농학석사학위논문

Roles of the Histone Acetyltransferase Rtt109 in Genome Integrity Maintenance of *Magnaporthe oryzae*

벼 도열병균 유전체 보전에 관여하는 히스톤 라이신 아세틸화 효소 Rtt109의 역할

2015년 2월

서울대학교 대학원

농생명공학부 식물미생물학전공

권 서 문

Master's Thesis in Agricultural Science

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February 2015

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ABSTRACT

Preservation of genome integrity is undoubtedly a fundamental function of life, ensuring the survival and propagation of specific genetic assemblages selected over time. The fungal-specific histone acetyltransferase Rtt109 is implicated in various chromatin functions influencing genome integrity and plasticity. In particular, Rtt109 plays a crucial role in nucleosome assembly associated with DNA replication and repair processes. This thesis explores the implications of Rtt109-mediated genome integrity maintenance in the plant pathogen *Magnaporthe oryzae*, regarding pathogenicity and mycological development. Deletion of Rtt109 led to a marked decrease in mycelial growth rate and a concomitant growth reduction *in planta*. The growth defect seems to be linked to the elevated level of DNA damage constitutively suffered by the mutant, even in the absence of genotoxins. Accordingly, expression of DNA damage repair genes and stress-related genes were increased. The deletion mutant was also rendered hypersensitive to various forms of genotoxic stress including ROS, which is integral to fungal

morphogenesis and the plant defense response. However, appressorium morphogenesis was unaffected by the absence of Rtt109 and the extent of growth reduction *in planta* was not beyond the innate mycelial growth defect. Therefore it is considered that the level of ROS and genotoxic stress experienced by *M. oryzae* during interaction with the host plant is not sufficient to hinder infectious development, even in the vulnerable state without Rtt109. Furthermore, it can be inferred that the intrinsic protective mechanisms together with the up-regulation of DNA damage and stress response genes may be sufficient to protect the mutant throughout the lifecycle. In conclusion, Rtt109-mediated genome preservation is required for normal growth and genotoxic stress resistance in *M. oryzae*. Although Rtt109 is not directly involved in pathogenicity, it is expected to have important contributions to genome regulation and evolution.

Keyword : Histone acetyltransferase, *Magnaporthe oryzae*
Student Number : 2013 -21161

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INTRODUCTION

Eukaryotic genomes are organised into a dynamic complex that is the chromatin, intricate with various protein and RNA components (Bernstein & Allis, 2005, Rodriguez-Campos & Azorin, 2007, Kornberg, 1977). The chromatin stores, maintains and determines the usage of genetic information. Proper modulation of the chromatin structure and composition is integral to transcriptional regulation, DNA replication and repair as well as chromosomal activities.

The basic unit of the chromatin is a nucleosome, which consists of approximately 146 base pairs of DNA wrapped around a core histone octamer (Luger et al., 1997). Histones are adorned with a variety of covalent post-translational modifications at different residues, which contribute to changes in the structure of the chromatin and affect the recruitment of effectors that mediate the transduction of these modifications into biological processes (Bannister & Kouzarides, 2011).

The combinatorial *modus operandi* of histone modifications and their interpretation by combination-specific 'reader' or effector proteins have provided the basis for the histone code hypothesis (Strahl & Allis, 2000, Rando, 2012). The 'writers' and the 'erasers' in this analogy are histone-modifying enzymes that attach and remove chemical groups at various histone residues. Although the term histone code has been used in the context of gene expression, it can be argued that there is a certain histone code directing nucleosome assembly associated with DNA

repair and replication (Li et al., 2008). H3K56 acetylation is one of such modifications, catalysed by the histone acetyltransferase (HAT) Rtt109 in fungi (Driscoll et al., 2007, Han et al., 2007a).

Rtt109 (Regulation of Ty1 transposition 109) was originally discovered as one of the repressors of the retrotransposon Ty1 in yeast (Scholes et al., 2001). It is the fungal-specific and less promiscuous structural ortholog of the metazoan p300/CBP, with a high specificity for H3K56 (Tang et al., 2008). The lysine-56 residue is located in the globular domain of the histone near the DNA entry-exit point of the nucleosome and interacts with the DNA phosphate backbone via a water molecule (Davey et al., 2002). H3K56 acetylation weakens this interaction and increases 'breathing' of the nucleosomal DNA for enhanced accessibility and flexibility, although it alone does not seem to affect chromatin compaction directly, but in combination with other factors (Neumann et al., 2009).

Interactions with histone chaperones Vps75 and Asf1 are pertinent to the catalytic activity and specificity of Rtt109 (Dahlin et al., 2014a, Fillingham et al., 2008). Vps75 directly forms a complex with Rtt109 and traffics it from the cytoplasm into the nucleus (Keck & Pemberton, 2011), where it acts on H3-H4 heterodimer bound by Asf1 (Han et al., 2007b). Newly synthesised histones are marked with H3K56ac for nucleosome incorporation (Masumoto et al., 2005). H3K56ac level peaks during S-phase for nucleosome assembly during DNA synthesis and is removed over G2/M

phase (Masumoto et al., 2005) by Hst3 and Hst4 histone deacetylases (HDACs) in *Saccharomyces cerevisiae* (Celic et al., 2006). Aside from DNA replication, H3K56 acetylation is important for DNA repair (Chen et al., 2008, Downs, 2008) and chromatin modulation at promoter regions (Williams et al., 2008, Rufiange et al., 2007). Hence correct regulation of both acetylation and deacetylation of H3K56 are important for genome integrity and transcriptional regulation.

During DNA replication and repair, the chromatin must be restructured and nucleosomes evicted to allow access to the respective machineries and then reassembled to restore the original chromatin structure (Chen et al., 2008, Li et al., 2008, Misteli & Soutoglou, 2009). The reassembly processes can be classified into replication-coupled (RCNA) and replication-independent nucleosome assembly (RINA) but the general consensus is that both mechanisms involve similar sets of proteins (Dahlin et al., 2014a). Rtt109 complexed with Vps75 acetylates new H3 at lysine-56 presented by Asf1 (Dahlin et al., 2014a). This modification enhances the association of the H3-H4 dimer to the CAF-1 chromatin assembly factor and the histone chaperone Rtt106 for efficient histone deposition onto the DNA (Li et al., 2008). The looser DNA binding by H3K56ac is thought to facilitate nucleosome repositioning by nucleosome remodellers during the nucleosome maturation process (Ransom et al., 2010).

Here we focus especially on the functions of Rtt109 during DNA repair. DNA damage usually leads to cell cycle arrest and requires H3K56ac of newly

incorporated nucleosomes to signal end of repair so the cell cycle may resume (Chen et al., 2008). In the absence of Rtt109, yeast cells are more susceptible to DNA damage and suffer from inefficient repair, leading to the accumulation of G2/M arrested cells (Driscoll et al., 2007). Lack of Rtt109 in yeast also leads to hyperrecombination, manifested as chromosomal translocations (Han et al., 2007a, Driscoll et al., 2007). Furthermore, Rtt109 affects double-strand break repair by non-homologous end joining (Jessulat et al., 2008) and homologous recombination (Munoz-Galvan et al., 2013), as well as mismatch repair (Kadyrova et al., 2013). Another interesting but less well understood function of Rtt109 is in suppressing the proliferation of repetitive sequences such as retrotransposons (Scholes et al., 2001) and ribosomal DNA (Ide et al., 2013).

All the functions of Rtt109 mentioned so far have been discovered in the budding yeast *S. cerevisiae*. Most of the findings on Rtt109 in other fungi have been in line with those previously found in the budding yeast, barring a few exceptional cases. Most remarkably, in the model filamentous fungus *Neurospora crassa,* H3K56ac by Rtt109 is required for quelling and DNA damage-induced small RNA (qiRNA) biogenesis via its participation in homologous recombination (Zhang et al., 2014).

In animal pathogens, Rtt109 has been studied as a promising target for antifungal development due to its fungal-specific occurrence (Wurtele et al., 2010). As a proof of concept, deletion of Rtt109 in both *Candida* and *Pneumocystis spp.* has reduced virulence and viability of these fungi, although an inhibitor effective *in vivo* is yet to

be found (Lopes da Rosa et al., 2013, Dahlin et al., 2014b). In *Candida albicans*, Rtt109 is important in tolerating toxic levels of reactive oxygen species (ROS) generated inside host macrophages and therefore pathogenesis (Lopes da Rosa et al., 2010).

ROS can cause multiple types of mutations and DNA strand breaks (Cooke et al., 2003). Plants also generate ROS in response to pathogen invasion, but it is highly debatable whether its function is solely restricted to plant defence signalling or additionally serves to attack the pathogens (Torres et al., 2006). Furthermore, using Rtt109 inhibitors as a chemical control is out of question in the field. However, it would be worth testing whether plant pathogens experience DNA damage during infection-related processes, which would imply generation of genetic diversity coupled with host-interaction or infectious development.

So what exactly is the relevance of chromatin modulation in plant pathogenic fungi? Indeed, understanding the mechanisms of pathogenicity is the primary goal of our research. Therefore the first important question is how regulation at the chromatin level contributes to the orchestration of myriad processes collectively exhibited as pathogenicity. This means chromatin level changes for morphological development (Ding et al., 2010), expression of virulence factors (Vu et al., 2013, Fernandez et al., 2014), toxin production (Visentin et al., 2012) etc., all carefully cued in response to host and environmental signals.

