proteins regulate carbon starvation repression in a TOR-dependent mechanism. TOR activation promotes the binding of 14-3-3 to Msn2 and Msn4 transcription factors. This interaction avoids the transport of Msn2 and Msn4 to the nucleus where they promote the expression of carbon starvation-associated genes (Beck & Hall, 1999).

Whereas the roles of 14-3-3 proteins have been thoroughly studied in other eukaryotes, little is known in filamentous fungi. Usually, 14-3-3 proteins are involved in fungal growth due to their regulation by the TOR pathway, and can regulate diverse processes in filamentous fungi, including germ tube formation in *A. nidulans* (Kraus *et al.*, 2002); cell cycle progression and cell morphology in *U. maydis* (Mielnichuk & Perez-Martin, 2008); secondary metabolite regulation in *Aspergillus flavus* (Ibarra *et al.*, 2018), correct arbuscule formation in *Rhizophagus irregularis* (Z. Sun *et al.*, 2018) and nitrogen sensing in *F. graminearum* (Brauer *et al.*, 2020).

Figure 1.10. Model diagram of the *M. oryzae* TOR signalling network based on *S. cerevisiae*. TOR1 is in the upper-left corner. TOR1 plays a key role in the pathway. It promotes cell growth stimulating ribosome synthesis and translation initiation through eIF4G and eIF2α factors. On the other hand, TOR1 controls blocking transcription of genes involved in stress response. This blocking is mediated by RIM15, MSN2/4 and 14-3-3 proteins. Arrows and bars indicate positive and negative interactions, respectively. Solid arrows denote direct interactions and dashed arrows indicate indirect interactions.

OBJECTIVES

Rbp35 is a component of the Cleavage Factor I (CFI) of the *M. oryzae* polyadenylation machinery, and possesses an uncommonly large 5' UTR. The main role of Rbp35 in *M. oryzae* is the selection of distal polyadenylation sites (PAS) in pre-mRNAs. The presence of two CFI complexes in *M. oryzae* suggests differences in the role played by these two complexes compared to yeast. To understand the role of the long 5'UTR of Rbp35 and the role of alternative polyadenylation in *M. oryzae* pathogenicity, the following three objectives have been pursued:

- 1- Characterisation of the potential regulatory roles exerted by the uORFs located within the 5'UTR intron of the *RBP35* gene using biochemical and genetic approaches.
- 2- Study of the relevance of alternative polyadenylation during *M. oryzae* infection by looking at the mRNA isoforms of the *14-3-3B* gene, whose most distal PAS is regulated by Rbp35.
- 3- Comparative study of *M. oryzae* and yeast Hrp1 proteins, components of a second CFI to understand if their function is conserved within the fungal kingdom.

2-MATERIAL AND METHODS

Fungal strains growth conditions and DNA extraction. The *Magnaporthe oryzae* wild-type isolate used in this study is the rice-infecting strain Guy11 (Leung *et al.*, 1988). Two mutant strains were used for the regulatory analyses of the *RBP35* gene: a T-DNA disruption mutant strain (M35) and a targeted gene replacement mutant ($\Delta rbp35$) of *RBP35* (Franceschetti *et al.*, 2011; Rodríguez-Romero *et al.*, 2015). Growth and maintenance of *M. oryzae* strains was carried out in complete media (CM) (Sweigard *et al.*, 1997). Growth test assays were performed in CM, minimal medium (MM), MM depleted of carbon (MM-C), MM depleted of nitrogen (MM-N), 1.5% Water Agar, MM supplemented with 0.2M Lithium Chloride, MM supplemented with 0.2, 0.4, 0.8M Sodium Chloride, MM with ammonium tartrate (10 mM) as unique nitrogen source (MMT) and MMT supplemented with rapamycin, a growth inhibitor (200 ng/mL). All these media have been previously published (A. J. Foster *et al.*, 2003; Tucker *et al.*, 2010).

M. oryzae genomic DNA was extracted from five day-old mycelium grown on static plates covered with 50 ml of liquid CM. Resultant mycelium was lyophilised at 4°C and pulverized. DNA extraction was performed using powdered mycelium and the NucleoSpin® Plant II (Macherey-Nagel) Kit.

***M. oryzae* annotated genes.** The gene names used in this study are based on the eighth genome annotation of *M. oryzae*, which can be found at EnsemblFungi (<http://fungi.ensembl.org/index.html>).

Binary vectors generated for *A. tumefaciens*-mediated transformation in *M. oryzae*

Targeted gene disruption of *RBP35* and *14-3-3B*. We used the MultiSite Gateway™ technology (Invitrogen) to generate all the constructs. To eliminate all the uORFs from the 5' UTR and create the new $\Delta\Delta rbp35$ strain, we constructed a new entry vector of *RBP35* 5' UTR as follows. We amplified by PCR the upstream region of the 5' UTR intron using the B4-RBP35 and B1R-RBP35 (no 5'UTR-intron) primers (Table 2.1), which already contain the attB4 and attB1r sites in their 3' ends. This 1-kb fragment was cloned into the pDONR 221 P4-1r vector, which contains the attP4 and attP1r sites, using the Invitrogen™ Gateway™ BP Clonase™ II enzyme generating the vector pGw1-RBP35-Intronless. The final construct was generated by multisite cloning with Invitrogen™ Gateway™ LR

Clonase™ II enzyme fusing the pDONR 221 (attP4-attP1r) vector to a pDONR 207 (attP1-attP2) containing the Hygromycin Resistance gene and a pDONR 221 (attP2r-attP3) vector containing the *RBP35* 3' UTR (pGw4-RBP35) generated in previous studies (Franceschetti *et al.*, 2011). The final construct was introduced in a pGKO2 binary plasmid that contains the Gateway destination cassette (attR4-ccdB-camR-attR3) (Khang *et al.*, 2005). Additionally, this plasmid contains a T-DNA with *HSVtk*, a Herpes Simplex Virus gene that encodes the thymidine kinase enzyme. This enzyme acts selecting negatively the transformants, avoiding ectopic T-DNA insertions in the genome (Khang *et al.*, 2005). Homologue recombination prevents the insertion of *HSVtk* whereas T-DNA random insertion integrates *HSVtk* in the fungal genome. *HSVtk* expression converts 5-fluoro-2'-deoxyuridine (f2dU) to toxic compounds that affects the fungal cell viability. A similar cloning approach was used to generate a gene deletion mutant in the *14-3-3B* gene. Primers used to create the *14-3-3B* gene deletion construct, which contains a hygromycin resistance cassette instead of the gene coding sequence, are in Table 2.1. Fungal transformants generated by *Agrobacterium*-mediated transformation (ATMT) were grown for selection in solid DCM medium. $\Delta\Delta rbp35$ and $\Delta 14-3-3b$ strains were selected simultaneously in DCM with 200 µg/ml hygromycin and f2dU (50µM). Complemented transformants were selected in DCM with sulfonylurea drug (100 mg/ml). Strains able to grow in this condition only contained a single insertion. Knock-out strains were checked by PCR using primers detailed in Table 2.1. All PCR reactions were performed with the Q5® High-Fidelity DNA Polymerase (NEB) following provider's protocol.

***RBP35* point mutation constructs.** We generated several variants of the *RBP35* and *14-3-3B* genes with the aim of studying different elements involved in their regulation.

In the case of *RBP35* we performed several point mutations in its 5' UTR. Using QuickChange™ site-directed mutagenesis protocol (Zheng *et al.*, 2004), we generated a point mutation in the uORF1 start codon (ATG→AAG) using primers FwORF1ATGtoAAG and RvORF1ATGtoAAG (Table 2.1). The pDONR 221 plasmid containing the *RBP35* upstream region and the coding sequence (GW3-RBP35) was used as template for amplification with these primers. The mutations in the conserved splice sites of the intron (5'ss and 3'ss) were carried out in three steps. In the first step, QuickChange™ site-directed mutagenesis Protocol

(Zheng *et al.*, 2004) was used for changing the 5' ss (GT mutation to CA) using primers of Table 2.1. Resulting circular products were treated with *DpnI* restriction enzyme for 1h at 37°C for template degradation. Final DNA product was transformed into *E. coli*. Purified plasmids were then sent for sequencing (Stab Vida).

We used a different approach for mutating the Branch Point (BP) and 3' ss conserved sites in the intron using the 'Round-the-horn site-directed mutagenesis' protocol (S. D. Moore & Prevelige, 2002). For BP and 3' ss mutations we designed a forward primer with the sequence we wanted to add in the construct in its 5' end (Table 2.1). The reverse primer was designed to anneal upstream of the region of interest. Both primers were treated with the T4 Polynucleotide Kinase (NEB) during 1h at 37°C for addition of 5'-phosphates to primers. As result of the PCR a 5'-phosphorylated linear PCR product was obtained from the GW3-RBP35 template with the mutation in its 5'. Linear product was treated with *DpnI* during 3h at 37°C for template degradation. Then, the DNA product was purified with NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel) kit after running in an 1% agarose gel. The purified fragment was self-ligated using a T4 DNA Ligase (NEB) overnight at 16°C and transformed into *E. coli* DH10B strain. Plasmids mutations were checked by sequencing (Stab Vida).

To study if the *uORF1* locus can synthesise a polypeptide we generated several constructs of *RBP35*. HA-Flag and GFP sequences were added to the *uORF1* 3' end by overlapping PCRs. Using as template GW3-RBP35 plasmid we amplified a first fragment from the attb4 sequence until the penultimate codon of the *uORF1* coding sequence - just before the stop codon - adding a flanking sequence containing the *HA-Flag* or *GFP* genes with the stop codon at their CDS end. The second fragment was amplified from *HA-Flag* and *GFP* sequences cloned in pDONR207, adding in their flanks an overlapping region with the *uORF1* coding sequence and its 3' UTR. Eventually, a third fragment was amplified from GW3-RBP35 adding an overlapping with HA-Flag or GFP in its 5'. These three fragments were used as template for a final PCR using B4-RBP35 and B1R-RBP35 primers (see Figure 2.1). The final gene construct containing the three joined fragments was cloned into a pDONR221 (attP4-attP1r) with the Invitrogen™ Gateway™ BP Clonase™ II enzyme and transformed into *E. coli* DH10B strain. A linker region of glycine was added between *uORF1* and HA-Flag/GFP sequences by the primers with the aim to reduce the possibility to alter protein structure. As a negative control, a construct of *uORF1* without the start codon and

fused to HA-Flag and GFP was generated. The primers used in the previous *uORF1*-tagged constructs were used to amplify these new fragments. For this experiment the template used for PCRs was the previously generated GW3-RBP35 (no *uORF1* ATG).

Figure 2.1. Gateway cloning system used for generating constructs in this study. At the top are schematically represented the fragments combinations used for the generation of knockout (KO), carboxy- and amino-tagged constructs. The first step represented is the BP reaction with the insertion by recombination of the fragments into the entry plasmids. Next, final LR reaction is represented with the recombination of the three fragments that are inserted in the binary vector for *M. oryzae* transformation.

For complementation assays in *S. cerevisiae* with the *HRP1* gene of *M. oryzae* we used the yeast inducible expression vector pYES2 (Invitrogen). *HRP1* cDNA was amplified with MD13-EcoRI-cdsMoHrp1 and MD14-XhoI-cdsMoHrp1 primers. The amplified *HRP1* cDNA fragment was inserted in the pYES2 vector through digestion and ligation.

14-3-3 point mutation constructs. *14-3-3B* gene was amplified by PCR without the stop codon and cloned into pDONR221 (attP4-attP1r). The full length of *14-3-3* 3' UTR was amplified and included all the polyadenylation sites (PAS), whereas the amplification of the short 3' UTR included exclusively the proximal PAS of the gene. Both constructs were fused to mCherry.

All the constructs derived from *RBP35*, *uORF1* and *14-3-3B* genes were introduced into the binary vector pSUR (Franceschetti *et al.*, 2011; Rodríguez-Romero *et al.*, 2015) that contains the destination Gateway cassette (attR4-ccdB-camR-attR3).

Plant material and infection assays. Seedlings of rice (*Oryza sativa* ssp. *indica*) cv. CO39 were grown until they reached the third leaf stage for leaf infection assays. Rice plants were grown in a Conviron growth chamber under controlled conditions, i.e. 25° C, 80% of relative humidity and a 16h-light/8h-dark photoperiod. Rice plants were watered with a modified Hoagland solution (1 mM ammonium phosphate; 4 mM potassium nitrate; 4 mM calcium nitrate; 2 mM magnesium sulfate; 4 mM ammonium nitrate; 0.01/0.01 Na₂ EDTA/FeSO₄·7H₂O) supplemented with 1 ml/l of trace elements solution (1.81gr/l manganous chloride; 2.86 gr/l boric acid; 0.22 gr/l zinc sulfate; 0.08 gr/l cupric sulfate; 0.106 gr/l molybdic acid). For leaf infections we used three pots with vermiculite as substrate and ten rice seeds in each one, i.e. thirty plants per experiment/biological replicate. *M. oryzae* conidia were recovered from eight days-grown plates with a sterilized solution of 0.25% gelatine in sterilized milliQ water. After spraying with 2 ml of 10⁵ conidia ml⁻¹ suspension each pot, infected plants were covered with a plastic bag and incubated at 20°C during 24h. Then, plants were uncovered and incubated for six days at 25°C with the same photoperiod and disease symptoms scored. The scoring of the disease symptoms was carried out using the image analysis software Assess 2.0 (The American Phytopathological Society).

Root infection assays in rice were performed using moist thick vermiculite. Vermiculite was prepared by immersing it during two hours in distilled water and draining it

in a sieve before sterilization. Thirty ml of sterilized vermiculite was introduced in a 50-ml centrifuge tube, and covered its surface with a *M. oryzae* mycelial plug. On top of the fungal plug another layer 5 cm thick of moist vermiculite was placed and five rice seeds were dropped in this layer. Finally, all was covered with another 5 cm layer of moist vermiculite. In order to keep the humidity during the fifteen days of incubation, the tube was sealed with Parafilm. Tubes were incubated in a chamber at 25°C and 16h-light/8h-dark photoperiod. *M. oryzae* root disease symptoms were scored as follows: 0 for non-pathogenic symptoms, 1 for weak symptoms, 2 for moderate symptoms and 3 for full disease/wild-type symptoms. Pictures of rice infections were taken with a Canon EOS 1200D camera.