There are also epigenetic phenomena to be discovered. A pathogen must be able to adapt or evolve rapidly against adverse changes in host or environmental conditions. This can be achieved by epigenetic means, establishing heritable expression patterns without changes to the inherent genome sequence. The best examples in plant pathogens would be the silencing of avirulence genes, leading to gain-of-virulence strains (Qutob et al., 2013) and silencing of infection-specific effector genes in axenic culture (Soyer et al., 2014).

Another subject of interest is chromatin implications in pathogen genome evolution. Chromatin architecture is inextricably linked to both genome integrity and plasticity. Inheritance of faithfully replicated genetic information is a fundamental requirement for life. Nevertheless, mechanisms allowing for genome plasticity are necessary for evolution under multiple selection pressures. This is especially true for pathogens that must stay a step ahead of the host in the co-evolutionary arms race to be able to survive.

Pathogens therefore utilise various mechanisms to generate diversity for adaptive evolution. A striking commonality among filamentous plant pathogens in particular is that many harbour a collection of transposable elements (Raffaele & Kamoun, 2012). Transposon activity not only results in insertional mutagenesis but also leads to repeat induced point mutations, increased recombination rates and epigenetic silencing of nearby genes (Raffaele & Kamoun, 2012, Biemont & Vieira, 2006). Fast-evolving genes such as effectors under positive selection tend to be localised in

regions of instability enriched with repetitive elements, whether they be gene sparse regions (Haas et al., 2009), isochore-like regions (Rouxel et al., 2011) or subtelomeres (Farman, 2007).

In the rice blast fungus, *Magnaporthe oryzae*, about a half of all known avirulence genes are located at the hypervariable subtelomeric regions (Farman, 2007), which is reminiscent of the localisation of surface protein-coding genes in animal pathogens (Wada & Nakamura, 1996, Horn & Cross, 1997, Freitas-Junior et al., 2000). Subtelomeres are known to evolve faster, with a high frequency of ectopic recombinations and transposon activity (Farman, 2007), facilitating the breakdown of R-gene resistance. The archetypal avirulence gene *Avr-Pita* family mostly resides in subtelomeres (Khang et al., 2008, Orbach et al., 2000). In the rice isolates, *Avr-Pita* is known to be translocated frequently, perhaps contributing to recurring gene loss and recovery in the pathogen population (Chuma et al., 2011).

It is also possible that the other repetitive sequences could participate in the generation of genetic diversity in *M. oryzae.* In the strain 70-15, the rDNA region on chromosome II is directly adjacent to the telomere and contains the retrotransposon MAGGY (Rehmeyer et al., 2006). In *Setaria* and wheat isolates, the a/virulence locus PWT1 is also tightly linked to the rDNA region immediately next to a telomere on chromosome II (Chuma et al., 2010). rDNA regions are known for their instability and frequent recombinations (Kobayashi, 2008) and these evidences give the impression that rDNA too could serve to amplify or delete

effector genes – this, however, remains to be tested.

We must be reminded that beside the underlying DNA sequence, the chromatin environment must be permissive of recombination and transposition activities. Rtt109 is implicated in recombination (Han et al., 2007b, Endo et al., 2010, Jessulat et al., 2008), suppression of retrotransposition (Scholes et al., 2001, Curcio et al., 2007), rDNA proliferation (Houseley & Tollervey, 2011) and small RNA biogenesis (Zhang et al., 2014).

Such were the signs that directed me to investigate the significance of Rtt109 in the rice blast fungus. However, the scope of this thesis is restricted to basic observations on the effect of Rtt109 deletion on mycological development, pathogenic phenotypes and fungal responses to DNA damage. I hope that this work is informative and serves as a foundation for future investigations regarding genomic stability and thereby attach some suggestions at the end of the discussion.

MATERIALS AND METHODS

I. Strains and Growth conditions

All *Magnaporthe oryzae* strains (Table 1) were grown at 25°C under constant light conditions on solid V8 medium (8% V8 juice (v/v), 1.5% agar powder (w/v), pH 6.7) unless otherwise stated. All conidia used in pathogenicity tests and observing pre-penetration development were harvested from 7 day-old cultures on solid V8 medium. Rice plants (*Oryza sativa* cv. Nakdong) were grown at 25°C under 16hr light and 8hr dark cycles. Protoplasts, DNA, RNA and protein were all extracted from 3-day-old mycelial cultures in CM broth (0.6% yeast extract, 0.6% tryptone, 1% sucrose; all w/v), grown from conidia harvested from 7-day-old cultures on V8A medium as the starting inoculum. Liquid cultures were incubated at 25°C with shaking at 150rpm in the dark.

II. Targeted deletion of *Rtt109* and genetic complementation

The knock-out construct was designed for targeted replacement of *Rtt109* (MGG_05969) ORF with hygromycin B phosphotransferase gene (HPH) cassette by homologous recombination. 1.361kb region flanking the 5' and 1.399kb sequence flanking the 3' of the ORF were amplified and fused to the HPH sequence by fusion PCR (Table 2). The construct was introduced to the wild-type KJ201 protoplasts by PEG-mediated transformation. Transformants were selected on solid TB3 media

(0.3% yeast extract, 0.3% casamino acid, 1% glucose, 20% sucrose and 0.8% agar powder; all w/v) containing 200ppm hygromycin B and screened by PCR then Southern blot to identify successful deletion mutants with a single integration event. Genetic complementation was carried out by re-introducing *Rtt109* ORF with the aforementioned flanking regions into Δ*rtt109* mutant protoplasts together with the pII99 plasmid containing the geneticin resistance gene. Complementation strains were screened first on solid TB3 medium supplemented with 800ppm geneticin and then by PCR. All strains were deposited at the Center for Fungal Genetic Resources at Seoul National University, Seoul, Korea (http://genebank.snu.ac.kr).

III. Western Blot analysis of H3K56 acetylation

Total protein (480μg per lane) extracted from mycelia was separated by 12% SDS-PAGE. Blots were probed with histone H3 acetyl-lysine K56 antibody (1:2000, Cat. No. 39281, Active Motif, CA, USA) and H3 antibody as loading control (1:2000, Cat. No. 39164, Active Motif, CA, USA). Pierce Fast Western Blot Kit, ECL Substrate (Pierce Biotechnology, Rockford, IL, USA) was used for probing and detection on X-ray films.

IV. Phenotypic complementation of *Saccharomyces cerevisiae rtt109Δ* mutant

MoRtt109 cDNA was PCR amplified and cloned into pYES2 (Life Technologies,

Carlsbad, CA), sequenced and introduced to *Saccharomyces cerevisiae rtt109*Δ strain Y01490 (Euroscarf, Frankfurt, Germany; Table 1). Transformants were selected on Sc-Ura medium and confirmed by PCR amplification of *M. oryzae Rtt109*. Yeast strains (Table 1) were grown to OD_{600}=0.5 then serially diluted tenfold upto 1/10000 for spot assay. 3µl each of the dilution series were spotted onto inductive solid Sc minimal medium (6.7% Yeast nitrogen base without amino acids, 2% Drop-out mix, 2% galactose and 1% raffinose) with or without 50mM hydroxyurea and incubated at 30°C for 3 days.

V. Cell wall staining and hyphal cell length measurements

7 day-old conidia were streaked on water agar slides and incubated for 14 hours at 25°C. Resulting hyphae were stained with calcofluor white and observed with Zeiss Axio Imager A1 fluorescence microscope (Carl Zeiss, Oberkochen, Germany). Distance between septa were measured on ImageJ.

VI. Genotoxin and ROS sensitivity tests

Plugs (4mm diam.) taken from the periphery of colonies grown on solid minimal medium (MM; 1% glucose, 0.6% $NaNO_3$, 0.05% KCl, 0.05% $MgSO_4$, 0.15% KH_2PO_4, 0.1% trace element (v/v), 0.1% vitamin supplement (v/v), pH 6.5; all w/v unless otherwise stated) were used to inoculate solid modified complete media (MCM; 1% glucose, 0.2% peptone, 0.1% yeast extract, 1% casamino acid, 0.6% $NaNO_3$, 0.05% KCl, 0.05% $MgSO_4$, 0.15% KH_2PO_4, 0.1% trace element (v/v), 0.1% vitamin

supplement (v/v), pH 6.5; all w/v unless otherwise stated) containing genotoxins and ROS stressors. Unsupplemented MCM was used as negative control. The following concentrations of DNA damaging agents and ROS stressors were tested: 0.1µM camptothecin, 0.001% methyl methane sulfonate, 10mM hydroxyurea, 2mU/ml bleomycin, 5mM hydrogen peroxide and 2mM methyl viologen. Plates were incubated at 25°C in the dark and radial growth was measured at 7 days post inoculation.

VII. Neutral comet assay

Neutral comet assay was carried out according to Olive & Banáth (Olive & Banath, 2006) with some modifications. Protoplasts were extracted to be used immediately and adjusted to 3-4x10^5/ml in 20% sucrose. As positive control for DNA double-strand breaks, KJ201 was treated with 5mU/ml bleomycin during incubation with *Trichoderma harzianum* lysing enzyme (Sigma-Aldrich, St. Louis, MO) in the protoplast extraction procedure. Comets were stained with 0.25µg/ml ethidium bromide and viewed under Zeiss Axio Imager A1 fluorescence microscope (Carl Zeiss, Oberkochen, Germany) with a filter set optimized for DsRed (20HE; excitation 546nm, emission 607nm). Comet images were analysed with CometScore (n > 100 for KJ201, Δ*rtt109* and *Rtt109c*; n = 50 for the positive control).