Protein extraction and immunodetection. Ten-day old *M. oryzae* strains grown on CM plates were cut out from agar and homogenized in a food blender (Waring Commercial, USA) with 150 ml of liquid CM. Mycelia, placed in 250 ml flasks, were incubated in darkness in a shaker at 25°C/ 120 rpm. After 48h mycelium was washed with sterile water and collected by filtering with an analytic filter paper. Resulting *M. oryzae* mycelium was incubated in 250 ml flask containing 150 ml of CM, MM-C, MMT or MMT + rapamycin (200 ng/ml). After 16h growing in darkness it was collected by filtering through a double layer of miracloth, washed with sterile water, grounded in liquid nitrogen and stored at -80°C. Proteins were extracted from 350-400 mg of mycelia (wet weight) using 1 ml of extraction buffer (50 mM Tris pH 7.5, 5 mM EDTA, 1% Triton X-100, 10% glycerol, 2 mM Phenylmethanesulfonyl Fluoride) and 1x Protease Inhibitor Cocktail (cOmplete™ EDTA-free Protease Inhibitor Cocktail). Cell debris was precipitated after 30 min of centrifugation (14000 rpm) at 4°C. Protein concentration was measured using Bradford reagent (Biorad).

Proteins were separated by electrophoresis in SDS-PAGE gel using a mini-PROTEAN Tetra Vertical Electrophoresis Gel (Biorad), and transferred into a PVDF Amersham™ Hybond® P membrane in a Mini Trans-Blot® Cell system (Biorad) during 1h at 400 mA.

For protein detection the following primary antibodies were used: anti-Rbp35 polyclonal antibody (Franceschetti et al., 2011), anti-mCherry polyclonal antibody (Rodríguez-Romero et al., 2015), anti-GFP monoclonal antibody (Invitrogen # A-11120) and the 14-3-3 (pan) polyclonal antibody (Cell Signaling #8312), which recognizes total levels

14-3-3 proteins. This antibody also detects all known isoforms of mammalian 14-3-3 proteins and is predicted to detect 14-3-3 homologues in other species. The secondary antibodies used in this study were the anti-rabbit HRP conjugated antibody (Abcam ab6721) and the anti-mouse HRP conjugated antibody (Abcam ab97023). Protein detection was carried out using Invitrogen™ iBright™ Imaging Systems.

RNA isolation. 100-150 mgr of wet weight powder of *M. oryzae* mycelium were resuspended by vortex (1 min) in 1 ml Phenol:Chloroform:IAA pH 8 and 500 µl TLES (Tris-HCl pH 8 100 mM; Lithium Chloride 100 mM; EDT pH 8 10 mM; SDS 1%). Then, an equal volume to the upper phase of 4 M LiCl was added, mixed gently and stored overnight at 4°C. After 30 min centrifugation at 14000 rpm the resulting pellet was resuspended in DEPC-treated H₂O and left with 3 M sodium acetate and 100% ethanol overnight at -80°C. The resulting total RNA (20-50 µg) was treated with TURBO™ DNase (Invitrogen) and store at -80°C.

mRNA quantification by quantitative polymerase chain reaction (qPCR). 500 ng of total RNA was reverse transcribed using PrimeScript™ RT reagent kit (Takara). The average threshold cycle (Ct) was normalized against actin transcript levels and relative quantification of gene expression was calculated using the $2^{\Delta\Delta C_t}$ method (Pfaffl, 2001). Primer efficiency was calculated using dilutions of a cDNA mixed from different conditions (CM, MM-C, MMT and MMT + rapamycin). qPCRs were carried out using 2.5 µl of a 1/5 dilution of the transcribed products using fast-start DNA master SYBR green I kit (Roche Diagnostics) in a final volume of 10 µl. The following PCR program was used: one cycle at 95°C for 4 min, followed by 45 cycles at 94°C for 30 s, 60°C for 30 s. The Ct (threshold cycle) provided a measure for starting copy numbers of the target genes. Three technical repetitions from three independent biological experiments were carried out to measure the transcript levels of each gene.

Yeast growth media and transformation. *Saccharomyces cerevisiae* strains were cultured in YPDA medium (1% yeast extract, 2% bacto-peptone, 20% glucose, 0.2% adenine). For selection of transformed colonies, *S. cerevisiae* strains were cultured in Dropout Base media

(DOB) MP Biomedicals™ (1.7 g Yeast Nitrogen Base, 5 g Ammonium Sulfate, 20 g Dextrose). Strains used as bait were selected with DOB medium supplemented with Complete Supplemented Mixture (CSM) minus Leucine (CSM-Leu, MP Biomedicals™). Strains used as prey were selected with DOB medium supplemented with CSM without Tryptophan (CSM-Trp) (MP Biomedicals™). The enrichment of *S. cerevisiae* diploids were carried out in DOB medium supplemented with CSM without leucine and tryptophan (CSM-Leu-Trp). Interaction assays were performed in DOB medium supplemented with CSM without leucine, tryptophan and histidine (CSM-Leu-Trp-His).

For transformation, yeast cells were previously grown in agitation during 48h at 28°C in liquid YPDA. Yeast previously grown in YPDA (3 ml) were centrifuged at 6000 rpm during 2 min. Centrifuged cells were cultured, without agitation, in dark 48h at 28°C with 10 µg/µl salmon sperm, 1000 ng of plasmid and 500 µl of filtered PAGE (40% PEG 4000; 0.1M Lithium acetate, TrisHCl pH 8, 0.5M EDTA). Washed cells with sterilized milliQ water were plated in DOB media (without the corresponding aminoacid).

For complementation assays in yeast using the *M. oryzae* *HRP1*, we used the *S. cerevisiae* wild-type (W3031Bα) and $\Delta hrp1-5$ thermosensitive mutant (MHY300) strains that were kindly provided by Helene Gaillard. Yeast strains were grown and maintained on YPD (1% yeast extract, 2% peptone, 2% glucose) or on synthetic selective medium supplemented with 2% galactose. Temperature-sensitive *hrp1* mutant was grown at 26°C-30°C (permissive temperature) or 35°C (restrictive temperature). Yeast strains were transformed using a modified lithium acetate method (Gietz *et al.*, 1995).

Yeast-two hybrid screen. Protein-protein interaction assays were carried out with ProQuest™ Two-Hybrid System (Invitrogen). *uORF1* was amplified from cDNA extracted from *M. oryzae* wild-type grown in carbon starvation using primers uORF1 Fw GwB1 Y2H and uORF1 Rv GwB2 Y2H (Table 2.1), which included attB1 and attB2 sites. *eIF4E*, *eIF4E-1* and *eIF4E-3* genes were amplified from cDNA extracted from *M. oryzae* wild-type grown in CM media with B1-B2 primers (Table 2.1). All genes were cloned in pDONR207, which contains the gateway cassette P1-P2™ by recombination using Invitrogen™ Gateway™ BP Clonase™ II enzyme. The *uORF1* sequence was cloned into the pDEST™ 32 plasmid using Invitrogen™ Gateway™ LR Clonase™ II enzyme. *eIF4E*, *eIF4E*, *eIF4E-1* and *eIF4E-3*

genes were cloned into pDEST™ 22. Genes in pDEST™ 32 were fused to the DNA binding domain of GAL4 (GAL4-BD) and used as bait while genes in pDEST™ 22 were fused to the GAL4 activation domain (GAL4-AD) and used as prey.

Plasmids pDEST™ 32 were introduced in the PJ694 yeast strain (MAT alpha, -Leu, -Trp, -Ura) and selected in DOB-Leu medium. pDEST™ 22 plasmids were introduced in the YM4271A yeast strain (MAT α , -Leu, -Lys, -Tyr, -Trp, -Ura) and selected in DOB-Trp medium. Transformed cells were checked by PCR and positive colonies were stored at -80°C. For protein-protein interaction assays, the mating of yeast strains was carried out in liquid YPDA media during 48h at 28°C in darkness. Then, diploid yeasts were kept in DOB-Leu-Trp for two days at 28°C in agitation for their enrichment. Next, yeast diploids were grown during two days in DOB -Leu, -Trp-His agar plates to detect positive interactions. The strength of the interaction was measured by adding different concentrations of 3AT (3-Amino-1,2,4-Triazol), an inhibitor of the HIS3 reporter gene. Final results were concluded using three biological experiments and using a positive and a negative control for every interaction tested.

Confocal microscopy. Visualization of fungal cells containing *mRFP* (mCherry variant) fused to *14-3-3B* gene was performed in a Leica TCS SP8 confocal microscope using the 561 nm laser line. The emission was captured with a 575-615 nm band-pass filter.

Polysomes fractionation and analysis. For polysomes fractionation *M. oryzae* mycelia were cultured in 250 ml flask in liquid CM for 48h. Then mycelia were filtered and washed with water and transferred into a 250 ml flask with CM or MM-C for 16h. In order to block ribosomal complexes, we added 0.1 mg/ml of cycloheximide in each flask and incubated in ice for 15 min. Mycelia were recollected and stored at -80°C. Protein and RNA extraction was performed in 2 ml tubes supplemented with crystal washed beads and 1 ml of ice-cold polysomal lysis buffer (10 mM NaCl, 2 mM Tris-HCl pH 7.5, 0.5 mM MgCl₂ and 1% of Triton X-100), 0.1 mg/ml of cycloheximide and 1x Protease Inhibitor Cocktail (cOmplete™ EDTA-free Protease Inhibitor Cocktail). Cells extracted were vortexed during 5 min always keeping them in ice. Lysate was cleared separated by centrifugation at 13,000 rpm during 20 min at 4°C. Supernatant was transferred into a 1.5 ml tube and additional centrifugation step at 14,000 rpm for 15 min at 4°C was performed for clearing the lysate. Lysate extract

corresponding to 800 µg of RNA were loaded onto 11 ml linear sucrose gradient (10% to 50% w/v). Sucrose gradient were carried out using polysomal lysis buffer without 1% of Triton X-100 and supplemented with 0.1 mg/ml of cycloheximide. Sucrose gradients with the lysate were loaded into a Beckman SW 4Ti rotor and centrifuged at 40,000 rpm for 160 min at 4°C. Gradient fractionation was carried out using a Bio-Rad BioLogic LP System and a long needle at the bottom of the sucrose gradient. Gradient was fractionated at 0.5 ml/min in 900 µl into fraction collectors. Fractions were measured by UV using Bio-Rad Econo UV monitor and obtained data was analysed by Origin 8 software (OriginLab). RNA was extracted of each fraction using the RNA extraction method previously described.

Table 2.1. List of primers used in this study.

General primers

M13F	5' CGCCAGGGTTTTCCCAGTCACGAC 3'
M13R	5' AGCGGATAACAATTTACACAGGA 3'

qPCR primers

q14.3.3B(MGG_13806) For	5' GCATTTGACGATGCCATTGC 3'
q14.3.3B(MGG_13806) Rev	5' TGCATGATAAGGGTGCTGTCTG 3'
q14.3.3BLong(MGG_13806) For	5' GCGTCGATCGAAAAAGATGGAG 3'
q14.3.3BLong(MGG_13806) Rev	5' TCTTTGGCAGTCATGACGAC 3'
5-Acting	5' CCTGGCACCGTCGTCGATGAAGG 3'
3-Acting	5' GCGAGGCGAGAATGGAACCACCG 3'
qPCR-RBP35F2	5' GGCTGCGAGGAGGAGTTGAAGG 3'
qPCR-RBP35R2	5' CCGCCTGCTGGGATGTGAGC 3'
Fw uORF1 qPCR NEW 3	5' CCCGACTGGTACTGGAAATGGCG 3'
Rv uORF1 qPCR NEW 4	5' ACGCGGTCAGGATTACAGGAGC 3'
Hrp1_exon4_Fw1	5' CTCAGCCCCGTGGCAACCTT 3'
Hrp1_exon4_Rv	5' GCCATCATCTGCGGCGTCAT 3'
Fw1 ScHRP1	5' ATGGTCCAAAGGGCCCATCA 3'
Rv1 ScHRP1	5' GACCACCACGGCCGTTTCTT 3'
Fw ACT1 Sc	5' CCTACGTTGGTGATGAAGCT 3'
Rv ACT1 Sc	5' GTCAGTCAAATCTCTAACC GG 3'

Primers used to confirm gene deletion mutants

14-3-3B 5' for	5' GATGAAAGCGGTAGGAAT 3'
14-3-3B 3' for	5' GCGCACGGACCGTGAACCCA 3'
CT74-1	5' AATTAACCCTCACTAAAGGGA 3'
5-HYGR	5' CCGAGCCGATGACTTACTGG 3'
HSVtk-Probe	5' GCTCCTCGATCAGAGTCTGAGGC 3'
M13	5' CAGGAAACAGCTATGACCATG 3'

Fw Rbp35 KO 5UTR	5' GCGCAATCGTCGGTAGC 3'
Rv Rbp35 KO 3UTR	5' GCGCTGTTGCCTTCAATGACG 3'

Primers used to generate deletion mutants

B4-RBP35	5' GGGGACAACCTTTGTATAGAAAAGTTGGCCTTCTCATTGGTGGTTAGCCACC 3'
B1R-RBP35(no intron)	5' GGGGACTGCTTTTTTTGTACAAACTTGACTCCGTGAACCTGATACACACG 3'
B4 14-3-3B (MGG13806)	5' GGGGACAACCTTTGTATAGAAAAGTTGCAGTGAATAAATTGCAGTAC 3'
B1RN 14.3.3B (MGG13806)	5' GGGGACTGCTTTTTTTGTACAAACTTGGTGTGAAATTTGTGAAATTTTTTC 3'
B2R (MGG13806)	5' GGGGACAGCTTCTTGTACAAAGTGGAAGCGCACGGACCGTGAACC 3'

Primers used for PCR-site directed mutagenesis, overlap extension constructs and mCherry protein fusion constructs

FwORF1ATGtoAA G	5' GGTTCACGGAGTGACGTAAGGTCAAAGTTCC 3'
RvORF1ATGtoAA G	5' CTTTGACCTTACGTACACTCCGTGAACCTGATAC 3'
FwRBP35UTRtoCA	5' GGTTCACGGAGTCAACGTATGGTCAAAGTTC 3'
RvRBP35UTRtoCA	5' CCATACGTTGACTCCGTGAACCTGATACACACG 3'
Fw noBP new	5' TGGCTGTGATATTGGGTTTCGG 3'
Rv noBP new	5' GAAATCAACATGATGGTTGCGC 3'
Fw RBP35AGtoGTnew	5' GTACACGCTCCTGTAAATCCTGACC 3'
Rv RBP35AGtoGTnew	5' GTGGTCTCCGAACCCAATATCAC 3'
B1RC-35	5' GGGGACTGCTTTTTTTGTACAAACTTGATTCCGGGCGAGGACGTTTGG CAC 3'
ORF1noStopRv	5' GCCGCCTGAAAAGAACGCCAACACAAGGAA 3'
ORF1-C-HAFw	5' TTTTCCTTGTGTTGGCGTTCTTTTCAGGCGGCTACCCCTACGACGTCC CC 3'
ORF1-C-HARv	5' TTTCCAGTACCAGTCGGGAAAACATGAAGATCACTTGTTCATCGTCGT CCT 3'
3'UTRORF1Fw	5' TCTTCATGTTTTCCCGACTGGTACTGGAAA 3'
B3-35	5' GGGGACAACCTTTGTATAATAAAGTTGGAAGACAAGATAAGATGAGT TGCC 3'
FwORF1-C-GFP	5' TTTTCCTTGTGTTGGCGTTCTTTTCAGGCGGCATGGTGAGCAAGGGC GAG 3'
RvORF1-C-GFP	5' TTTCCAGTACCAGTCGGGAAAACATGAAGATTACTTGTACAGCTCGT CCA 3'
B1RC 14.3.3. nonstop	5' GGGGACTGCTTTTTTTGTACAAACTTGGCGAAGCGGGGGCGGCGGC 3'
B3 14.3.3B (MGG13806) Long	5' GGGGACAACCTTTGTATAATAAAGTTGAGGATGGGAAATGGGGGAAAC C 3'

B3 (MGG13806) Short	14.3.3B	5' GGGGACAAC TTTGTATAATAAAAGTTGCAAAATAGAATGGTGTCTCGGT TC 3'
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Primers used for yeast two-hybrid experiments

uORF1 Fw GwB1 Y2H	5' GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATGGTCAAAGTT CCG CG 3'
uORF1 Rv GwB2 Y2H	5' GGGGACCACTTTGTACAAGAAAGCTGGGTGTTATGAAAAGAAC GCC AAC 3'
eIF4E Fw GwB1 Y2H	5' GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATGGCCGCCCGC CCTAC 3'
eIF4E Rv GwB2 Y2H	5' GGGGACCACTTTGTACAAGAAAGCTGGGTGTCACCTGTCCGCAC CGGG 3'
eIF4E-1 Fw GwB1 Y2H	5' GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATGTCTACCCAA GT CGA 3'
eIF4E-1 Rv GwB2 Y2H	5' GGGGACCACTTTGTACAAGAAAGCTGGGTGTTAGACTGTGAAC TTG GC 3'
eIF4E-3 Fw GwB1 Y2H	5' GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATGGATAACCTA TG GAG 3'
eIF4E-3 Rv GwB2 Y2H	5' GGGGACCACTTTGTACAAGAAAGCTGGGTGCTATTGAGTCGCG CC GCT 3'
Y2H Rbp35-B1	5' GGGGCAAGTTTGTACAAAAAAGCAGGCTCAATGGCCGAGGAGG A TTTC 3'

Primers used for MoHRP1 complementation experiments in yeast

MD13-EcoRI-cdsMoHrp1	5' CGCTGAATTCATGACGGAACCTGAGGC 3'
MD14-XhoI-cdsMoHrp1	5' CGCTCTCGAGGCCGCGGGCATATG 3'

3-RESULTS

Results presented in section 3.2 are included in a manuscript published in New Phytologist (2019) 221: 399-414. doi:10.1111/nph.15405.

Virulence- and signaling-associated genes display a preference for long 3'UTRs during rice infection and metabolic stress in the rice blast fungus

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3.1. Study of the regulation of the *RBP35* gene, a non-essential component of *M. oryzae* polyadenylation machinery involved in alternative polyadenylation

3.1.1. The ratio of Rbp35A and Rbp35B differs depending on the growth conditions and the incubation time

Rbp35 is a component of the polyadenylation machinery that plays a central role in *M. oryzae* pathogenicity (see Introduction, section 1.5). This protein is highly regulated, which suggests its relevant role in the fungal cell. Rbp35 has two protein isoforms, A and B that interact and form part of the fungal Cleavage Factor I (CFI) (Franceschetti *et al.*, 2011). Rbp35A isoform is less stable than Rbp35B and it cannot be detected in fungal cells grown in a carbon-free liquid medium (MM-C). Regulatory domains present in the C-terminus of Rbp35A trigger its degradation and regulate its nuclear localisation (Franceschetti *et al.*, 2011; Rodríguez-Romero *et al.*, 2015). Another level of regulation of Rbp35 is exerted by the uORFs located within the 5'UTR intron. The *uORF1* mRNA is induced in carbon-starved fungal cells, i.e. cells grown in MM-C (Franceschetti *et al.*, 2011). The *uORF1* can override the growth defects of *M. oryzae* in minimal media supplemented with ammonium tartrate as unique nitrogen source (MM^T) and rapamycin (Rodríguez-Romero *et al.*, 2015).

Figure 3.1. Protein levels of Rbp35A are lower than Rbp35B in fungal cells grown in minimal media. Cellular concentration of Rbp35A and Rbp35B during a time course experiments. Western blot of crude protein extracts from mycelia grown in liquid **A)** MM-C, **B)** MM^T and **C)** MM^T supplemented with rapamycin (200 ng/ml).

Based on these results, we decided to investigate further the involvement of uORF1 in the regulation of Rbp35 due to its ability to interfere with the *M. oryzae* target of rapamycin (TOR) signalling pathway (see Introduction, section 1.5), and the relevance of this pathway in fungal growth and pathogenesis (A. Gonzalez & Hall, 2017; Marroquin-Guzman & Wilson, 2015; G. Sun *et al.*, 2018). We started this analysis by looking at the involvement of uORF1 in the maintenance of Rbp35 protein levels in the fungal cell.

Carbon and nitrogen starvation media are used on pathogenicity research studies since these conditions are often encountered by plant pathogens during host colonisation (Wilson *et al.*, 2012). As previously mentioned, MM-C and MM^T are also two fungal media that promote uORF1 expression and function. To compare Rbp35A steady-state protein levels between MM-C and MM^T, first we carried out Western blots of crude protein extracts of fungal mycelium grown on these two media on a time course experiment (Figure 3.1). Under these two conditions, Rbp35A protein is not detected at late time points. In MM-C, Rbp35A is equimolar to Rbp35B and starts to disappear at 4h – 6 h (Figure 3.1A). In MM^T the amounts of Rbp35A detected are minimal, much lower than Rbp35B and nearly absent after 1.5 h (Figure 3.1B). The use of ammonium as a sole nitrogen source triggers the decay of the Rbp35 full length isoform (Rbp35A) but not of Rbp35B. Addition of rapamycin does not alter the production and decay patterns of Rbp35 in MM^T (Figure 3.1C). These results confirm that i) Rbp35A is more unstable than Rbp35B in these two minimal media, ii) the ratio of the two isoforms varies depending on the medium, and iii) Rbp35A and Rbp35B cellular levels remain unchanged in cells that have been in contact with rapamycin.

3.1.2. The 5' UTR intron contains cis-acting elements that inhibit the translation of the RBP35 protein-coding sequence

To understand the involvement of uORF1 in Rbp35 regulation, we used two different *rbp35* mutants, the T-DNA insertional mutant M35 and the deletion mutant $\Delta rbp35$ generated in previous studies (Franceschetti *et al.*, 2011) (Figure 3.2), and introduced different *RBP35:mCHERRY* constructs into these strains (Figure 3.3). The M35 mutant does not produce any of the three expected native transcripts in the *RBP35* locus, e.g. *uORF1*, *uORF2* and a functional *RBP35 CDS* due to a tandem T-DNA insertion in this region. However, M35 produces a truncated mRNA that contains part of the *RBP35* coding sequence (CDS) and

produces a non-functional protein that is recognised by the Rbp35 antibody (Rodríguez-Romero *et al.*, 2015). The deletion mutant $\Delta rbp35$ produces the *uORF1* mRNA but not *uORF2* or *RBP35 CDS* (Figure 3.2). We generated a third mutant in this locus, the double deletion mutant $\Delta\Delta rbp35$, which cannot produce any of these three transcripts as an additional control to ensure that the truncated protein of Rbp35 in M35 is not interfering in subsequent experiments.

Figure 3.2. Features of the *rbp35* mutants used in this study. **A)** In M35, two T-DNAs are inserted in tandem in the *RBP35* locus: the first T-DNA (T-DNA1) is located upstream of the *uORF1* and the second T-DNA (T-DNA2) is within the coding sequence of *RBP35*. As a consequence of the T-DNA2 insertion, a truncated *RBP35* mRNA encoding for a non-functional Rbp35 protein is produced in this mutant. **B)** In the $\Delta rbp35$ deletion mutant, the partial deletion of the *RBP35* coding sequence and the insertion of the hygromycin gene avoids the synthesis of a *RBP35* transcript. However, a polyadenylated transcript is produced from the 5'UTR region in this locus due to the presence of an intact *uORF1* (Rodríguez-Romero *et al.*, 2015). **C)** The double deletion mutant $\Delta\Delta rbp35$ lacks the three transcripts: *uORF1*, *uORF2* and *RBP35* (this study).

The influence of uORF1 in *M. oryzae* TOR pathway is dependent on the presence of Rbp35 in the cell (Rodríguez-Romero *et al.*, 2015). Therefore, we analysed mRNA and protein levels of *RBP35* in the complemented *rbp35* mutants to investigate any correlation between the disappearance of Rbp35A and the presence of *uORF1* in MM^T and rapamycin treatment. *RBP35* expression remains constant independently of the *rbp35* mutant background, and the lack of uORF1 does not influence *RBP35* transcript levels (Figure 3.4A-B). The addition of rapamycin does not promote any change in *RBP35* transcript levels. As expected, the *RBP35:mCherry* with the promoter of the ribosomal *RP27* protein and *NOS* terminator shows nearly a 10-fold increase expression compared to the constructs whose expression is driven by the native promoter of *RBP35*.

Figure 3.3. Schematic representation of the *RBP35* constructs used for the analysis of uORF1. **A)** C-terminal fusion of *RBP35* to mCherry, indicating the elements present in the 5'UTR intron. Orange letters represent conserved splicing sites (ss): 5'ss (GT), branch point (BP; GCTTAC) and 3'ss (AG). The polyadenylation site (PAS) of uORF1 is outside the 5' UTR intron. Start (ATG) and stop (TGA) codons of uORF2 are also represented, the latter inside *RBP35* coding sequence (CDS). **B)** *RBP35* construct with a strong promoter (Pr_{RP27}) and terminator (Ter_{NOS}). **C)** *RBP35* intro-less construct has a native UTR but lacks the 5' UTR intron due to a 211-bp deletion (Rodríguez-Romero *et al.*, 2015).

Next, we compared the results of mRNA expression with Rbp35 protein levels. As previously observed, Rbp35B is not affected by nutritional stresses (Figure 3.4C-D), whereas Rbp35A is hardly detected or absent in M35 and $\Delta rbp35$ strains carrying the native *RBP35* gene (Figure 3.4C-D, *Native-5'UTR* lanes). By contrast, M35 and $\Delta rbp35$ mutants expressing Pr_{RP27}+Ter_{NOS} and 5'UTR *Intron-less* constructs show moderate/good steady-state levels of Rbp35A in MM^T, indicating that the lack of uORF1 increases the translation of the immediately downstream coding gene *RBP35*. The presence of uORF1 in $\Delta rbp35$ - a strain that expresses uORF1- does not interfere with Rbp35A expression of the $\Delta rbp35/RBP35:mCherry(Pr_{RP27}+Ter_{NOS})$ strain (Figure 3.4D, Pr_{RP27}+Ter_{NOS} lanes), which suggest that uORF1 is acting *in cis*. When we force the fungal cell to express the 5'UTR intron constitutively (*Intron-less* construct), a clear reduction of Rbp35A expression levels in the $\Delta rbp35/Intron-less$ is observed strain compared to M35/*Intron-less* in MM^T, suggesting that uORF1 may have also an effect *in trans*. The addition of rapamycin to the medium seems to influence negatively Rbp35A cellular concentration in M35 and $\Delta rbp35$ expressing *RBP35:mCherry(Pr_{RP27}+Ter_{NOS})*. It also affects to $\Delta rbp35/5'UTR Intron-less$ but

not to M35/5'UTR *Intron-less*. It is possible that this is an effect due to the aberrant Rbp35 protein present in the M35 background or to an interaction between uORF1 and rapamycin that modulates Rbp35A steady-state levels. These results also point uORF1 as a potential translational repressor of Rbp35.

Figure 3.4. The 5' UTR contain cis-acting elements that inhibit Rbp35 translation. A-B) qPCR analysis of *RBP35* mRNA levels from mycelia grown in MM^T and MM^T with rapamycin. Primers used amplified *RBP35* CDS (n=3; mean ± SE; letters indicated significance at P < 0.05 using Fisher's analysis of variance/ANOVA). **C-D)** Western blots of proteins extracted from mycelia grown in MM^T (minimal medium containing 10 mM of ammonium tartrate as unique nitrogen source) and MM^T with rapamycin. The constructs introduced in M35 and *Δrbp35* contain a carboxy-terminal fusion of Rbp35 to mCherry, reason why the Rbp35B isoform in blots treated with anti-mCherry antibody (Ab) is not shown. Presence (+) or absence (-) of uORF1 in the background of the strain and of the constructs tested is indicated in red.

3.1.3. uORF1 and RBP35 mRNA levels differ at early stages of leaf and root infection

The level of *RBP35* transcript in the wild-type strain is constant under all laboratory conditions tested and only varies slightly during plant infection (Franceschetti *et al.*, 2011). During leaf colonisation, the *RBP35* mRNA concentration shows a 4-fold increase during

the first steps of plant invasion, i.e. at 8h during the peg formation and appressorium-mediated penetration. Thus, we also set up experiments in order to decipher the expression pattern of *uORF1* during *M. oryzae* plant colonization. The qPCR primers were designed to anneal the transcript sequence of *uORF1*, i.e. the 5' primer after the stop codon inside the 5'UTR intron and the 3' before the polyadenylation site of the *uORF1* transcript. Primers efficiency was validated performing qPCRs in CM and MM-C.

A

B

Figure 3.5. *RBP35* and *uORF1* mRNA levels reveal differences between leaves and roots. qPCRs using RNA extracted from infected leaf and root tissues at 8, 24, 48, 72, 96 and 120 h post-inoculation (hpi). The qPCR primers amplify *RBP35* CDS and *uORF1* 3'UTR. (n=3; mean $\pm$ SE; letters indicated significance at $P < 0.05$ using Fisher's analysis of variance /ANOVA). The abundance of *RBP35* and *uORF1* varies during rice infection.