VIII. Pathogenicity tests

For spray inoculation, 10ml of conidial suspension adjusted to 10^5 spores/ml was

sprayed onto 2-week old rice plants (*Oryza sativa* cv. Nakdongbyeo). Inoculated rice were incubated in the dew chamber for 18 hours (25°C, 100% humidity, and constant dark), then returned to the original rice growing conditions as stated above and collected at 7 days post inoculation. Conidial suspension of $3x10^4$ spores/ml and 4-week old rice plants were used for wound and sheath inoculation. 20µl of conidial suspension was dropped onto puncture-wounded leaves then lesion size was measured at 5 days post inoculation using ImageJ software. Excised rice sheaths were filled with conidial suspension and incubated for 48 hours at 25°C. Fungal growth inside the rice sheath epidermal cells was observed under a light microscope (n≥132).

IX. Observations on asexual and pre-penetration development

Conidiation ability was measured using 7-day old cultures on solid V8 medium. The entire colony was scraped in sterile distilled water and conidia were counted in a haemocytometers. Total number of collected conidia was estimated and divided by colony growth area to determine the number of conidia produced per cm^2. Pre-penetration development was observed using conidial suspensions of $3x10^4$ spores/ml dropped onto hydrophobic coverslips. Number of conidia in different stages of pre-penetration development was counted at 2, 4, 8 and 12 hours later (n≥100). Stages of pre-penetration development were broadly divided into ungerminated conidium, germinated conidium, incipient appressorium and mature,

melanised appressorium.

X. qRT-PCR and RNA sequencing

RNA extracted from 3-day old mycelia in the aforementioned growth conditions was used for both qRT-PCR and RNA sequencing. qRT-PCR was performed using Rotor-Gene® SYBR® Green PCR Kit and Rotor-GeneQ (Qiagen, Hilden, Germany). Primers used are listed in Table 2. Paired-end sequencing of mRNAs was carried out on the Illumina platform. TopHat was used to map the reads to the annotated *M. oryzae* transcripts, followed by analysis of transcript abundance and differential expression between the strains using Cufflinks. Expression levels were calculated as FPKM. RNA sequencing results were processed and analysed by Dr. Gir-Won Lee and Dr. Junhyun Jeon.

Table 1. List of strains used

Strain	Description	Source
KJ201	*M. oryzae* Wildtype strain	CFGR
Δ*rtt109*	*M. oryzae Rtt109* deletion mutant with KJ201 background	This study
Rtt109c	*M. oryzae* genetic complementation strain of the above Rtt109 deletion mutant	This study
BY4741 (Acc. No. Y00000)	*S. cerevisiae* reference strain *MAT a; his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0*	EuroScarf
*Scrtt109*Δ (Acc. No. Y01490)	*S. cerevisiae Rtt109* deletion mutant with BY4741 background. BY4741; MAT a; his3*Δ* 1; leu2*Δ* 0; met15*Δ* 0; ura3*Δ* 0; YLL002w::kanMX4	EuroScarf
*Scrtt109*Δ-*Mo*Rtt109	*S. cerevisiae* genetic *Rtt109* deletion mutant complemented with *pYES2-MoRtt109*	This study

Table 2. List of primers used

Name	Sequence 5' -> 3'	Description
MGG_05969_5F	GTCCTCCGCAGTGATGGGTATT	Fusion PCR for KO construct design
MGG_05969_5R	GCACAGGTACACTTGTTTAGAGAAGTTATATGCGAACTCATGCCATTG	
MGG_05969_3F	CCTTCAATATCATCTTCTGTCGAGATTGCGACTTGGCTATGTTAACAA	
MGG_05969_3R	CTAAATGGTCGCTTATCGAGTGCT	
MGG_05969_5N	ACACGTTATACAGTTGTTGCTGCGA	
MGG_05969_3N	TGGACCACAGCTAGGTCATATCCG	
MGG_05969_ORF_F	ATGACAACAACTATGGCTACCATG	*MoRtt109* ORF screening
MGG_05969_ORF_R	TTGTCCTTGTTCTTTTTTTCTGACCAG	
Rtt109_Ycompl_seq	TCCCAGGCAGTGTTGAGTA	Sequencing prior to yeast complementation
MGG_05969_KpnI_ORF_F	GGTACCATGACAACAACTATGGCTACCATGG	*MoRtt109* ORF primers with restriction sites for cloning into pYES2 for yeast complementation
MGG_05969_BamHI_ORF_R	GGATCCTCATTGTCCTTGTTCTTTTTTTCTGACC	
MGG_05969_qRT_F	ATGAGGTTGGTCTTGGGATG	qRT-PCR primer for *MoRtt109* transcript
MGG_05969_qRT_R	GCGGTCTCTTCCTCTTAATCAG	
MGG_15576_qRT_F	GAAACCCATAGGCGGTAACA	qRT-PCR primer for *Rhm51* (*Rad51*) transcript
MGG_15576_qRT_R	CTGGTAGACATGGACTGTCATAAA	

Name	Sequence 5' -> 3'	Description
MGG_08585_qRT_F	GGAAGTATTCTCAACCCTCAGTT	qRT-PCR primer for *Rhm52* (*Rad52*) transcript
MGG_08585_qRT_R	CGGCCTGTACTGATTCCTATTC	
MGG_03549_qRT_F	TGTAACCACCCTGACCTACT	qRT-PCR primer for *Rhm54* (*Rad54*) transcript
MGG_03549_qRT_R	TACCACGGCTTGATTTCTCTATC	
MGG_12899_qRT_F	GACAGCGAGGTGGACATTAT	qRT-PCR primer for *MoLig4* (DNA ligase IV) transcript
MGG_12899_qRT_R	TCCGAAGCATTGAGGAGATG	
MGG_10157_qRT_F	CTCACAAAGCACAGGCTACT	qRT-PCR primer for *MoKU80* transcript
MGG_10157_qRT_R	CGTATTCTTGAGCCTCCTCATC	

RESULTS

I. Identification of Rtt109 ortholog in *Magnaporthe oryzae*

The *Rtt109* ortholog in *Magnaporthe oryzae* (MGG_05969) was identified by BLASTP. *M. oryzae* Rtt109 amino acid sequence was aligned with orthologous Rtt109 sequences from some model fungal species and a human pathogen I-TASSER, in which Rtt109 functionality as a H3K56 acetyltransferase had been confirmed experimentally: *Saccharomyces cerevisiae* (Driscoll et al., 2007, Han et al., 2007a), *Neurospora crassa* (Zhang et al., 2014), *Aspergillus nidulans* (as yet uncharacterized) and the human pathogen *Candida albicans* (Lopes da Rosa et al., 2010) (Figure 1). The residues involved in catalysis, acetyl-coA binding (Tang et al., 2008) and autocatalysis (Stavropoulos et al., 2008, Albaugh et al., 2011) that had been experimentally validated in *Saccharomyces cerevisiae* Rtt109 were all conserved in the predicted amino acid sequence of *M. oryzae* Rtt109 (Figure 1).

Then the structure of *M. oryzae* Rtt109 was predicted *in silico* and compared with the previously determined structure of *S. cerevisiae* Rtt109 (Tang et al., 2008) using I-TASSER (Yang et al., 2014). Despite only 24% overall amino acid sequence similarity between the two orthologs, they share a high structural similarity with a Tm score of 0.878 (Figure 2). Overall, the presence of catalytically significant residues combined with the structural similarities strongly suggests functional conservation.

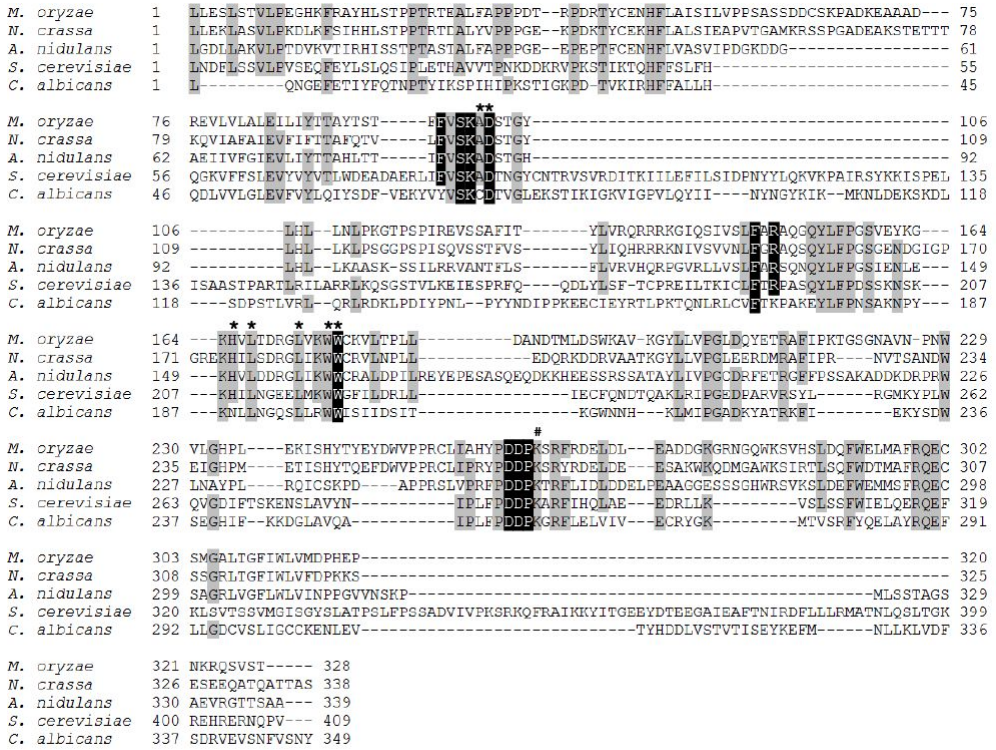


Figure 1. Conserved catalytic residues of Rtt109 orthologs

Rtt109 family signature sequences (IPR013178) of *M. oryzae* Rtt109 and Rtt109 proteins in other fungi were aligned by MUSCLE software (Edgar, 2004) on MEGA ver. 6.06. Key catalytic residues (highlighted in black) are conserved in *M. oryzae* Rtt109 as well as those involved in acetyl-coA-binding (*) and autocatalysis (#).