We measured transcripts levels of *uORF1* and *RBP35* during a time-course infection experiment extracting the RNA from leaves and roots (Figure 3.5A). On leaves, the expression pattern of *RBP35* is nearly constant at all selected time points except at 24hpi. No increase is observed at 8hpi of the leaf infection as previously shown (Franceschetti *et al.*, 2011), possibly due to the difference in the efficiency of qPCR primers used in our study. The *uORF1* expression levels is three times higher than *RBP35* at 24hpi (Figure 3.5A), the step in which the fungal cell is getting adapted to intracellular growth before colonising adjacent cells.

The expression pattern on roots of these two transcripts is different to the observed pattern on leaves. Both *RBP35* and *uORF1* show a significant increased expression during the first 24hpi on roots although *uORF1* expression is lower than *RBP35* expression, which is the opposite situation found on leaves. This may be linked to the different mechanisms and developmental processes of *M. oryzae* during penetration of plant tissues, hyphopodium on roots versus appressorium on leaves. In addition, the levels of *uORF1* on roots at late time points of infection (120hpi) are higher than *RBP35* (Figure 3.5B), an effect also observed on leaves but stronger on underground tissues. The low levels of *Rbp35* at early stages of infection correlate perfectly with the requirement of inhibition of TOR for appressorium-mediated plant penetration in *M. oryzae* (Marroquin-Guzman *et al.*, 2017; Marroquin-Guzman & Wilson, 2015).

3.1.4. The $\Delta rbp35$ and $\Delta\Delta rbp35$ mutants show different colony morphologies in complete medium

Previous studies helped to understand the role of *Rbp35* in the fungal cell using either the T-DNA mutant M35 or the *RBP35* deletion mutant $\Delta rbp35$ (Figure 3.3). As previously explained, the M35 mutant does not produced the *uORF1* transcript but is still capable of generating a truncated and non-functional *Rbp35* protein whereas the $\Delta rbp35$ deletion mutant can produce the *uORF1* transcript. To better understand the involvement of 5'UTR elements present in the *RBP35* gene, we also generated the double deletion mutant $\Delta\Delta rbp35$ that lacks the 5'UTR intron and the *RBP35* coding sequence (Figure 3.6A). PCR analysis reveals that the recombination event has taken place successfully in the $\Delta\Delta rbp35$ genome, and that no additional T-DNA fragments are found in the genome (Figure 3.6B). Interestingly, the

$\Delta\Delta rbp35$ mutant shows a similar growth rate but slightly different colony morphology compared to $\Delta rbp35$ in CM agar plates (Figure 3.6C), with lower pigmentation levels, reduced conidia production (Figure 3.6D) and thinner mycelia.

Figure 3.6. Generation of $\Delta\Delta rbp35$, a deletion mutant of *M. oryzae* unable to produce *uORF1* and *RBP35* transcripts. **A)** Schematic representation of the targeted gene deletion strategy of *uORF1* and *RBP35*. **B)** Agarose gel showing the expected single PCR products that confirm the *RBP35* gene replacement. PCR reactions were carried out using genomic DNA extracted from $\Delta\Delta rbp35$ and the following pair of primers: Fw RBP35 KO 5UTR/CT74-1 (lane 1), Rv RBP35 KO 3UTR/5' Hygr (lane 2), HSVTK/M13 (lane 3) and HSVTK/M13 (lane 4). Absence of a PCR product of the thymidine kinase (TK) gene in lane 3 indicates the lack of an ectopic insertion (see material&methods). Lane 4: positive control (C+) of the PCR (TK). **C)** Growth assays and **D)** sporulation rates of *M. oryzae* wild-type (WT) strain and the deletion mutants $\Delta rbp35$ and $\Delta\Delta rbp35$. CM, complete medium (n=3; mean $\pm$ SE; letters indicated significance at $P < 0.05$ using Fisher's analysis of variance /ANOVA). Presence (+) or absence (-) of *uORF1* and *Rbp35* in the background of the strain tested is indicated in red.

3.1.5. The mutation in the *uORF1* start codon does not alter *Rbp35* protein expression

Regulatory elements present in the 5' UTR have been extensively studied in eukaryotes, and have been found always acting *in cis* of a major adjacent ORF (see Introduction, section 1.7). In *M. oryzae*, the 5'UTR intron of *RBP35* contains a full uORF (*uORF1*) and the partial sequence of a second uORF (*uORF2*) (Figure 3.2A). Proteins potentially encoded by *uORF1* and *uORF2* are 36- and 71-aa long, respectively (Rodríguez-Romero *et al.*, 2015). A genome-wide mapping analyses of 3'ends of *M. oryzae* transcriptome has revealed that these two

uORFs are polyadenylated (Rodriguez-Romero *et al.*, 2019), and that the polyadenylation site (PAS) of uORF1 is found outside the intron.

Figure 3.7. Generation of constructs mutated at the 5'UTR region of *RBP35*. **A)** Top panel: original construct of the C-terminal fusion of *RBP35*:*mCherry* used to generate mutated variants; elements present in the 5'UTR intron are shown (white box). The conserved sequences of 5' and 3' splicing sites (ss) are represented in green; 5'ss (GT), branch point (GCTTAC) and 3'ss (AG). The polyadenylation site (PAS) of *uORF1* is located outside the intron. The start codon (ATG) of *uORF2* is inside the intron and the stop codon (TGA) is located at the 5' end of the *RBP35* CDS. Middle panel: *RBP35* locus showing conserved splicing regions and their mutations; 5'ss (GT→CA), branch point (GCTTAC→TGGCCA) and 3'ss (AG→GT). Bottom panel: *RBP35* construct indicating the point mutation carried out in the *uORF1* start codon (ATG→AAG). **B)** Predicted consensus branch point in *M. oryzae* introns.

When the *RBP35:mCherry* construct without the 5'UTR intron is introduced into $\Delta rbp35$ and M35, only the deletion mutant $\Delta rbp35$ fully recovers the wild-type phenotype on water agar plates, which are used to measure fungal autophagy activity (Rodríguez-Romero *et al.*, 2015). This can only be explained by the presence of *uORF1* in the genome of $\Delta rbp35$. Importantly, this experiment suggests that *uORF1* is able to act *in trans* over *RBP35* CDS to manipulate the TOR signalling pathway (Rodríguez-Romero *et al.*, 2015), a feature that is very unusual for an *uORF* (Hood *et al.*, 2009).

To investigate this in more detail, we created different constructs of the *RBP35* gene (Figure 3.7). First, we generated a genetic construct that cannot undergo the splicing of the 5'UTR intron. Eukaryotic introns contain three conserved elements necessary for its processing: the 5' splicing site (5'ss), the branch point (BP) and the 3' splicing site (3'ss). The two conserved nucleotides of the 5'ss in eukaryote introns are GT, and they are also conserved in the 5'UTR intron of *RBP35*. We mutated these two nucleotides into CA (Figure 3.7A). A genome-wide bioinformatic analyses of *M. oryzae* introns identified that the canonical sequence of the BP is RCURAC (Marconi, 2017) (Figure 3.7B). We found that this sequence corresponds to GCTTAC nucleotide sequence in the 5' UTR intron of *RBP35* within the *uORF2* (Figure 3.7A). This sequence is near the 3' end of the intron and was mutated into TGGCCA. Finally, the two conserved nucleotides of the 3'ss (AG) were changed to GT. The final construct with the mutated 5'ss, BP and 3'ss regions was named “5'UTR-Intron⁺”.

The start codon of the *uORF1* was also changed from ATG→AAG. This point mutation should be able to avoid a peptide synthesis from *uORF1* without altering the splicing of the intron (Figure 3.7A). These two constructs, 5'UTR-Intron⁺ and *uORF1*^{ATG→AAG} were subsequently introduced into the new deletion mutant $\Delta\Delta rbp35$.

To understand how the point mutations in the 5'UTR region are acting over the main ORF (*RBP35*), we analysed the mRNA and protein expression of *RBP35*. In the wild-type strain, *RBP35* mRNA levels are very similar in CM or in MM-C while the expression of *uORF1* transcript shows a 3-fold increase in MM-C (Figure 3.8A), which correlates with previous results (Rodríguez-Romero *et al.*, 2015). In $\Delta\Delta rbp35/uORF1^{ATG\rightarrow AAG}$, the mRNA levels of *RBP35* CDS in CM double compared to the wild-type whereas *uORF1*^{ATG→AAG} transcript levels are similar in both strains. The expression of *RBP35* and *uORF1* transcripts

is approximately at the same level in $\Delta\Delta rbp35/5'UTR-intron^+$ strain in opposition to their difference in expression in carbon-starved wild-type cells. It is surprising the different expression pattern of the *RBP35* locus in $\Delta\Delta rbp35/Native-5'UTR$, possibly due to the insertion of the construct in a different locus. Overall, these results suggest that the ATG→AAG mutation of the *uORF1* and the alteration of conserved splicing sites of the 5'UTR intron promote a misregulation in the expression of *uORF1* and *RBP35*.

Figure 3.8. *RBP35* 5'UTR intron contains *cis*-acting elements that regulate Rbp35 expression. A) *RBP35* and *uORF1* mRNA levels detected by qPCR from mycelia grown in CM and carbon-starved cells (MM-C). The qPCR primers have been designed to amplify *RBP35* CDS and *uORF1* 3'UTR. (n=3; mean $\pm$ SE; letters indicated significance at $P < 0.05$ using Fisher's analysis of variance /ANOVA). **B)** Western blots of crude protein extracts from mycelia grown in CM and MM-C. Presence (+) or absence (-) of *uORF1* in the background of the tested strains is indicated in red.

To analyse if *uORF1* can modulate Rbp35 translation, we carried out Western blots to correlate the mRNA levels of *uORF1* and *RBP35* CDS with Rbp35 protein expression (Figure 3.8B). In MM-C, the Rbp35A isoform is hardly detected in the wild-type, as well as in $\Delta\Delta rbp35$ complemented with the *Native-5'UTR* construct and *uORF1*^{ATG→AAG}. In CM and MM-C, $\Delta\Delta rbp35/5'UTR-intron^+$ displays nearly undetectable levels of Rbp35A. When we

compare these results with mRNA expression, the wild-type, $\Delta\Delta rbp35$ /Native-5'UTR and $\Delta\Delta rbp35$ /*uORF1*^{ATG→AAG} strains show similar Rbp35 protein levels, indicating that the *uORF1* point mutation has no effect on Rbp35 synthesis. The $\Delta\Delta rbp35$ /5'UTR-intron⁺ strain is the only one that produces wild-type levels of both *RBP35* and *uORF1* mRNAs but shows almost no detectable levels of Rbp35A and Rbp35B in CM and in MM-C. This confirms that the 5'UTR-intron⁺ construct contains *cis*-acting elements repressing Rbp35 synthesis, and indicates that either the *uORF1*^{ATG→AAG} point mutation has not disrupted its potential function as translational repressor or there are other *cis* elements regulating Rbp35 synthesis within the intron.

Figure 3.9. Complementation experiments of *RBP35* constructs with *uORF1* fused to the *GFP* gene. **A)** Representation of different *RBP35* constructs with a C-terminal GFP fusion of *uORF1*. **B)** Growth tests of the different strains containing intact and mutated *uORF1* fused to GFP in CM. The *RBP35*:mCherry construct that contains the *uORF1*^{ATG→AAG}:GFP cannot restore $\Delta\Delta rbp35$ developmental defects.

3.1.6. In vivo expression of *uORF1:GFP* construct suggests that *uORF1* is translated

We have observed an effect *in trans* of the *uORF1* acting over the TOR pathway, and an effect *in cis* that reduces the translation of *RBP35* CDS in the $\Delta\Delta rbp35/5'$ UTR-intron⁺.

To understand how uORF1 can be acting upon Rbp35, we looked at the ability of uORF1 to produce a functional protein. In other organisms, uORFs and small ORFs can synthesise peptides with important roles in the cell (see Introduction, section 1.7). Small peptides are difficult to detect by Western blotting. Therefore, we generated a *M. oryzae* strain that contains a green fluorescent protein (GFP) protein fused to the C-terminus of the uORF1, $\Delta\Delta rbp35/uORF1:GFP$, and as a control we used another *M. oryzae* strain that contains the same fusion with a mutation in uORF1 start codon, $\Delta\Delta rbp35/uORF1^{ATG\rightarrow AAG}:GFP$ (Figure 3.9A). Importantly, these two strains show distinct phenotypes. The $\Delta\Delta rbp35/uORF1:GFP$ strain fully recovers pigment production compared to the wild-type although growth is slightly affected (Figure 3.9B). Remarkably, $\Delta\Delta rbp35/uORF1^{ATG\rightarrow AAG}:GFP$ displays a phenotype similar to the double mutant $\Delta\Delta rbp35$ (Figure 3.9B). This indicates that the GFP fusion has not altered uORF1 function and that uORF1 is possibly being translated in the fungal cell.

We also looked at mRNA and protein expression levels of Rbp35 and uORF1:GFP in these strains. The amount of *RBP35* mRNA in $\Delta\Delta rbp35/uORF1:GFP$ and $\Delta\Delta rbp35/uORF1^{ATG\rightarrow AAG}:GFP$ strains is higher than in the wild-type, and present similar patterns to those displayed by the complemented strain with the *Native-5'UTR* construct (Figure 3.10A). Looking at their Rbp35 protein levels, Rbp35 expression is lower in $\Delta\Delta rbp35/uORF1:GFP$ compared to the complemented strain with the *Native-5'UTR* construct (Figure 3.10B) even though *RBP35* mRNA levels are similar (Figure 3.10A). We also confirm that *uORF1* is translated since the GFP protein is detected in both media, and at higher levels in MM-C in correlation with uORF1 higher expression in this medium. The second band observed in MM-C can be due to a processing of the protein in carbon-starved cells. Rbp35 or uORF1:GFP are not detected in the $\Delta\Delta rbp35/uORF1^{ATG\rightarrow AAG}:GFP$ strain, which explains its phenotype.