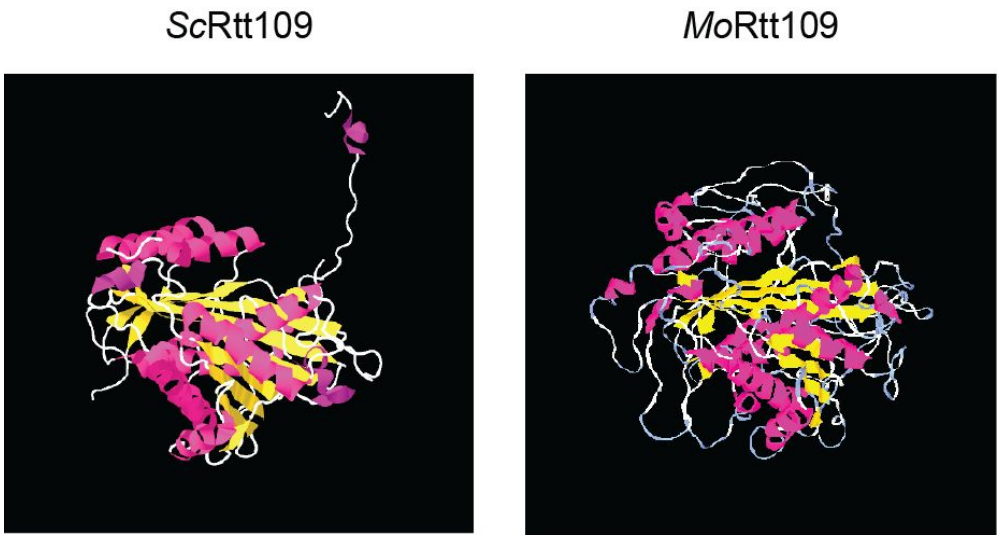


Figure 2. Structural homology between the yeast Rtt109 and the predicted *M. oryzae* Rtt109 proteins

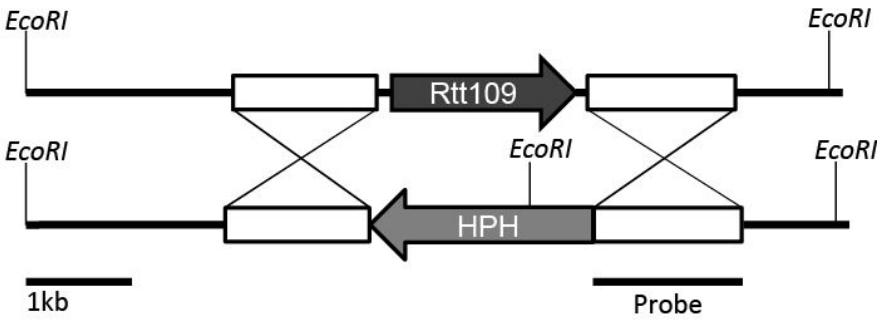
The experimentally determined yeast Rtt109 (*Sc*Rtt109) structure was compared with the predicted *M. oryzae* Rtt109 (*Mo*Rtt109) using I-TASSER (Yang et al., 2014). The structures show a high degree of similarity (TM score = 0.878). Contributed by Dr. Junhyun Jeon.

II. Evidence of *M. oryzae* Rtt109 functionality as a H3K56 acetyltransferase

To test for *Rtt109* functionality in *M. oryzae*, a deletion mutant was generated by targeted gene replacement via homologous recombination (Figure 3). Acetylation at histone H3 lysine-56 residue was abolished in the deletion mutant then restored upon reintroduction of the gene (Figure 4a). This is in accordance with the Rtt109 deletion results in other fungal species (Driscoll et al., 2007, Lopes da Rosa et al., 2010, Xhemalce et al., 2007, Dahlin et al., 2014b, Zhang et al., 2014), attesting that Rtt109 function is indeed conserved in *M. oryzae* as expected. Furthermore, heterologous expression of *M. oryzae Rtt109* in the yeast *rtt109*Δ strain recovered its tolerance to hydroxyurea (Figure 4b), a genotoxic agent and DNA replication inhibitor (Driscoll et al., 2007, Han et al., 2007a).

In *S. cerevisiae*, H3K56 acetylation by Rtt109 largely depends on the presence of the histone chaperone Asf1 and the deletion of this chaperone results in phenotypes similar to Rtt109 deletion, including hypersensitivity to genotoxins (Driscoll et al., 2007). Therefore it is assumed that *M. oryzae* Rtt109 was able to functionally interact with the yeast histone chaperones to restore tolerance to hydroxyurea. It seems quite probable that such interactions pertinent to chromatin dynamics during DNA replication (Han et al., 2007a, Li et al., 2008) and repair processes (Masumoto et al., 2005, Chen et al., 2008) as discovered in yeast are also likely to be conserved in the rice blast fungus also.

(A)



(B)

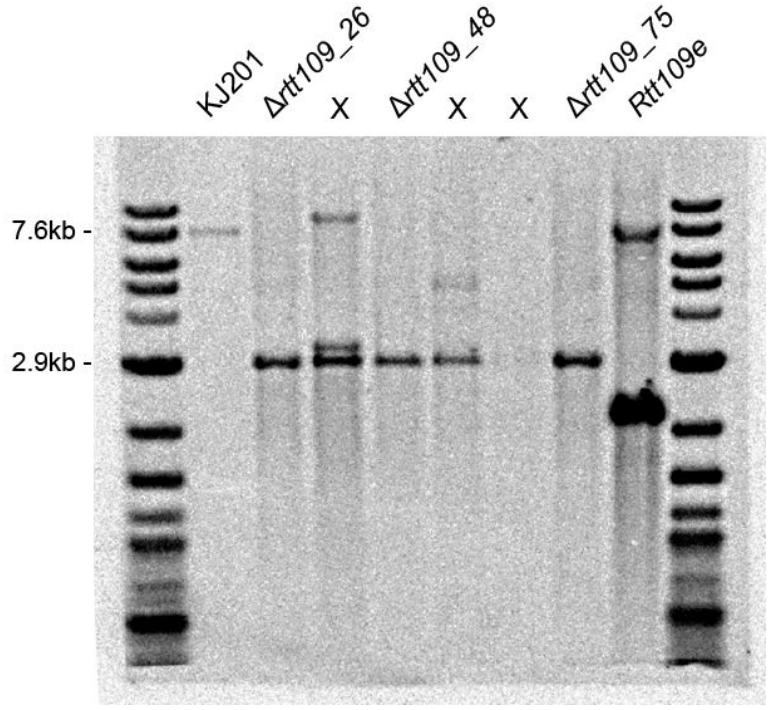


Figure 3. Deletion of *Rtt109* by targeted gene replacement.

(A) Strategy for the replacement of *Rtt109* (MGG_05969) with hygromycin B phosphotransferase gene cassette (HPH).

(B) Identification of correctly replaced transformants (Δ*rtt109_26, 48* and *75*) by Southern blot. Expected wildtype and deletion mutant band sizes are 7.6kb and 2.9kb, respectively.

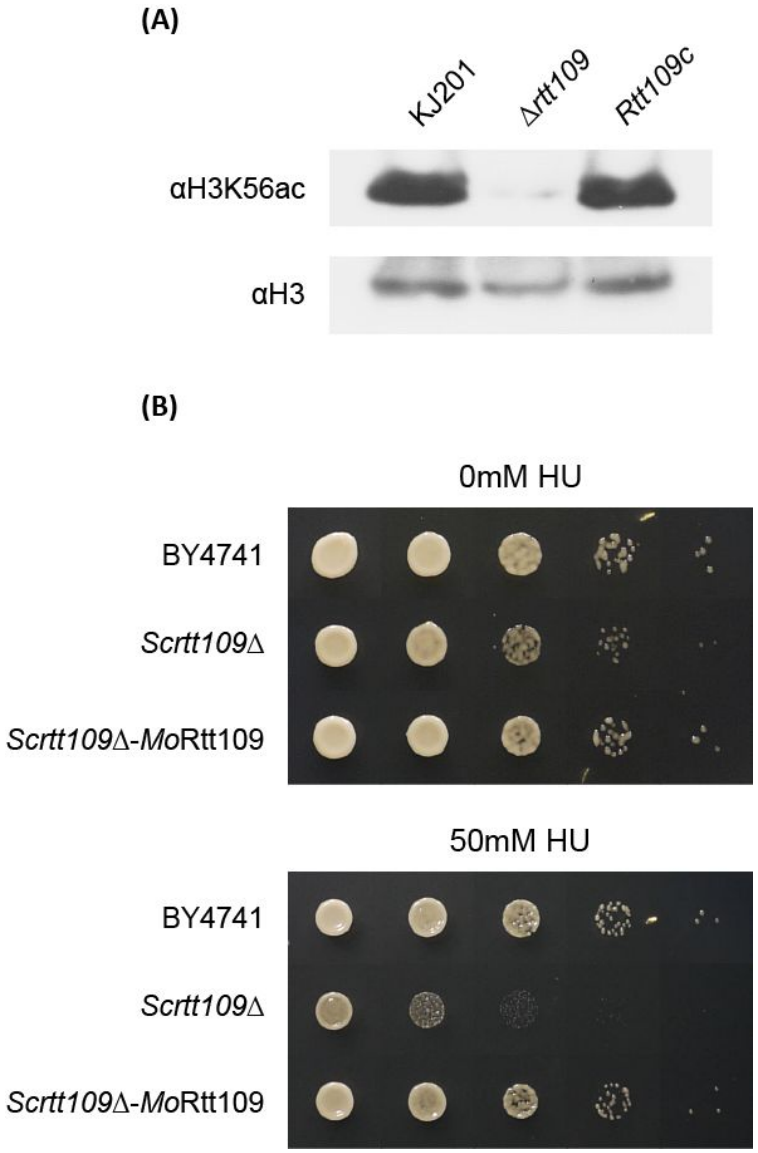


Figure 4. Confirmation of Rtt109 ortholog functionality in *M. oryzae*

(A) Western blot analysis of H3K56ac levels against H3 loading control. H3K56 acetylation is abolished in Δ*rtt109* and restored upon reintroduction of the gene.

(B) Genetic complementation of the yeast *Scrtt109*Δ mutant with the *M. oryzae Rtt109* gene, restoring the growth rate and hydroxyurea (HU) tolerance. Spotted cultures were serially diluted tenfold up to 1/10000 from left to right.

III. Deletion of *Rtt109* leads to reduced mycelial growth and increases DNA double strand breaks

One of the most evident phenotypic defects of the Δ*rtt109* mutant was the reduction in mycelial growth. Radial growth of the mutant at 7dpi was reduced to approximately 50-60% of the wild type on complete medium (CM; Figure 5a). To ascertain the nature of the growth defects, conidia were germinated on CM agar to observe hyphal growth and septation. Cell length was found to be significantly affected in the mutant, suggesting that cell elongation is delayed (Figure 5b). However, reduction in cell size (83% of the wildtype) alone cannot explain the overall mycelial growth defect (50~60% of the wildtype) of the mutant, suggesting that cell cycle progression may also be delayed.

Yeast cells lacking Rtt109 have reduced growth rate due to frequent double strand breaks (DSBs) and consequently cell cycle arrest (Driscoll et al., 2007). Therefore the extent of DSB damage was examined by neutral comet assay (Olive & Banath, 2006). Percentage of DNA in the tail portion of the comet is a representation of the extent of DSBs present in each protoplast examined. The deletion mutant suffered an elevated level of DSBs compared to the wild type strain (Figure 6), which would manifest as growth retardation especially since the DNA damage sensors and cell cycle checkpoints are intact.

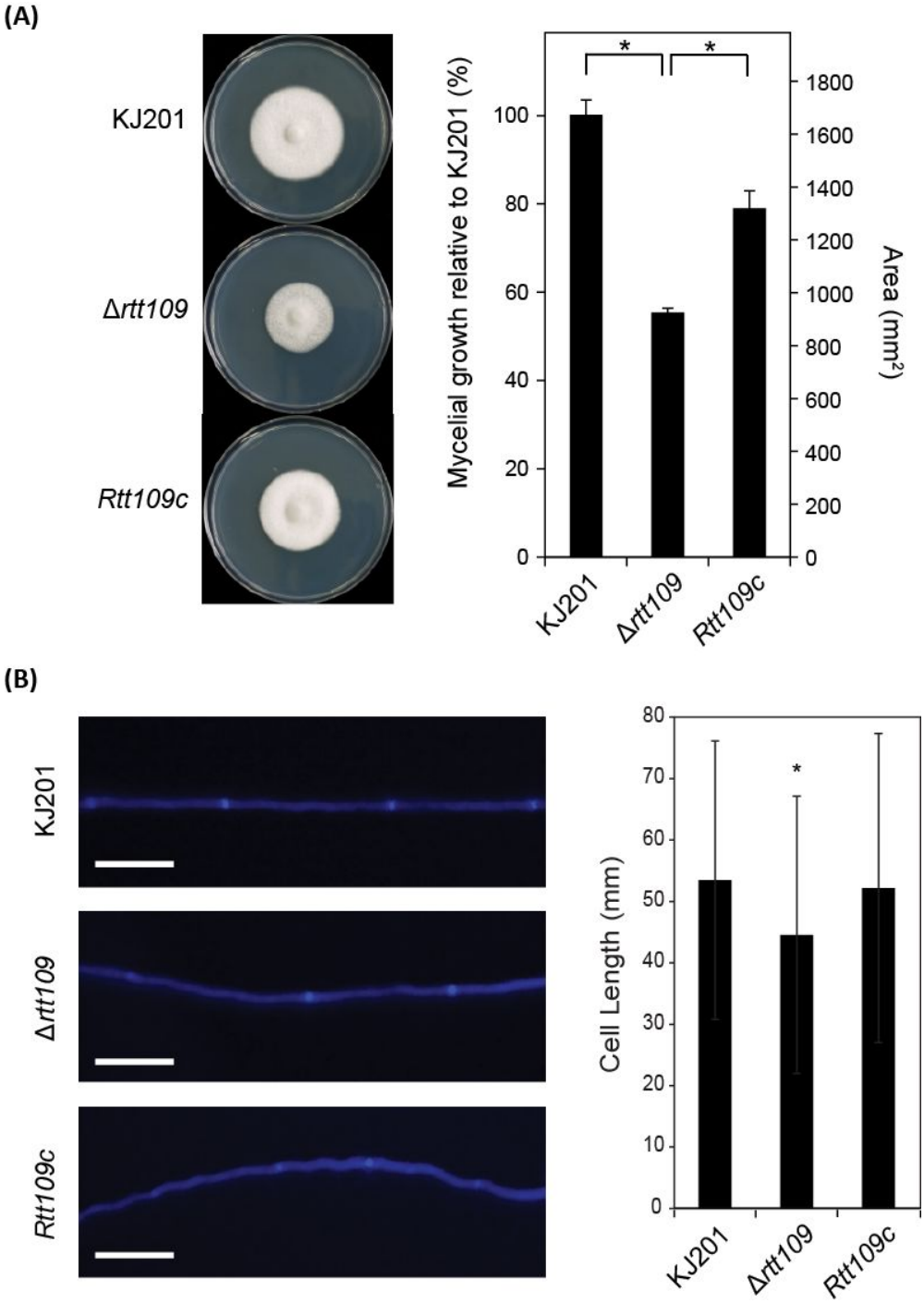

(A)
KJ201
Δrtt109
Rtt109c
Mycelial growth relative to KJ201 (%)
Area (mm²)
0
20
40
60
80
100
0
200
400
600
800
1000
1200
1400
1600
1800
*
*
KJ201
Δrtt109
Rtt109c
(B)
KJ201
Δrtt109
Rtt109c
Cell Length (mm)
0
10
20
30
40
50
60
70
80
*
KJ201
Δrtt109
Rtt109c

Figure 5. Hyphal and mycelial growth morphologies

(A) Mycelial growth on complete agar medium (CM) on day 7. Mutant growth is reduced to approximately 55% of the wildtype.

(B) Cell length measured as the distance between adjacent septa. Cell length and septation are significantly shorter in the mutant ($p<0.05$). Hyphae derived from conidia were stained with calcofluor white. Scale bar = 20μM.

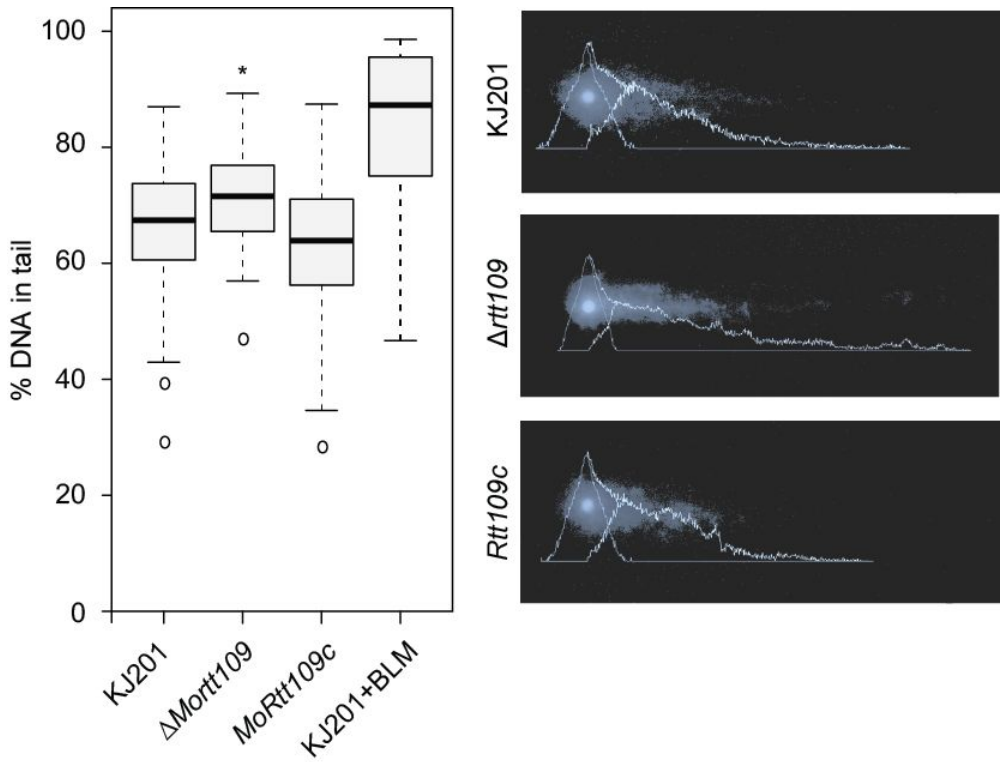


Figure 6. Endogenous DNA damage levels

Alkaline comet assay to detect level of double strand breaks (DSBs). Basal level of endogenous DNA damage is significantly elevated in Δ*rtt109* compared to KJ201 and *Rtt109c* ($p < 0.001$). KJ201 treated with 5mU BLM has been used as a positive control.