3.1.7. The 5' UTR intron and uORF1 are important for *M. oryzae* response to nutritional stress

We analysed the capacity of *5'UTR-Intron*⁺ and *uORF1*^{ATG→AAG} constructs to recover $\Delta\Delta rbp35$ developmental defects in CM (Figure 3.11 and Table 3.1). The single deletion mutant $\Delta rbp35$, which lacks the *RBP35* CDS but contains the *uORF1* gene in its genome,

$\Delta\Delta rbp35$ /Native-5'UTR and wild-type strains were used as controls. The $\Delta\Delta rbp35$ mutant shows similar growth compared to $\Delta rbp35$ in CM, with some differences in colony morphology and pigment production as previously described in section 3.1.4 (Figure 3.6). The introduction of the *RBP35* construct with a native 5'UTR (Native-5'UTR) in $\Delta\Delta rbp35$ promotes the full recovery of the growth, colony morphology and sporulation. The $\Delta\Delta rbp35$ /5'UTR-Intron⁺ strain shows a phenotype similar to the $\Delta\Delta rbp35$ mutant, which reflects the low levels of Rbp35 produced in this strain (Figure 3.8). The $\Delta\Delta rbp35$ /uORF1^{ATG→AAG} strain is able to recover the wild-type pigmentation and sporulation levels in *M. oryzae* (Figure 3.11B), but not wild-type growth, supporting that uORF1 alone is able to modulate *M. oryzae* development since the amount of Rbp35 protein detected in this strain reach to wild-type levels.

Figure 3.11. Mutations in the *RBP35* 5'UTR influence *M. oryzae* growth and colony morphology. **A)** Growth assays and **B)** conidia production in CM of wild-type (WT), $\Delta rbp35$, and $\Delta\Delta rbp35$ complemented with different constructs of *RBP35*. The $\Delta\Delta rbp35$ /uORF1^{ATG→AAG} strain recovers pigment production and conidiation but not WT growth; $\Delta\Delta rbp35$ /5'UTR-Intron⁺ shows a similar growth and conidiation compare to $\Delta\Delta rbp35$. (n=3, mean±SE; letters indicated significance at P < 0.05 using Fisher's analysis of variance /ANOVA). Presence (+), absence (-) or decreased levels (↓) of uORF1 and Rbp35 in the background of the tested strains is indicated in red.

Next, we tested these strains in different minimal media (Table 3.1 and Supplementary Figures 1.1-1.3). Nutrient-deficient medium such as water agar forces the fungus to use autophagy for growth. In this medium, an enhanced growing capacity is found in $\Delta rbp35$ compared to the wild-type strain, which indicates that $\Delta rbp35$ displays an accelerated autophagy due to the downregulation in this mutant of the TOR signalling pathway (Franceschetti *et al.*, 2011). Growth of the double $\Delta\Delta rbp35$ mutant in water agar is reduced compared to the single $\Delta rbp35$ mutant but higher than the wild-type and $\Delta\Delta rbp35/Native-5'UTR$, which corroborates that uORF1 alone can alter autophagic processes. The differences in growth rate shown by $\Delta\Delta rbp35/uORF1^{ATG\rightarrow AAG}$ compared to $\Delta\Delta rbp35/5'UTR-Intron^+$ in water agar supports a major role for uORF1 peptide in the *M. oryzae* autophagic response. These results suggest that both uORF1 and Rbp35 are required for a correct growth in water agar and autophagy, and that their involvement is accumulative.

	WT (Guy11) uORF1+ Rbp35+	$\Delta rbp35$ uORF1+ Rbp35-	$\Delta\Delta rbp35$ uORF1- Rbp35-	$\Delta\Delta rbp35/RBP35:mCherry$		
				<i>Native-5'UTR</i> uORF1+ Rbp35+	<i>uORF1^{ATG→AAG}</i> uORF1- Rbp35+	<i>5'UTR-Intron⁺</i> uORF1+ Rbp35↓
CM	100 a	78.9 b	76.5 b	97.9 a	77.9 b	76.6 b
WA	64.3 d	98.4 a	79.5 b	59.9 d	78.2 b	68.9 c
MM-N	125.1 a	112.2 b	103.4 c	124.2 a	124.5 a	119.4 a,b
MM-C	93.6 b	105.6 a	101.3 a	85.0 b	103.8 a	98.1 a
MM ^T	100 a	81 b	65.9 c	93.6 a	67.8 c	74.5 b,c
MM ^T +R	66.5 b	89.5 a	88.3 a	59.2 b	86.4 a	85.7 a

Table 3.1. *uORF1* and the 5' UTR intron play a role in autophagy (WA), the response to metabolic stresses (MM, MM-N, MM-C, MM^T) and the TOR signalling pathway (MM^T+R). Growth assays of wild-type (WT), $\Delta rbp35$, and $\Delta\Delta rbp35$ complemented with different constructs of *RBP35* (n=3, mean±SE). Wild-type and $\Delta\Delta rbp35$ growth behaviours are indicated in bold black and red, respectively. Letters indicate the significance of the growth test in each medium at P < 0.05 using Fisher's analysis of variance/ANOVA. The $\Delta\Delta rbp35/uORF1^{ATG\rightarrow AAG}$ strain only recovers wild-type phenotype in nitrogen-free minimal medium (MM-N). The $\Delta\Delta rbp35/5'UTR-Intron^+$ strain shows wild-type growth in water agar (WA) and carbon-free minimal medium (MM-C). Presence (+), absence (-) or decreased levels (↓) of uORF1 in the background of the tested strains is indicated in red.

We further analysed the involvement of uORF1 in the response to other nutritional stresses, i.e. carbon and nitrogen starvation (Table 3.1 and Supplementary Figure 1.2), which

play a central role during *M. oryzae* plant infection (Marroquin-Guzman *et al.*, 2017; Marroquin-Guzman & Wilson, 2015). In MM-N, $\Delta\Delta rbp35$ grows less than $\Delta rbp35$ although this difference is not highly significant when applying an ANOVA test, suggesting a positive involvement of uORF1 alone in fungal growth during nitrogen starvation. The $uORF1^{ATG\rightarrow AAG}$ and $5'UTR\text{-}Intron^+$ constructs can fully restore $\Delta\Delta rbp35$ growth to wild-type levels, which supports the comparable role played by Rbp35 and uORF1 in the response to the lack of nitrogen in the medium.

In carbon-starved cells, only the $\Delta\Delta rbp35\text{-}Native\text{-}5'UTR$ shows wild-type growth whereas $\Delta rbp35$, $\Delta\Delta rbp35$, $\Delta\Delta rbp35/uORF1^{ATG\rightarrow AAG}$ and $\Delta\Delta rbp35/5'UTR\text{-}Intron^+$ strains display a faster growth due to the accelerated autophagy already detected in these strains in water agar. This confirms that both uORF1 and Rbp35 play a role in metabolic adaptation to carbon-free medium.

To further confirm the potential role of uORF1 in *M. oryzae* through the TOR signalling pathway, these strains were tested in MM^T with and without rapamycin (Table 3.1 and Supplementary Figure 1.3). As expected, the addition of rapamycin to the medium causes a reduction in the colony size of the wild-type and $\Delta\Delta rbp35\text{-}Native\text{-}5'UTR$ strains due to the inhibition of the TOR pathway. Remarkably, the $\Delta\Delta rbp35/uORF1^{ATG\rightarrow AAG}$ or $\Delta\Delta rbp35/5'UTR\text{-}Intron^+$ strains behave like $\Delta\Delta rbp35$. This result supports that uORF1 and Rbp35 have the ability to modulate TOR signalling pathway if only these two proteins are concomitantly present in the cell.

3.1.8. The 5'UTR of RBP35 regulates *M. oryzae* plant infection

Environmental nutrient sensing is carried out in eukaryotic cells by the TOR signalling cascade. Nutrient sensing and the TOR pathway are also essential for *M. oryzae* plant colonisation (Marroquin-Guzman *et al.*, 2017; Marroquin-Guzman & Wilson, 2015; G. Sun *et al.*, 2018). Therefore, we set up to study the plant infection behaviour of the different mutant strains generated in this study.

Differences are observed in rice roots between the single and double *rbp35* deletion mutants; $\Delta rbp35$ causes more necrotic symptoms than $\Delta\Delta rbp35$. Additionally, a single point mutation in the *uORF1* is enough to reduce disease severity (Figure 3.12A), and the $\Delta\Delta rbp35/5'UTR-Intron^+$ strain cannot recover the wild-type disease symptoms although is still able to produce necrosis in a similar degree to $\Delta rbp35$. These results corroborate that *uORF1*-mediated regulation of Rbp35 is not only important for nutrient sensing but also for *M. oryzae* plant colonisation.

Figure 3.12. Relevance of *RBP35* 5'UTR for *M. oryzae* plant colonisation. Infection experiments using wild-type (WT) strain, the $\Delta rbp35$ and $\Delta\Delta rbp35$ deletion mutants and $\Delta\Delta rbp35$ complemented with three *RBP35*:*mCherry* constructs on roots (A) and leaves (B). The *RBP35*:*mCherry* construct with its native 5' UTR fully restores $\Delta\Delta rbp35$ disease symptoms in both tissues. $\Delta\Delta rbp35/uORF1^{ATG\rightarrow AAG}$ strain shows full virulence in leaves but not in roots. The 5'UTR-Intron⁺ construct is unable to completely restore pathogenicity in both tissues (three biological replicates; mean percentage $\pm$ SE; letters and stars indicated significance at $P < 0.05$ using Fisher's analysis of variance /ANOVA). Total number of leaves and seeds analysed in infections experiments are indicated. Presence (+) or absence (-) of *uORF1* in the background of the tested strains is indicated in red.

As expected, the $\Delta\Delta rbp35/Native-5'UTR$ strain is able to produce wild-type symptoms. Regarding leaf infections, we observed some differences in the pathogenic behaviour of these fungal strains compared to roots. Both $\Delta rbp35$ and $\Delta\Delta rbp35$ deletion mutants produce less symptoms on leaves (Figure 3.12B). Disease severity of $\Delta\Delta rbp35/uORF1^{ATG\rightarrow AAG}$ is higher than the wild-type and $\Delta\Delta rbp35/Native-5'UTR$ strains (Figure 3.12B), suggesting an additional effect of the lack of uORF1 in the cell. The reduced disease symptoms of $\Delta\Delta rbp35/5'UTR-Intron^+$ are in accordance with the reduced amount of Rbp35 protein present in this strain (Figure 3.8B).

3.1.9. Polysome profile analysis suggests that uORF1 can interfere with the location of ribosomes along the RBP35 mRNA molecule

The position of polysomes along the mRNA molecule can suggest if a gene is being actively translated whereas the attachment of ribosomes subunits indicates a stalling of ribosomes on the mRNA molecule (see Introduction, section 1.7). We performed a polysome profile with the strains included in this study to find evidences that can help to understand if uORF1 can influence Rbp35 translation.

Figure 3.13. A) Polysomal profiling of wild-type (WT), $\Delta rbp35$ and $\Delta\Delta rbp35$ strains. MW: molecular weight. **B)** Quantitative PCRs of *RBP35* and *uORF1* using mRNAs extracted from polysomal fractions 1–8 (n=3; error bars represent $\pm$ SE; letters indicated significance at $P < 0.05$ using Fisher's analysis of variance/ANOVA).

First, we observed that the polysomal profiles of wild-type and the $\Delta rbp35$ mutant are similar whereas $\Delta\Delta rbp35$ profile displays an enrichment in polysomes with high molecular weight in two different fungal growth media, i.e. CM and MM-C (Figure 3.13A and Supplementary Figure 1.4). This may suggest that uORF1 could play a general inhibitory role in the translation of *M. oryzae* proteins. It is known that the stalling of ribosomes is a common role for some uORFs (Wethmar, 2014).

Next, we looked at the amount of *RBP35* and *uORF1* mRNAs found in the polysomal fractions (Figure 3.13B). In CM, the wild type strain contains 72.4% of *RBP35* mRNAs in the fraction of polysomes whereas the remaining 27.6% of *RBP35* mRNAs are in the ribosomal subunits fractions (40S/60S and 80S). This pattern is not affected by depleting glucose to the medium. We observed no significant differences between the wild-type strain Guy11 and $\Delta\Delta rbp35/Native-5'UTR$.

The *RBP35* mRNA is highly enriched in the polysomal fraction of the $\Delta\Delta rbp35/uORF1^{ATG\rightarrow AAG}$ strain in CM (93.8%) whereas *uORF1* mRNA abundance (53.7%) remains similar to wild-type and $\Delta\Delta rbp35/Native-5'UTR$. It seems that the loss of the ATG in the uORF1 is favouring the movement of ribosomes along the *RBP35* mRNA molecule and possibly increasing its translation rate. However, this effect is lost in MM-C, a nutritional condition that increases uORF1 mRNA expression levels.

The more striking results are displayed by the $\Delta\Delta rbp35/5'UTR-intron^+$ strain. We observe that *RBP35* mRNA abundance (46.9% in CM and 31.9% in MM-C) in polysomal fractions is much lower than the one found in any of the tested strains although the amount of *uORF1* mRNAs (65.3% in CM and 66.9% in MM-C) remain similar. Overall, $\Delta\Delta rbp35/uORF1^{ATG\rightarrow AAG}$ shows differences compared to wild-type and $\Delta\Delta rbp35/Native-5'UTR$ only in CM while the $\Delta\Delta rbp35/5'UTR-intron^+$ strain shows lower ratios of *RBP35* mRNA in polysomal fractions in both media.

3.1.10. *uORF1* predicted peptide contains a potential motif present in proteins that interact with eukaryotic initiation factor eIF4E

We carried out a BLASTP search using the uORF1 predicted 36-aa sequence to identify potential motifs conserved in this protein. Interestingly, the uORF1 contains a conserved binding element **YCSSLLFF** similar to the characterised binding site of the eIF4E-binding

protein p20 of *S. cerevisiae* (Y**TIDEL**FQ), which has the ability to bind the eukaryotic initiation factor eIF4E. The consensus sequence in eukaryotes is Yxxxx**LØ** (where Ø is a hydrophobic aminoacid) (Figure 3.14A). In *M. oryzae*, there are three predicted eIF4E proteins - eIF4E (MGG_12301), eIF4E-1 (MGG_04647) and eIF4E-3 (MGG_08170) - in contrast to *S. cerevisiae*, which have only the isoform Cdc33 (Brenner *et al.*, 1988) or to humans, where four isoforms of eIF4E have been identified (Fischer, 2009). The yeast two hybrid assays show that neither of the *M. oryzae* eIF4E proteins or Rbp35 interact *in vitro* with the uORF1 peptide (Figure 3.14B).