IV. Δ*rtt109* is hypersensitive to genotoxins and reactive oxygen species

There is evidence that the rice blast fungus experiences DNA damage throughout its lifecycle (Ndindeng et al., 2010) and must deal with endogenous (Egan et al., 2007, Ryder et al., 2013) and exogenous (Torres et al., 2006) ROS stresses. I questioned whether the tolerance to genotoxins and oxidative stress would be affected in the absence of Rtt109. Since it is difficult to test this in planta, I first tested the sensitivity of mycelia in vitro with four genotoxins, bleomycin (BLM), camptothecin (CPT), hydroxyurea (HU) and methyl methanesulphonate (MMS), and two ROS stressors, hydrogen peroxide (H_2O_2) and methyl viologen (MV). Sensitivity was inferred from radial growth area measurements on complete medium containing genotoxins or ROS stressors as a percentage of the growth on the untreated medium (Figure 7a). Δ*rtt109* displayed significantly greater sensitivity compared to the wild type in the presence of the four genotoxins and the ROS stressors tested. Mutant sensitivity to CPT and MMS was particularly pronounced, which was almost fully recovered by genetic complementation. Results have been less consistent with other stress media, but the mutant generally showed greater sensitivity. Unexpectedly on methyl viologen medium, the complemented strain showed extreme sensitivity (Figure 7b).

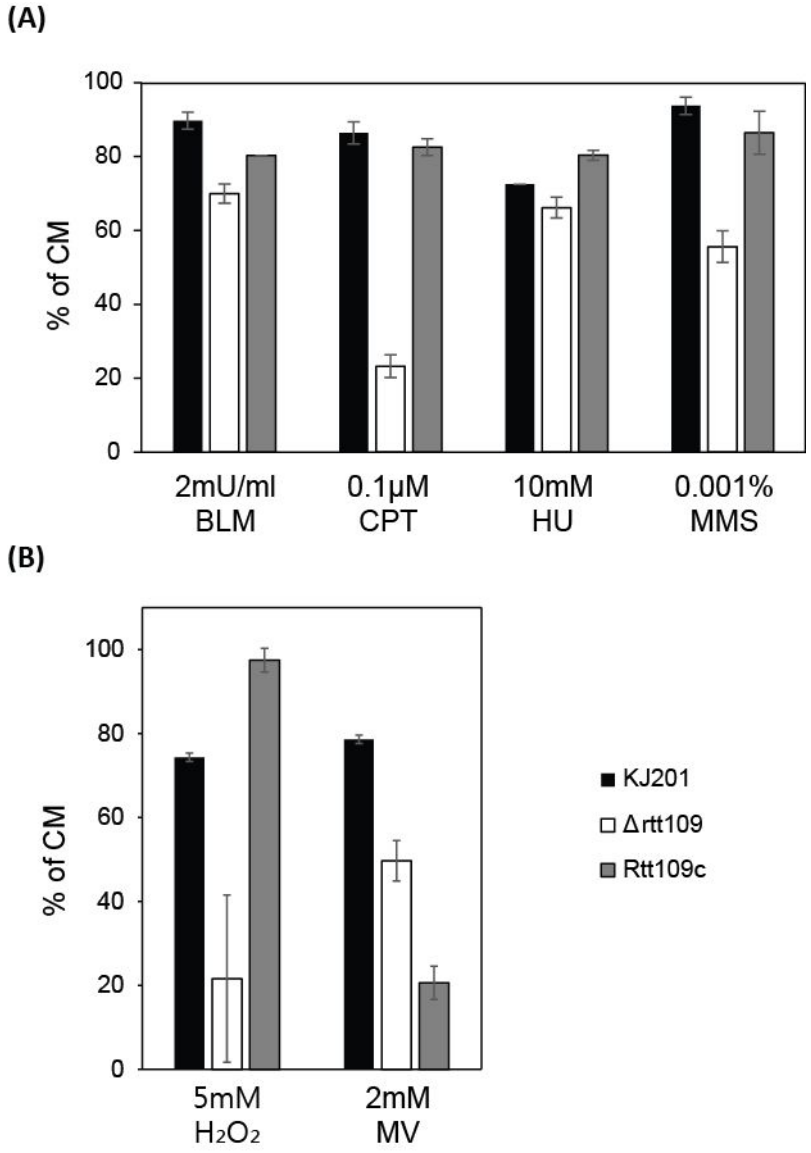


Figure 7. Mycelial sensitivity to genotoxins and ROS stressors

Radial growth area measured on CM agar supplemented with genotoxins (A) and ROS stressors (B) shown as a percentage of the respective strains grown on untreated CM. Δ*rtt109* is hypersensitive to genotoxins and ROS stressors compared to the wild-type.

V. Pathogenicity is compromised in Δ*rtt109*

Lesions produced by *rtt109*Δ are much smaller, being around 54% of the wild-type lesions in size (Figure 8). In the broadest sense, there could be two non-mutually exclusive reasons for this: delayed pre-penetration development and/or reduced invasive growth. As *M. oryzae* conidia must undergo a round of DNA replication and mitosis to form appressoria (Veneault-Fourrey et al., 2006, Saunders et al., 2010), I hypothesized that Δ*rtt109,* which is more prone to DNA damage and potentially cell cycle arrest, would have a higher proportion of germlings delayed in appressorium development. However, germination rate, appressorium initiation and maturation of Δ*rtt109* were unaffected (Figure 9a). Other than that, conidiation on V8 agar medium was decreased in the Δ*rtt109* mutant, perhaps due to reduced growth rate (Figure 9b).

Then, invasive growth was examined by rice sheath inoculation, where the majority of Δ*rtt109* invasive hyphae had only colonized the first cell at 48 hours post inoculation, while a greater proportion of the wild-type and *Rtt109c* hyphae had proceeded to adjacent cells and further (Figure 10). It seems highly plausible that the innate reduced growth rate of Δ*rtt109* as shown in axenic culture is accountable for the slow invasive growth during infection, although it cannot be ruled out that there are other external factors affecting the mutant growth additionally *in planta*.

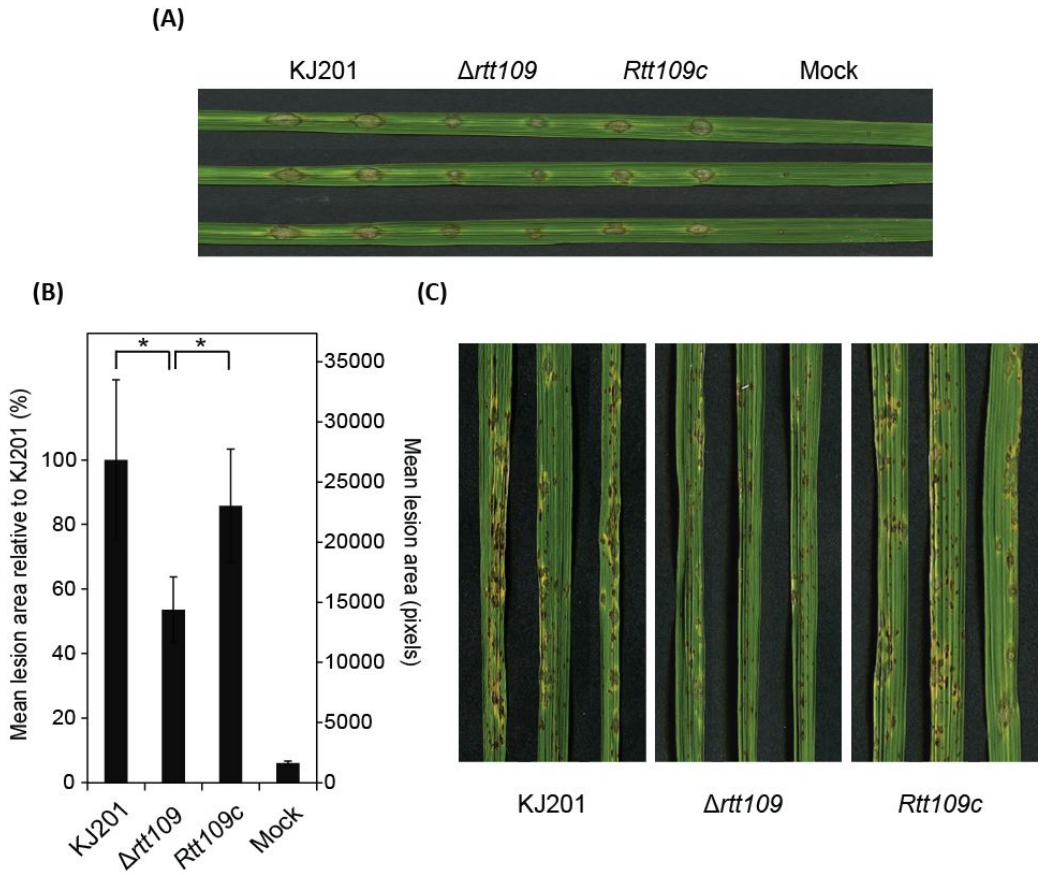
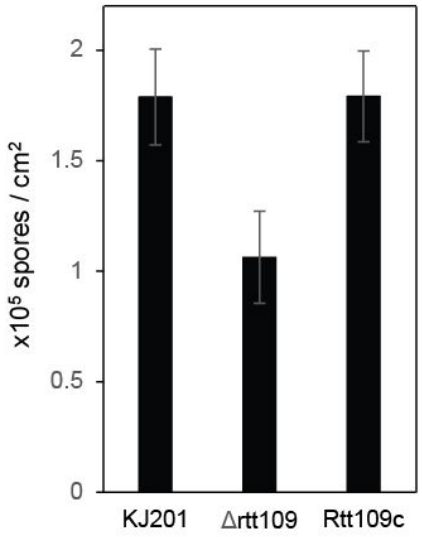


Figure 8. Pathogenicity phenotypes

(A) and (B) Wound-inoculated rice leaves on 5 dpi. Lesions produced by the Δ*rtt109* mutant are considerably smaller (~54%) compared to the wildtype lesions and pathogenicity is increased again in the complementation strain.

(C) Spray inoculated rice leaves on 7 dpi.

(A)


2
1.5
1
0.5
0
x10⁵ spores / cm²
KJ201
Δrtt109
Rtt109c

(B)

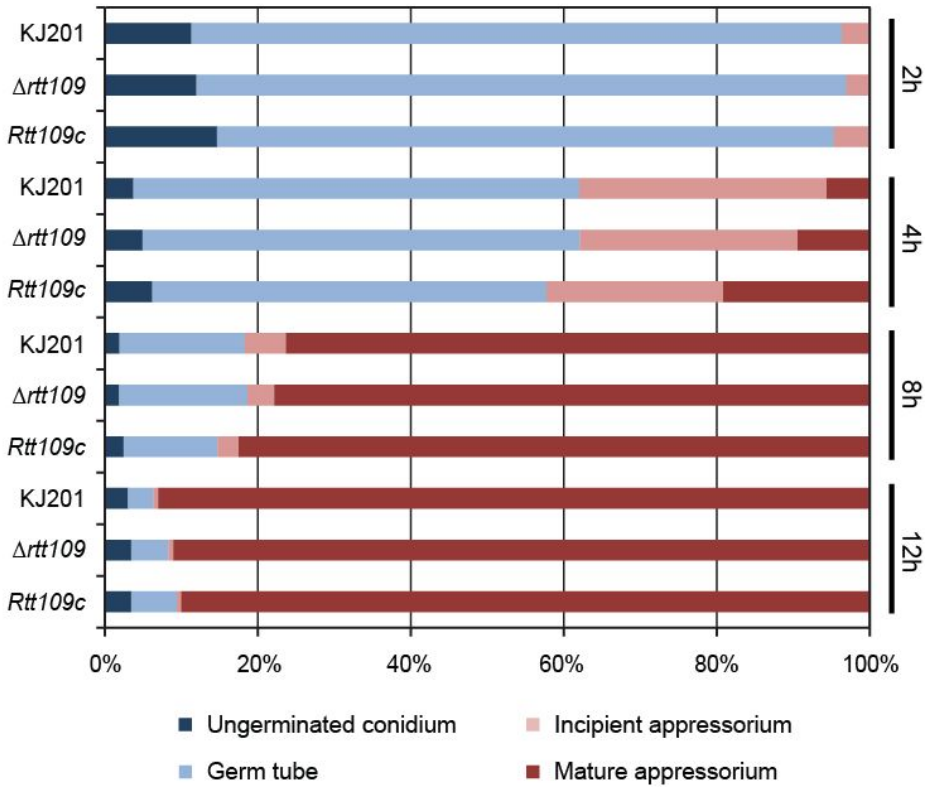

KJ201
Δrtt109
Rtt109c
KJ201
Δrtt109
Rtt109c
KJ201
Δrtt109
Rtt109c
KJ201
Δrtt109
Rtt109c
2h
4h
8h
12h
0%
20%
40%
60%
80%
100%
Ungerminated conidium
Germ tube
Incipient appressorium
Mature appressorium

Figure 9. Conidiation and pre-penetration development

(A) Conidiation density estimates from 7-day old colonies on V8 medium. Conidiation is significantly reduced in Δ*rtt109.*

(B) Progression of pre-penetration development. Germlings at four different developmental categories have been counted at 2, 4, 8 and 12 hours after being placed on hydrophobic coverslips (n=100 for each timepoint). The mutant is not delayed in pre-penetration development.

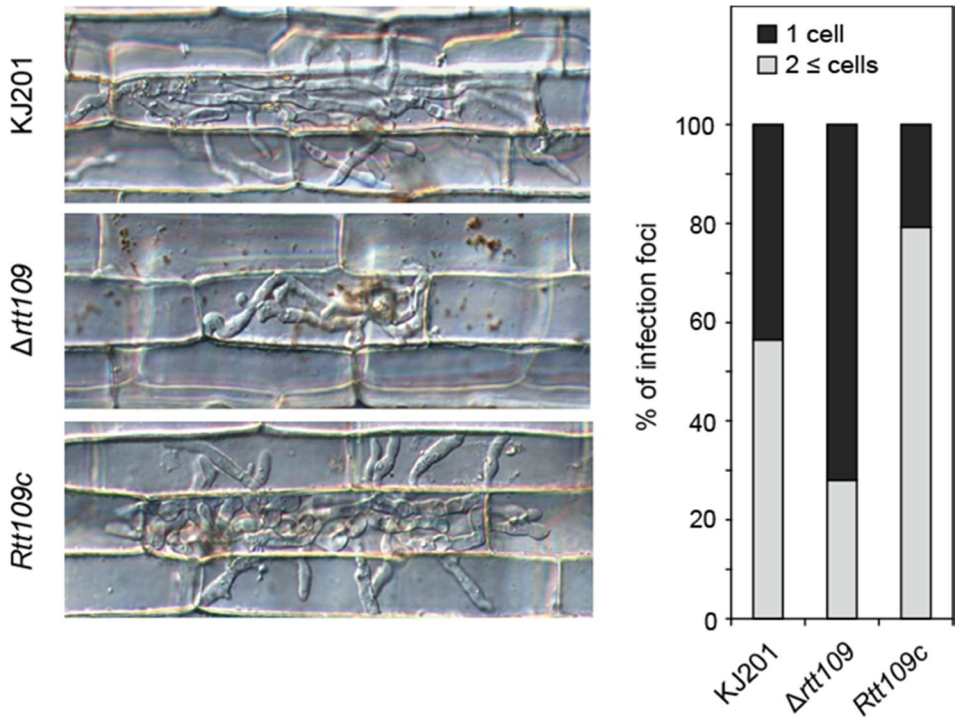


Figure 10. *In planta* invasive growth

Observation of invasive growth in rice sheath cells at 48 hours post inoculation. Mutant invasive growth is delayed but not restricted.

VI. Deletion of *Rtt109* leads to gross changes in expression of DNA metabolism and repair genes

There are two major DSB repair pathways: homologous recombination (HR) and non-homologous end joining (NHEJ) (Shrivastav et al., 2008). Since the level of DSBs was higher in Δ*rtt109*, an increase in expression of DSB repair genes was anticipated. Expression of some DSB repair genes previously studied in *M. oryzae* were checked by qRT-PCR (Figure 11) and re-confirmed by RNA sequencing (Table 3), all of which showed up-regulation in the Δ*rtt109* mutant. *Rhm51* (Ndindeng et al., 2010), *Rhm52* and *Rhm54* (Elegado et al., 2006) are Rad genes involved in homologous recombination and *MoLig4* (Kito et al., 2008) and *MoKu80* (Villalba et al., 2008) are DNA ligase IV and Ku80 orthologs responsible for NHEJ. Additionally, RNA sequencing revealed differential expression of 1641 genes between Δ*rtt109* and the wildtype, of which 908 were up-regulated and 733 were down-regulated. Notably, GO term analysis of the up-regulated genes, revealed enrichment of various DNA metabolism and repair related genes (Table 4) and down-regulated terms were found to be mostly associated with amino-acid metabolism and growth (Table 5).

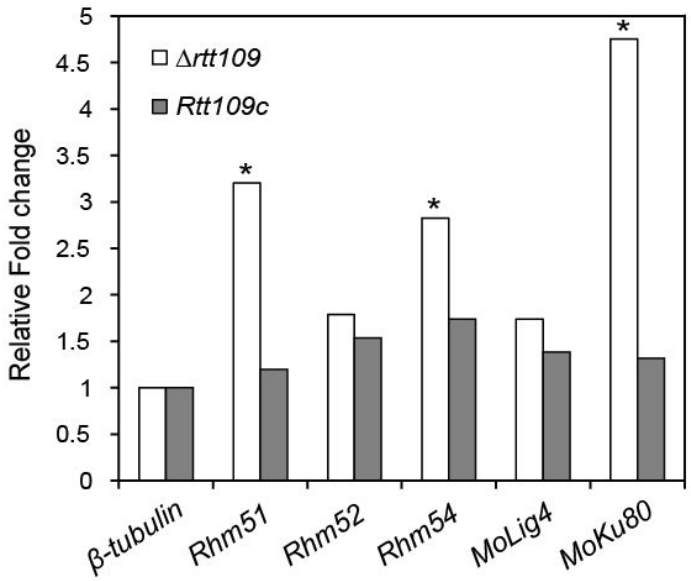


Figure 11. Expression of DNA double-strand break (DSB) repair genes

qRT-PCR analysis of homologous recombination and non-homologous end joining gene expression. Expression levels in the mutant (white bars) and the complementation strain (grey bars) are shown as fold change in relation to KJ201 (2^-ddct).