Figure 3.14. uORF1 does not interact with Rbp35 or eIF4E proteins *in vitro*. **A)** uORF1 predicted 36-aa polypeptide. The square box includes the conserved eIF4E element found in yeast cdc33. Letters in red show conserved aminoacids within the 4E-binding domain. Letters green show serine 20 and serine 25 predicted to be phosphorylated with NetPhos3.1. **B)** Yeast two hybrid assays using uORF1 as a prey and as a bait the three *M. oryzae* eIF4E proteins (MGG_12301, MGG_04647 and MGG_08170) and Rbp35. Yeast strains transformed with empty vectors are used as negative control of the interactions. DOB (-L-W) medium is used as a control of yeast growth for all strains. DOB (-L-W-H) medium is used as selective medium for interactions. Resistance to 3AT is an indication of the strength of the positive interaction. C+: positive control of the experiment to show that the media and conditions were properly developed.

3.2. Characterisation of the role of alternative polyadenylation in the regulation of 14-3-3b function, a protein regulated by Rbp35 and required for *M. oryzae* plant infection

3.2.1. Protein expression of 14-3-3a and 14-3-3b are not affected by RBP35 5' UTR elements

Rbp35 is a component of the polyadenylation machinery required for alternative polyadenylation of mRNAs involved in fungal development and pathogenicity (Rodriguez-Romero *et al.*, 2019). Lack of Rbp35 in the fungal cell results in the shortening of mRNAs regulated by Rbp35. The altered phenotype of $\Delta rbp35$ compared to $\Delta\Delta rbp35$ in the presence of rapamycin suggests that uORF1 can also modulate the TOR pathway in *M. oryzae*. Some small ORFs can play a role regulating i) protein complex formation in protein membranes, ii) protein levels or iii) protein function (see Introduction, section 1.7). Therefore, we aimed to identify among the proteins whose mRNAs are regulated by Rbp35 and play a role in the TOR cascade if they can also be regulated by uORF1. One of the key components of the TOR pathway are the 14-3-3 proteins, which facilitate the phosphorylation of proteins involved in

A

B

Figure 3.15. 14-3-3 proteins are not affected by RBP35 5'UTR elements. **A)** Two different Western blots of proteins extracted from mycelia grown on CM or MM-C. Coomassie blue stain was used as loading control. 14-3-3 (pan) polyclonal antibody detects 14-3-3A and 14-3-3B proteins in eukaryotes. **B)** Schematic representation of 14-3-3-SL with proximal, middle and distal PAS, and the 14-3-3B-S construct with a deletion of the distal PAS.

signalling cascades (Yaffe, 2002). The *14-3-3b* transcript is alternatively polyadenylated by Rbp35 (Franceschetti *et al.*, 2011) (Figure 3.15A), and represents a good candidate for this analysis.

Looking at the protein profile of the two *M. oryzae* 14-3-3 proteins in the background of the *M. oryzae* strains used in this study (Figure 3.15B), no detectable changes in protein expression are observed for 14-3-3a and 14-3-3b, which suggests that the unexpected phenotype of $\Delta rbp35$ in the presence of rapamycin may not be due to the 14-3-3 proteins (Figure 3.15B).

Figure 3.16. Analysis of 14-3-3:mCherry constructs in *M. oryzae*. **A**) Agarose gel (top) and schematic diagram (bottom) showing targeted gene deletion strategy of *14-3-3B*. Single PCR products of expected band sizes confirmed *14-3-3B* gene replacement using genomic DNA from $\Delta 14-3-3b$ strain and the following pair of primers: 14-3-3B 5' For/CT74-1 (lane 1), 14-3-3B 3' Rev/5' Hygr (lane 2), and HSVTK/M13 (lane 3). Absence of a PCR product in lane 3 indicated the lack of an ectopic insertion due to inactivity/mutations of the thymidine kinase (TK) gene. **B**, **C**) Growth of wild-type (WT) and the $\Delta 14-3-3b$ mutant (n=3, mean±SE). The mutant is slightly affected in fungal growth under all conditions tested. **D**) Growth of WT and $\Delta 14-3-3b$ complemented either with the full length sequence of *14-3-3B*, which contains all mRNA isoforms (-SL) or with a short 3'UTR isoform that includes only the first PAS (-S). Pictures in A and B were taken at 8 dpi.

3.2.2. *Rbp35* regulates alternative polyadenylation of *14-3-3B* mRNA

The main role described for *Rbp35* is the selection of distal PAS in pre-mRNAs of *M. oryzae* (Franceschetti *et al.*, 2011; Rodríguez-Romero *et al.*, 2015; Rodríguez-Romero *et al.*, 2019). Some of these genes have critical roles in *M. oryzae* cell biology. The 14-3-3 proteins are important scaffold proteins of many signalling pathways in eukaryotic cells, including the TOR cascade. One of the two 14-3-3 proteins of *M. oryzae*, 14-3-3b, was previously identified for being regulated by *Rbp35* (Franceschetti *et al.*, 2011). Subsequently, a genome-wide polyadenylation sites mapping has found three PASs in *14-3-3B* mRNA (Rodríguez-Romero *et al.*, 2019), with the distance between the first and the last PAS being ~550 nt (Figure 3.15A). To investigate if the regulation of the length of the 3'UTR of *14-3-3B* can alter its protein function, we generated the deletion mutant $\Delta 14-3-3b$ (Figure 3.16A). We complemented this mutant with a *14-3-3B:mCherry* fusion construct that either contains its native 3'UTR (*14-3-3B-S/L* for short and long 3'UTR; Figure 3.15A) or includes only the first PAS (*14-3-3B-S* for short 3'UTR) (Figure 3.15A).

Figure 3.17. Relevance of long *14-3-3B* mRNA isoform to *M. oryzae* plant colonization. Infection experiments using wild-type (WT) strain and the $\Delta 14-3-3b$ mutant complemented either with the full length sequence of *14-3-3B*, which contained short or long 3'UTRs (short and long, S/L) or with a short 3'UTR (S). *14-3-3B-S* was unable to restore $\Delta 14-3-3b$ disease symptoms (three biological replicas; mean percentage $\pm$ SE). Total number of leaves analyzed for WT (n = 54), $\Delta 14-3-3b$ (n = 24), $\Delta 14-3-3b/14-3-3B-S$ (n = 45) and $\Delta 14-3-3b/14-3-3B-S/L$ (n = 49); number of seeds analyzed in root infections are indicated in the bottom panel (n).

The $\Delta 14\text{-}3\text{-}3b$ mutant growth is slightly affected in CM, MM and under different stresses including osmotic stress (Figure 3.16B and C). The $14\text{-}3\text{-}3B\text{-}S$ and $14\text{-}3\text{-}3B\text{-}S/L$ constructs restore $\Delta 14\text{-}3\text{-}3b$ growth defects in MM supplemented with NaCl (Figure 3.16D), indicating that the C-terminal mCherry tag and the 3'UTR length are not affecting protein function. Infection tests reveal that $\Delta 14\text{-}3\text{-}3b$ is strongly impaired in leaf and root infection (Figure 3.17). A strong symptom reduction is observed in $\Delta 14\text{-}3\text{-}3b$ carrying the short 3'UTR construct whereas the strain with $14\text{-}3\text{-}3B\text{-}S/L$ shows wild-type disease symptoms, suggesting a relevant function for the full 3'UTR length of $14\text{-}3\text{-}3B$ during infection. In addition, the $14\text{-}3\text{-}3B\text{-}S/L$ construct retains the ability to reduce its expression levels in MM-C; however, this preference is lost in $14\text{-}3\text{-}3B\text{-}S$ (Figure 3.18A), suggesting the existence of a region within the long 3'UTR responsible of this effect.

Figure 3.18. Relevance of long $14\text{-}3\text{-}3B$ mRNA isoform in $14\text{-}3\text{-}3B$ translation efficiency. **A)** qPCR analysis of $14\text{-}3\text{-}3B$ mRNAs ($n = 3$; mean $\pm$ SE). **B)** Western blotting of proteins extracted from mycelia grown on CM or MM-C. Coomassie stain and rpS6 antibody (Ab) were used as loading controls. $14\text{-}3\text{-}3B\text{-}S$ has lost its regulation in MM-C. **C)** Confocal images of conidia expressing $14\text{-}3\text{-}3B\text{-}S$ and $14\text{-}3\text{-}3B\text{-}S/L$ fused to mCherry in the *M. oryzae* $\Delta 14\text{-}3\text{-}3b$ mutant background. These images were taken with identical confocal settings.

3.2.3. The length of the 3' UTR regulates *14-3-3B* mRNA and protein levels

We found less *14-3-3B-S* mRNAs compared with *14-3-3B-S/L* despite the short isoform being more stable (Figure 3.18A), and the protein is barely detectable in the $\Delta 14-3-3b/14-3-3B-S$ strain (Figure 3.18B). The 45% reduction of mRNA levels and the failure of short *14-3-3B* transcripts to translate efficiently could explain this drastic reduction in protein expression in $\Delta 14-3-3b/14-3-3B-S$ (Figure 3.18B). Confocal imaging confirmed *14-3-3b* protein abundance in the $\Delta 14-3-3b/14-3-3B-S/L$ strain and located *14-3-3b* in the cytoplasm within aggregates near the spore cell wall (Figure 3.18C).

3.3. Hrp1, another protein component of *M. oryzae* polyadenylation machinery

3.3.1. Hrp1 function differs between *M. oryzae* and *S. cerevisiae*

In yeast, the deletion of the *HRP1* is lethal in contrast to *M. oryzae* where the $\Delta hrp1$ mutant is viable although it shows a compromised viability (Rodríguez-Romero *et al.*, 2015). This suggests that common components of the polyadenylation machinery of filamentous fungi and budding yeast can possess different roles. Significantly, Hrp1 protein is absent in plants or humans. Thus, characterisation of *M. oryzae* Hrp1 (MoHrp1) can provide a deeper understanding of the polyadenylation machinery and of the polyadenylation mechanisms in filamentous fungi, which ultimately could help to identify novel targets for pathogen control.

Figure 3.19. *S. cerevisiae* Hrp1 and *M. oryzae* Hrp1 (MoHrp1) proteins. MoHrp1 has 47% aa identity compared to *S. cerevisiae* Hrp1 and the RNA recognition motifs (RRMs) have a 59% of conservation. Arginine-glycine-glycine (RGG) tripeptides are involved in protein localisation and degradation. Serine arginine residues (SR) are present in proteins involved in RNA splicing (Zahler *et al.*, 1992). *MoHRP1* locus has five exons (E1 to E5) and four introns (I1 to I5). Three polyadenylation sites (PAS) have been identified in *MoHRP1*. The first PAS is located inside the second intron (731 nt from the exon 2) and the second and the third PAS within the 3'UTR (390 and 757 nt from exon 5, respectively).

Hrp1 is relatively well conserved between yeast and *M. oryzae* but the presence of more RGG modules and SR elements in MoHrp1 suggests higher levels of protein regulation (Figure 3.19).

Figure 3.20. *S. cerevisiae* HRP1 cDNA partially restores developmental defects of *M. oryzae* *Δmohrp1*. **A)** Complementation assay of *M. oryzae* *Δmohrp1* with *MoHRP1* cDNA fused to HA-FLAG or GFP, and *S. cerevisiae* HRP1 (*Sc HRP1*) cDNA under two different ribosomal promoters, *M. oryzae* *RP27* and *S. cerevisiae* *RBL2*. Fungal growth is compared to *M. oryzae* wild-type (WT). The *Δmohrp1* mutant shows a severe reduction in growth. Pictures of mycelium were taken at 10 days post-inoculation in CM plates grown at 25°C. **B)** Quantitative PCR analysis of *MoHRP1* and *S. cerevisiae* HRP1 mRNAs (n = 3; mean ± SE; letters indicated significance at P < 0.05 using Fisher's analysis of variance/ANOVA).

We started generating several constructs of *MoHRP1* and *S. cerevisiae* HRP1 (*ScHRP1*) to study its role in *M. oryzae* and in yeast. The *MoHRP1* gene was tagged with HA-Flag in N- and C-terminal ends and with GFP in its N-terminal end. These constructs can restore *M. oryzae* growth defects when introduced into the *M. oryzae* *Δmohrp1* mutant (Figure 3.20A). The mRNA levels of *HA-FLAG:MoHRP1* construct are similar to the expression of previously studied *Δmohrp1/HRP1:HA-FLAG* (Rodríguez-Romero *et al.*, 2015) (Figure 3.20B). The expression levels of *GFP:MoHRP1* and *MoHRP1:GFP* are

similar to the wild-type strain (Figure 3.20B). The expression levels corroborate the ability to restore $\Delta mohrp1$ growth phenotype.

The constructs generated with *S. cerevisiae* *HRP1* were cloned under the control of the *M. oryzae* strong promoter *RP27* and *S. cerevisiae* strong promoter *RBL2*. Both strains containing these constructs show less growth recovery compared to *M. oryzae* $\Delta mohrp1$ complemented with *HA-FLAG:MoHRP1* or *GFP:MoHRP1* (Figure 3.20A). A quantitative PCR assay confirmed the correct expression of *S. cerevisiae* *HRP1* in *M. oryzae* (Figure 3.20B). This result confirms that *S. cerevisiae* *HRP1* is not fully functional and restores only partially *M. oryzae* growth.

Figure 3.21. *M. oryzae* *MoHRP1* gene cannot restore growth of *S. cerevisiae* *hrp1-5* sensitive mutant at non-permissive conditions. **A)** Temperature sensitivity assay of *S. cerevisiae* W303Ba wild-type (WT) and *S. cerevisiae* MHY300 *hrp1-5* strains containing empty plasmid (-), or a plasmid with *M. oryzae* *MoHRP1* (*MoHRP1*) and *S. cerevisiae* *HRP1* (*ScHRP1*; positive control) cDNAs. Five microliters of tenfold serial dilution from overnight culture in SD minus uracil were spotted on SD-URA plates. *S. cerevisiae* W3031Ba and *hrp1-5* strains were kindly provided by Prof. Aguilera. **B)** Quantitative PCR analysis of *MoHRP1* from yeast *hrp1-5* strain grown in YPDA. Primers used in these studies amplify a conserved region of the *HRP1* CDS (n=3; mean $\pm$ SE; letters indicated significance at $P < 0.05$ using Fisher's analysis of variance/ANOVA)

Reciprocally, to investigate if *M. oryzae* *MoHRP1* is able to recover the phenotype defects caused by the lack of Hrp1 in yeast, we used a *S. cerevisiae* *HRP1* thermosensitive mutant, MHY300 *hrp1-5* that is not able to growth at 35°C (Gaillard & Aguilera, 2014). The yeast *hrp1-5* mutant complemented with *S. cerevisiae* *HRP1* recovers wild-type growth at non-permissive temperatures whereas *hrp1-5* with *M. oryzae* *MoHRP1* does not (Figure 3.21A). We checked that the *M. oryzae* *MoHRP1* transcript is being expressed to rule out cloning artefacts (Figure 3.21B). This result supports that MoHrp1 and *S. cerevisiae* Hrp1

are not fully interchangeable, and suggests an evolutionary divergence of the role played by this protein in the fungal kingdom.

3.3.2. *M. oryzae* Hrp1 interacts with Rbp35 and itself

In yeast, it has been largely studied that Hrp1 plays a central role in the cell, and it is involved not only in mRNA polyadenylation but also in mRNA transport and non-mediated decay (C. Gonzalez *et al.*, 2000; Kessler *et al.*, 1997; Perez-Canadillas, 2006). To fulfil these roles, yeast Hrp1 is able to translocate between the nucleus and the cytoplasm due to the methylation of several of its RGG domains. Hmt1 is a member of heterogeneous nuclear riboproteins (hnRNPs) in yeast and it is able to methylate Hrp1. *M. oryzae* MoHrp1 contains several RGG domains and a clear orthologue of Hmt1 (MoHmt1) exists in its genome. Thus, we carried out yeast two hybrid experiments using as bait MoHrp1, Rbp35 and MoHmt1 (Figure 3.22). We do not observe an interaction between MoHrp1 and MoHmt1 although MoHrp1 interacts with itself and with Rbp35. This suggest that Rbp35 and Hrp1 can be part of the same complex within the multicomponent polyadenylation machinery. It also indicates that MoHrp1 possibly needs to form dimers or oligomers to carry out its cellular functions.

Figure 3.22. *M. oryzae* Hrp1 interacts *in vitro* with Rbp35 and itself. Yeast strains transformed with empty vectors were used as negative control of the interactions. Yeast two hybrid assays of *MoHRP1*. DOB (-L-W) medium was used as control of yeast growth for all strains. DOB (-L-W-H) medium was used as selective medium for interactions. Addition of 3AT concentrations was used for measuring the strength of the positive interactions.

GENERAL DISCUSSION

Plant pathogenic fungi represent a serious threat to food security (Fisher *et al.*, 2012). The rice blast fungus *Magnaporthe oryzae* is a dangerous pathogen that produces important economic losses every year (Hossain, 2004). Fungal blast is a severe disease aggravated by the increased restrictions in the use of antifungal chemicals and the great capability of *M. oryzae* to overcome new resistant rice varieties in a very short time (FAO, 2013; Strange & Scott, 2005). Moreover, a new *M. oryzae* isolate causing disease on wheat has been reported, increasing considerably the negative impact of this organism in other staple crops (Cruz & Valent, 2017). Therefore, the identification of potential targets that can be used to produce biotechnological tools to control rice and wheat blast has become a research priority.

A potential fungal target for pathogen control was identified using a *M. oryzae* random T-DNA insertional library (Tucker *et al.*, 2010). Root infections tests identified the pathogenicity-deficient T-DNA mutant (M35) that encodes a novel component of the pre-mRNA 3' end processing machinery in the rice blast fungus, namely Rbp35 (MGG_02741) (Franceschetti *et al.*, 2011). Rbp35 is an RNA-binding protein required for fungal virulence in leaves and roots. Rbp35 lacks of clear orthologues in yeast, plants, or metazoans and has the potential to be considered as a fungal target for disease control. *RBP35* has an unusual large 5' UTR with an intron localised in this region harbouring two upstream open reading frames (Rodríguez-Romero *et al.*, 2015). This feature is unique in the *M. oryzae* genome (Franceschetti *et al.*, 2011; Marconi, 2017).

Previously, growth assays performed in the presence rapamycin, an inhibitor of the TOR kinase demonstrated the interdependence between uORF1 and Rbp35 (Rodríguez-Romero *et al.*, 2015). The uORF1 of *RBP35* can modulate the TOR signalling pathway and therefore cellular growth when Rbp35 is present in the cell (Rodríguez-Romero *et al.*, 2015). Under normal conditions, Rbp35 is processed in its carboxy terminal end resulting in the presence of two isoforms, Rbp35A (full length) and Rbp35B (Franceschetti *et al.*, 2011). The two Rbp35 isoforms differ in the Met-Asp-Gly domain and in a nuclear localisation signal present in the Rbp35A C-terminus. This region is required for translational repression of Rbp35, i.e. maintaining Rbp35 steady-state protein levels in the cell and for nuclear localisation (Rodríguez-Romero *et al.*, 2015). Methionine-rich domains have been described in human ribonucleoproteins with a role in pre-mRNA maturation (Datar *et al.*, 1993).

In this study, we focus on investigating mechanisms that regulate two key components of the *M. oryzae* polyadenylation machinery, Rbp35 and Hrp1, which are involved in alternative polyadenylation of genes required for fungal development and pathogenicity. We investigate how these regulatory mechanisms can modulate *M. oryzae* plant virulence. In addition, we detail how alternative polyadenylation of a specific set of genes, *14-3-3A* and *14-3-3B* regulate *M. oryzae* full virulence.

We started this work by looking at the involvement of an upstream open reading frame (uORF1) located in the 5'UTR intron of the *RBP35* gene and its ability to modulate Rbp35 cellular levels. We observe a gradual disappearance of Rbp35A when the fungus grows on MM-C (carbon-free medium) or MM^T (minimal medium) while Rbp35B levels remain intact. This gradual fading of Rbp35A is slower in MM-C compared to MM^T and is not rapamycin-dependent. The other two polyadenylation components involved in alternative polyadenylation, Hrp1 and Cf25 also disappear in carbon-starved cells (Rodríguez-Romero *et al.*, 2015). Therefore, the stability of Rbp35B points this protein as a key component of the 3' end processing machinery necessary for fungal survival in adverse environments, whereas Rbp35A, Hrp1 and Cf25 are dispensable.

Since the uORF1 can override the growth defects of *M. oryzae* in MM^T with rapamycin in the presence of Rbp35 (Rodríguez-Romero *et al.*, 2015), we looked at the mRNA and protein levels of Rbp35 and uORF1 using the same conditions in the wild-type strain, the two deletion mutants M35 and $\Delta rbp35$, and their complemented strains with *RBP35:mCherry* (*Native-5'UTR*, *Pr_{RP27}+Ter_{NOS}* and *5'UTR Intron-less*). The *RBP35* mRNA levels are not affected by the deletion of the 5' UTR intron or the presence of uORF1, discarding a potential implication of uORF1/uORF2 in the non-mediated decay (NMD) pathway, a mechanism frequently associated to the presence of uORFs (Hug *et al.*, 2016). The NMD detects the presence of premature stop codons in the mRNA populations and triggers their degradation to avoid the production of aberrant proteins. Then, we looked at the Rbp35 protein levels produced by these strains. We found a negative correlation between presence of uORF1 and Rbp35A protein. The two mutants, M35 and $\Delta rbp35$ that contain *Pr_{RP27}+Ter_{NOS}* and *5'UTR Intron-less* constructs and therefore lack uORF1, show high levels of Rbp35A compared to wild-type, *M35/Native-5'UTR* and $\Delta rbp35$ /*Native-5'UTR* strains, which supports the effect *in cis* displayed by uORF1 in detriment of the expression of the

main CDS found downstream of uORF1, i.e. *RBP35* CDS. The behaviour of these four strains also differs when rapamycin is added to the medium. The steady-state levels of the Rbp35A protein under rapamycin treatment are reduced in all of them except in M35/5'UTR *Intron-less*. The inhibition of TOR reduces canonical protein synthesis, thus this reduction in Rbp35A levels can be an expected outcome of the drug. However, the lack of response to this drug observed only in M35/5'UTR *Intron-less* suggests that this result can also be linked to the role of uORF1 and Rbp35 regulating this signalling cascade. In this context, the presence of an aberrant Rbp35 protein in M35 background could be masking the effect of rapamycin. Nevertheless, the difference of Rbp35A cellular concentration in M35/5'UTR *Intron-less* compared to $\Delta rbp35/5'UTR$ *Intron-less* corroborates the ability of uORF1 to regulate *in trans* Rbp35A protein levels. It is possible that uORF controls Rbp35 synthesis *in trans* by promoting translation reinitiation through phosphorylation of the eukaryotic initiation factor eIF3h regulated by the TOR pathway (Rodríguez-Romero *et al.*, 2015; Schepetilnikov *et al.*, 2013), which could explain this unusual function of uORF1 when the TOR pathway was inhibited, i.e. adding rapamycin to the media in fungal growth assays. Yeast *CLN3*, *FOL1*, *HOL1*, *IMD4*, *INO2* genes and *Aspergillus nidulans* *CPA* contain uORFs that also regulate their translation (Hood *et al.*, 2009).

To our knowledge *RBP35* 5' UTR intron is the first intron described so far with the ability to control the translation of a virulence gene. Thus, we analysed *RBP35* and *uORF1* mRNA on rice leaves and roots during *M. oryzae* infection. We observed that their messenger levels correlate tightly during fungal growth *in planta* or defined stress media, supporting the idea that the observed posttranscriptional mechanism regulating *RBP35 in vitro* is also active during fungal plant infection. Overall, expression patterns on leaves and roots are relatively similar although some differences are observed at 24hpi and 120hpi. On leaves, *uORF1* mRNA is upregulated at 24hpi compared to *RBP35* mRNA. This difference suggests that *uORF1* role is more influential during the metabolic changes that the fungus undergoes due to the adaptation to intracellular growth, i.e. inside the host cell, whereas no differences are detected between both transcripts in the subsequent infection steps. On roots, this expression pattern is reversed, both *uORF1* and *RBP35* mRNAs are upregulated at 24hpi but *uORF1* expression levels are lower than *RBP35*. Previous time course experiments in rice leaves demonstrated that *RBP35* mRNA is expressed at all stages of fungal infection, and shows an

increase of *RBP35* mRNAs during the first 8hpi (Franceschetti *et al.*, 2011). In our conditions, this upregulation takes place at 24hpi. In both situations, these results indicate a more significant role of Rbp35 and uORF1 at early stages of plant infection, i.e. appressorium- and hyphopodium-mediated penetration and metabolic adaptation within the first infected cell. Here, our results indicate that *M. oryzae* produces higher levels of *RBP35* and *uORF1* mRNAs at 24hpi to support the metabolic change required to adapt from an environment poor in nutrients outside the plant growing at the expense of stored resources within the fungal spore to a rich environment inside the plant cell. In correlation with these results, a genome-wide RNA sequencing approach showed that distal poly(A) sites (PAS) selection during leaf and root infection increases at 8h and 24h and that proximal PAS selection increases the following hours (Rodriguez-Romero *et al.*, 2019), which further supports a predominant role of Rbp35/uORF1 at the early stages of infection regulating longer 3'UTRs.

In addition to M35 and $\Delta rbp35$, we generated a new *rbp35* mutant, i.e. $\Delta\Delta rbp35$ that lacks the *uORF1* gene in the genome to help us understand the implication of the 5' UTR intron in fungal development. The intron deletion does not cause differences in growth rate in CM compared to $\Delta rbp35$ but the colour and hypha density differ from $\Delta rbp35$. This feature provides additional evidences to support that *RBP35* 5' UTR elements are involved in *M. oryzae* development.

We generated different constructs with mutations in the 5' UTR intron (*uORF1*^{ATG→AAG} and 5'UTR-intron⁺) and introduced them into $\Delta\Delta rbp35$ to better understand how elements contained within the intron can regulate *RBP35* expression. We observed that *uORF1* and *RBP35* mRNA levels are influenced by the loci where the constructs are inserted in the genome. Despite of this, this analysis suggests that the ATG→AAG mutation of the *uORF1* and the alteration of conserved splicing sites of the 5'UTR intron promote a misregulation in the expression of both *uORF1* and *RBP35* mRNAs. The observed increased expression of uORF1 correlates with reduced levels of the Rbp35A protein, confirming the anticipated role of uORF1 in regulating Rbp35 synthesis. The detection of the uORF1:GFP protein fusion by Western blotting further supports the interference of uORF1 during ribosomal scanning.

In general, the main role described for uORFs is the translational repression of the main ORF (Arribere & Gilbert, 2013). When uORFs are overlapping with the main ORF, this repression is even greater (Johnstone *et al.*, 2016; McGeachy & Ingolia, 2016). This is the case of yeast GCN4 uORF3, which inhibits GCN4 translation when is expressed under non-stress conditions (Sundaram & Grant, 2014). Probably, this effect is not observed in *M. oryzae* uORF1 due to the distance of almost 200 nt to *RBP35* start codon, which possibly is allowing a normal ribosomal scanning reaching the main ORF. It cannot be discarded that the uORF2, which overlaps with *RBP35* CDS, can be regulating Rbp35 translation through a cap-independent translation mechanism such as Internal Ribosome Entry Site (IRES). In humans, several uORFs in FGF9 gene can block translation probably through unfolding of the IRES structure in the 5' UTR (Chen *et al.*, 2014). The mutations we have introduced in the 5' *UTR-Intron+* can also be affecting the secondary structure of the 5' UTR and therefore translation of either *uORF2* or the main ORF.

To our knowledge, all the effects of uORFs over the main ORF described until now have been observed *in cis*. However, we have observed the influence of uORF1 in the fungal cell due to its ability to act *in trans*. Autophagy, nitrogen/carbon metabolism, TOR signalling and pathogenicity are affected when we introduced mutations in the 5' UTR or uORF1. Autophagy is crucial for *M. oryzae* penetration, conidial components degradation, storage product utilization in the appressorium and cell death (N. J. Talbot & Kershaw, 2009), and it is one of the biological processes regulated by the TOR pathway. Regarding nitrogen and carbon metabolism, *M. oryzae* *ASD4* and *ABL1* are genes required for sensing nitrogen and glucose levels, respectively, and are involved in appressorium development and function (Marroquin-Guzman *et al.*, 2017; Marroquin-Guzman & Wilson, 2015). Their role is also carried out through the TOR pathway, which is inhibited during appressorium formation. Interestingly, their polyadenylation sites are affected in $\Delta rbp35$ (Rodriguez-Romero *et al.*, 2019), which could explain the altered growth of $\Delta\Delta rbp35/uORF1^{ATG\rightarrow AAG}$ and $\Delta\Delta rbp35/5'UTR-intron^+$ in MM-N and MM-C.

Due to the role of the 5' UTR elements in recruiting ribosomes, facilitating or blocking their scanning through the mRNA molecule and influencing the context of start codons for translation initiation, we investigated the distribution of *uORF1* and *RBP35* mRNAs in polysomes. Ribosomal profiles show that *RBP35* mRNA is more frequently found

in polysomes than *uORF1* transcript. This correlates with our model where *uORF1* is repressed in CM but upregulated under carbon starvation (Figure 4.1).

The study of uORFs is an emergent field of interest in several organisms like humans due to its implication in the translation of oncogenes. The study of these elements is difficult due to the low conservation of uORF sequences between organisms but novel techniques such as ribo-seq is helping with their identification. One of the difficulties to identify and assign a role to uORFs is that normally their peptide sequence is not conserved among them. It seems that the large majority of uORFs are not under selective pressure to maintain the amino acid sequence they encode, which means that their potential function is largely independent of a conserved peptide (Johnstone *et al.*, 2016). In zebrafish, only the 0.3%, in mouse 1.1% and in humans 1.7% of uORFs showed a high degree of conservation, reason why is very difficult to identify uORFs in the genomes (Johnstone *et al.*, 2016). In our case, the analysis of the primary sequence of the predicted 36-aa peptide of uORF1 reveals the presence of a domain with homology to the eIF4E-binding domain of the two 4E-binding proteins described in yeast, Caf20p and Eap1p. These proteins can modulate negatively translation initiation through its binding to the translation initiation factor eIF4E, and blocking the recruitment of this factor by the translation initiation complex (Castelli *et al.*, 2015). Therefore, we performed yeast two hybrid assays against the three identified eIF4E orthologues in *M. oryzae* (MGG_12301, MGG_04647, MGG_08170) and Rbp35. In these assays, none of the three proteins nor Rbp35 show an interaction with uORF1. We cannot discard yet this uORF1 potential role in translation initiation regulation until we carry out *in vivo* pulldown experiments to confirm if uORF1 can bind *M. oryzae* eIF4E proteins. Post-translational modifications are often found in RNA-binding proteins, and the lack of conservation of these modifications in a yeast system can also be responsible for masking the *in vivo* interaction of uORF1 with eIF4E proteins and Rbp35. Several RNA-binding proteins containing the RGG tripeptide are methylated by arginine methylases (C. Xu & Henry, 2004).

In *M. oryzae*, approximately 52% of the genes are alternatively polyadenylated and the short mRNA isoforms normally are more stable and more efficiently translated than mRNAs with longer 3'UTRs (Rodriguez-Romero *et al.*, 2019). During plant colonisation, a subset of *M. oryzae* mRNAs show a lengthening of its 3'UTR, which correlates with the important role that Rbp35 has regulating distal PAS selection during infection. One of the genes regulated by Rbp35 is *14-3-3B* (Franceschetti *et al.*, 2011). The *14-3-3* proteins act as scaffold proteins of several signalling cascades (Morrison, 2009), including the TOR pathway (Beck & Hall, 1999). Whereas in other organisms its role in nervous system diseases or in cancer has been studied in exquisite detail, their roles in filamentous fungi is starting to be understood (Berg *et al.*, 2003; Morrison, 2009).

Figure 4.1. Model explaining mRNA and protein levels of uORF1 and Rbp35 during *M. oryzae* adaptation to distinct nutritional environments and their influence in poly(A) site selection (PAS) selection. 1. Rbp35A and Rbp35B are found at equimolar amounts in a nutrient-rich medium (CM), and downregulated *uORF1* mRNA produces less peptide levels (in blue). 2. Glucose deprivation (MM-C) promotes Rbp35A degradation whereas Rbp35B levels remains unaltered. *RBP35* mRNA levels do not change by nutritional stress. *uORF1* mRNA is upregulated and produces more protein, which increases root virulence. Rbp35 and uORF1 acts together down-regulating the TOR pathway, which in turn derepresses autophagy. When the cell moves into a nutritionally rich environment, uORF1 is degraded and its mRNA levels reduced. 3. Rbp35 regulates distal polyadenylation site (PAS) selection of *14-3-3B* mRNA, which increases steady-state mRNA and protein levels. 14-3-3b is an important component of TOR pathway and probably regulated by phosphorylation.

Under normal conditions, the TOR pathway represses the expression of carbon-starvation related-genes through the 14-3-3 proteins. Regularly, these proteins are

phosphorylated and their phosphorylation status regulate their functions in the cell (Yaffe, 2002). In *M. oryzae*, 5'UTR elements are not influencing 14-3-3 protein levels although we did not have a system in place to see if the phosphorylation status of the two *M. oryzae* 14-3-3 proteins is affected by Rbp35 or uORF1. We also analysed the influence of the 3'UTR length in the role of *M. oryzae* 14-3-3b, which is regulated by Rbp35-mediated alternative polyadenylation. The lack of 14-3-3b in the cell affects *M. oryzae* response to nitrogen and carbon starvation, and osmotic stress. In *F. graminearum*, the 14-3-3 proteins are involved in nitrogen metabolism but it is unclear if they do so through the TOR pathway, the glutamine synthetase pathway or both (Brauer *et al.*, 2020). The 14-3-3 genes of *F. graminearum* (Brauer *et al.*, 2020) or arbuscular mycorrhizal fungi (Z. Sun *et al.*, 2018) can restore yeast 14-3-3 mutant defects, which indicates their high degree of conservation. In yeast, 14-3-3 proteins are involved in the regulation of cell cation homeostasis by interacting with the $\text{Na}^+(\text{K}^+)/\text{H}^+$ antiporter Nha1 (Zahradka *et al.*, 2012). Taking this into account, it is not surprising that NaCl is the medium where we have observed differences in phenotype due to the lack of *14-3-3B* mRNA in *M. oryzae*. The *M. oryzae* 14-3-3b localises in the cytoplasm of the spore but also forms aggregates near the cell wall. This localisation can be due to the role of 14-3-3 proteins modulating membrane antiporters (Zahradka *et al.*, 2012).

We have seen that the length of the 3'UTR regulates *14-3-3B* mRNA and protein levels in *M. oryzae*. Interestingly, regulation of a distal PAS selection in *14-3-3B* mRNA has also a strong effect on *M. oryzae* full virulence on leaves and roots. The significant role of *M. oryzae* 14-3-3b in pathogenicity is also conserved in other filamentous fungi. Both *F. graminearum* 14-3-3 proteins are involved in fungal development but only the 14-3-3b (Bmh2) is regulating virulence in *F. graminearum* (Brauer *et al.*, 2020). One of the functions described for 14-3-3 proteins is cell division modulation in fission yeast (Mishra *et al.*, 2005; Ozoe *et al.*, 2002). In *M. oryzae*, cell division progress is essential for appressorium formation and plant penetration (Oses-Ruiz *et al.*, 2017).

The other specific component of the fungal CFI complex, *M. oryzae* Hrp1 (MoHrp1) also plays a crucial role in the biology of this fungus (Rodríguez-Romero *et al.*, 2015). This protein has been thoroughly described in *S. cerevisiae*. The yeast Hrp1 is an RNA-binding protein well conserved in filamentous fungi. Yeast Hrp1 is involved in 3'-end processing and

mRNA export (Barnwal *et al.*, 2012; Kessler *et al.*, 1997; C. Xu & Henry, 2004), and together with Nab4 control the mRNA levels through NMD pathway (C. Gonzalez *et al.*, 2000).

The existence of a second CFI in filamentous fungi, i.e. Rbp35 and CFI25 in addition to Hrp1 suggest that these proteins might have evolved acquiring or losing functions. Unlike yeast, $\Delta mohrp1$ mutant is not necessary for fungal survival although the lack of MoHrp1 results in a seriously compromised fungal strain (Rodríguez-Romero *et al.*, 2015). Here, the complementation experiments of *S. cerevisiae* *HRP1* and *MoHRP1* further support that these two genes are not interchangeable, and therefore, either they play different roles or have additional roles in the fungal cell. In our group we have shown that the RNA motif recognised by MoHrp1 is different to the one recognised by *S. cerevisiae* Hrp1, the UAUUA sequence (Perez-Canadillas, 2006). The *MoHRP1* gene contains four introns. The size of the second intron is unusually large for a *M. oryzae* gene, 1.2 kb and contains a PAS (Marconi, 2017; Rodríguez-Romero *et al.*, 2019), which could generate a shorter protein isoform (119 aa). Attempts to identify the existence of this second isoform in *M. oryzae* have been unfruitful (unpublished results). This upstream PAS can also be involved in reducing the levels of *HRP1* transcripts through NMD.

Rbp35 can have strong effects in the biology and virulence of *M. oryzae*. Here, we investigate the importance of *M. oryzae* uORF1 and detail its involvement in fungal development and plant virulence. We also report the first biological evidence of how PAS selection is important in the mRNA of *14-3-3B* for *M. oryzae* virulence and study the conservation of Hrp1, a second CFI in *M. oryzae* that also interacts with Rbp35. Our results suggest that alternative polyadenylation allows a fine tuning of the proteins such as *14-3-3B* that are required to adapt fungal growth, possibly producing long mRNAs with higher turnover and translation rates. This area of research can help to identify novel targets to control fungal diseases and will provide new insights into the evolution of the polyadenylation mechanisms in eukaryotes.

CONCLUSIONS

- 1- The 5'UTR intron contains at least two *cis*-acting elements that inhibit the synthesis of *RBP35*, uORF1 and uORF2. This translation inhibition is supported by the alterations observed in the polysome profile of the *RBP35* mRNA in the strain that retains the 5' UTR intron.
- 2- The mutation of the *uORF1* start codon does not alter Rbp35 protein expression, which suggest that uORF2 is sufficient to repress Rbp35 synthesis. Nevertheless, uORF1 can interfere with the location of ribosomes along the *RBP35* mRNA molecule.
- 3- Several evidences highlight the capacity of uORF1 to have a *trans*-acting function in *M. oryzae*:
 - A. The *rbp35* single and double deletion mutants differ in their colony morphology and sporulation rates.
 - B. *In vivo* expression of the uORF1:GFP construct suggests that uORF1 is translated. The predicted peptide is 36 aa long and contains a potential motif present in proteins that interact with eukaryotic initiation factor eIF4E.
 - C. Growth tests suggest that both uORF1 and Rbp35 act co-ordinately in the adaptive response of *M. oryzae* to nitrogen and carbon-free media through their ability to modulate TOR signalling pathway.
 - D. The $\Delta\Delta$ *rbp35* mutant that contains the mutation of the *uORF1* start codon is more virulent than the wild-type strain. This capacity of uORF1 to modulate *M. oryzae* infection correlates with the differences observed in *uORF1* and *RBP35* mRNA levels at early stages of colonisation of plant tissues.
- 4- The deletion of the distal polyadenylation site of *14-3-3B* mRNA, a gene regulated by Rbp35 alters mRNA/protein levels and disease severity. This protein is also important for fungal development and osmotic stress resistance.
- 5- MoHrp1 and *S. cerevisiae* Hrp1 are not interchangeable, which suggests an evolutionary divergence of the role played by this protein in the fungal kingdom. Supporting this, MoHrp1 interacts with Rbp35, a protein that has no clear orthologues in yeast.

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SUPPLEMENTARY FIGURES

Supplementary Figure 1.1. Mutations in the *RBP35* 5'UTR influence *M. oryzae* growth and colony morphology. Growth assays in CM (A), water agar (B) of wild-type (WT), $\Delta rbp35$, and $\Delta\Delta rbp35$ complemented with different constructs of *RBP35* (n=3, mean±SE). A) the $\Delta\Delta rbp35/uORF1^{ATG\rightarrow AAG}$ strain recovers pigment production but not WT growth; $\Delta\Delta rbp35/5'UTR-Intron^+$ shows a similar growth compare to $\Delta\Delta rbp35$. B) The percentage represents the growth in water agar against the growth in CM. $\Delta\Delta rbp35/uORF1^{ATG\rightarrow AAG}$ shows a similar growth compared to $\Delta rbp35$, and $\Delta\Delta rbp35/5'UTR-Intron^+$ grows like the WT strain.

Supplementary Figure 1.2. Mutations in the *RBP35* 5'UTR influence *M. oryzae* growth and colony morphology. Growth assays in MM (A), MM-N (B), MM-C of wild-type (WT), $\Delta rbp35$, and $\Delta\Delta rbp35$ complemented with different constructs of *RBP35* (n=3, mean±SE). **A)** the $\Delta\Delta rbp35/uORF1^{ATG\rightarrow AAG}$ and $\Delta\Delta rbp35/5'UTR-Intron^+$ strains do not recover WT growth; both strains show a similar growth compare to $\Delta\Delta rbp35$. **B)** The percentage represents the growth in MM-N against the growth in MM. $\Delta\Delta rbp35/uORF1^{ATG\rightarrow AAG}$ shows a similar growth compared to WT, and $\Delta\Delta rbp35/5'UTR-Intron^+$ grows like the $\Delta rbp35$ strain. **C)** The percentage represents the growth in MM-C against the growth in MM. the $\Delta\Delta rbp35/uORF1^{ATG\rightarrow AAG}$ strain shows a similar growth compared to $\Delta\Delta rbp35$. the $\Delta\Delta rbp35/5'UTR-Intron^+$ strain shows a similar growth compare to WT. Pictures and colony measurements were taken at 8 days.

A Minimal Medium/Ammonium tartrate

B Minimal Medium/Ammonium tartrate + Rapamycin

Supplementary Figure 1.3. Lack of uORF1 or constitutive expression uORF1 can alter the TOR signalling pathway. A) Growth of wild-type (WT), $\Delta rbp35$, $\Delta\Delta rbp35$ and complemented strains with *RBP35* variants in MM^T (n=3, mean±SE). All the strains are affected in growth by ammonium tartrate uptake. *RBP35* variant slightly recover the mutants phenotype. **B)** Growth of wild-type (WT), the $\Delta rbp35$, the $\Delta\Delta rbp35$ mutant and complemented strains with *RBP35* variants in MM^T supplemented with rapamycin. The percentage represents the growth in MM-N against the growth in MM^T (n=3, mean±SE). WT and $\Delta\Delta rbp35/RBP35$ are affected negatively in growth by rapamycin. By contrast, both deletion mutants show a lack of sensitivity to rapamycin. Complemented strains with mutations on uORF1 or *RBP35* 5'UTR Intron also have an altered response to rapamycin. Images were taken at 5 dpi.

Supplementary Figure 1.4. Polysomal profiling of $\Delta\Delta rbp35$ /Native-5'UTR, $\Delta\Delta rbp35$ /*uORF1*^{ATG→AAG} and $\Delta\Delta rbp35$ /5'UTR-Intron⁺ strains. MW: molecular weight.