	Gene	qRT-PCR 2^-ddct	RNA-seq Log2(Δ*rtt109*/KJ201)
Homologous recombination (HR)	***Rhm51***	3.2	7.1
	Rhm52	1.8	2.3
	Rhm54	2.8	4.6
Non-homologous End Joining (NHEJ)	***MoLig4***	1.7	2.0
	MoKu80	4.8	10.4

Table 3. Expression of DNA double-strand break (DSB) repair genes

DSB repair gene up-regulation in the Δ*rtt109* mutant relative to the wildtype according to qRT-PCR and RNAseq results.

Table 4. Top 20 most significantly enriched GO Terms for up-regulated genes

	GO.ID	Terms	Annotated	Significant	Expected	ClassicFisher
1	GO:0006281	DNA repair	99	21	7.38	8.60E-06
2	GO:0006974	response to DNA damage stimulus	108	22	8.05	1.10E-05
3	GO:0006259	DNA metabolic process	194	32	14.46	1.30E-05
4	GO:0006303	double-strand break repair via non-homologous end joining	3	3	0.22	0.00041
5	GO:0003333	amino acid transmembrane transport	28	8	2.09	0.00073
6	GO:0033554	cellular response to stress	145	22	10.81	0.00096
7	GO:0006865	amino acid transport	30	8	2.24	0.0012
8	GO:0006950	response to stress	219	29	16.32	0.00153
9	GO:0000726	non-recombinational repair	4	3	0.3	0.00155
10	GO:0015849	organic acid transport	33	8	2.46	0.00234
11	GO:0046942	carboxylic acid transport	33	8	2.46	0.00234
12	GO:0044046	interaction with host via substance released outside of symbiont	69	12	5.14	0.00446
13	GO:0052047	interaction with other organism via secreted substance involved in symbiotic interaction	69	12	5.14	0.00446
14	GO:0052048	interaction with host via secreted substance involved in symbiotic interaction	69	12	5.14	0.00446
15	GO:0052051	interaction with host via protein secreted by type II secretion system	69	12	5.14	0.00446
16	GO:0052211	interaction with other organism via protein secreted by type II secretion system involved in symbiotic interaction	69	12	5.14	0.00446
17	GO:0051701	interaction with host	89	14	6.63	0.00566
18	GO:0044403	symbiosis, encompassing mutualism through parasitism	91	14	6.78	0.00691
19	GO:0044419	interspecies interaction between organisms	91	14	6.78	0.00691
20	GO:0006302	double-strand break repair	12	4	0.89	0.00929

Table 5. Top 20 most significantly enriched GO Terms for down-regulated genes

(B)	GO.ID	Term	Annotated	Significant	Expected	ClassicFisher
1	GO:0044710	single-organism metabolic process	1598	195	131.05	1.10E-11
2	GO:0043581	mycelium development	691	105	56.67	2.90E-11
3	GO:0007275	multicellular organismal development	696	105	57.08	4.50E-11
4	GO:0032501	multicellular organismal process	696	105	57.08	4.50E-11
5	GO:0044707	single-multicellular organism process	696	105	57.08	4.50E-11
6	GO:0044767	single-organism developmental process	743	108	60.93	2.30E-10
7	GO:0048856	anatomical structure development	737	107	60.44	3.10E-10
8	GO:0032502	developmental process	755	108	61.92	6.20E-10
9	GO:0055114	oxidation-reduction process	918	123	75.28	2.00E-09
10	GO:0043436	oxoacid metabolic process	280	53	22.96	2.90E-09
11	GO:0006082	organic acid metabolic process	283	53	23.21	4.30E-09
12	GO:0006520	cellular amino acid metabolic process	214	44	17.55	5.30E-09
13	GO:0019752	carboxylic acid metabolic process	273	51	22.39	9.50E-09
14	GO:0044281	small molecule metabolic process	667	89	54.7	8.40E-07
15	GO:1901566	organonitrogen compound biosynthetic process	251	43	20.58	1.80E-06
16	GO:0044283	small molecule biosynthetic process	180	34	14.76	2.50E-06
17	GO:0044711	single-organism biosynthetic process	188	35	15.42	2.50E-06
18	GO:1901605	alpha-amino acid metabolic process	124	26	10.17	5.60E-06
19	GO:0009081	branched-chain amino acid metabolic process	19	9	1.56	6.80E-06
20	GO:0009082	branched-chain amino acid biosynthetic process	15	8	1.23	7.40E-06

DISCUSSION

Presented in this thesis are the effects of Rtt109 on pathogenicity, mycological development and genome integrity of *Magnaporthe oryzae*. The most salient but unfortunately unoriginal finding is that in the rice blast fungus, Rtt109 is required for proper maintenance of genome integrity and resistance to genotoxic and ROS stresses, as previously established in yeast (Driscoll et al., 2007, Han et al., 2007a) and in other fungi (Han et al., 2007b, Lopes da Rosa et al., 2010, Kottom et al., 2011, Dahlin et al., 2014b). However, there are some curious results contrary to prior expectations that will be brought to attention. Otherwise, gene expression analysis offers some unanticipated leads which could complement the studies suggested for the future at the end of this section.

I. *M. oryzae* requires Rtt109 for genome stability and genotoxin resistance

Since Rtt109 is conserved across the fungal kingdom and functions in such a fundamental process as genome integrity maintenance, we can expect a similar set of phenotypic consequences from its deletion in different species. Although the precise enzymatic activity of Rtt109 in *M. oryzae* has not been tested here, the sequence and structural conservation (Figures 1 and 2) along with the genetics evidence (Figure 4) provide a convincing indication that it is a histone acetyltransferase targeting H3K56.

The most pronounced developmental phenotype of the *M. oryzae* Δ*rtt109* mutant is mycelial growth reduction (Figure 5a). As the mutant hyphal cell length is not significantly shorter than the wild-type cells, it can be inferred that delayed cell division but not cell elongation is accountable for growth reduction in Δ*rtt109* (Figure 5b). In the budding yeast, deletion of Rtt109 is known to result in reduced growth rate primarily due to increased frequency of double-strand breaks (DSBs) leading to G2/M arrest (Driscoll et al., 2007). *M. oryzae* Δ*rtt109* mycelia were indeed found to constitutively suffer a slightly elevated level of DSBs, as detected by neutral comet assay (Figure 6). Whether this damage directly leads to G2/M arrest and manifests as a growth defect has not been tested in this study. However, hypersensitivity of the mutant strain to the four genotoxic agents suggests that Rtt109 must play an integral role in DNA damage repair (Figure 7a). Thus the suboptimal DNA repair efficiency must at least be partially accountable for the growth reduction in the absence of Rtt109.

Of the genotoxins tested, bleomycin is radiomimetic, causing double and single strand breaks (Povirk, 1996). Camptothecin traps topoisomerase I on nicked DNA, which leads to double strand breaks after collision with transcription or replication machineries (Pommier et al., 2003). Hydroxyurea is an inhibitor of ribonucleotide reductase (RNR), depleting dNTP pools and stalling DNA replication forks (Koc et al., 2004). Methyl methanesulfonate is a alkylating agent, which causes base mispairing and replication blocks leading to both single and double strand breaks

(Lundin et al., 2005). The Δ*rtt109* mutant showed increased sensitivity to all four genotoxins tested but particularly to camptothecin and methyl methanesulphonate (Figure 7a). It is difficult to explain why the mutant is more sensitive to these genotoxins as they all ultimately result in double strand breaks and can have unknown pleiotropic effects.

II. Rtt109 is required for full tolerance to ROS but is dispensable for pre-penetration development and pathogenicity

Deletion of Rtt109 has also rendered *M. oryzae* sensitive to the oxidative stressors hydrogen peroxide and methyl viologen (Figure 7b). Hydrogen peroxide in the medium is an exogenous source of ROS, whereas methyl viologen raises endogenous ROS levels as well as upsetting other cellular processes (Fukushima et al., 2002). Regarding the pathogenic lifestyle of the blast fungus, this could have two implications: increased sensitivity to endogenous ROS generated during pre-penetration development (Egan et al., 2007, Ryder et al., 2013) and exogenous plant-derived ROS which may serve as a direct insult against the pathogen (Torres et al., 2006).

Pre-penetration development requires an endogenous ROS burst mediated by NADPH oxidases (Egan et al., 2007, Ryder et al., 2013), which could contribute to DNA damage (Cooke et al., 2003). Furthermore, initiation of appressorium development and maturation respectively requires passage through the S-phase and mitotic entry (Saunders et al., 2010). The lack of Rtt109 in yeast is known to

cause DSBs and consequently more frequent G2/M arrest (Driscoll et al., 2007). The *M. oryzae* Δ*rtt109* strain does suffer from an increased level of DNA damage (Figure 6). Therefore it was expected that a greater proportion of Δ*rtt109* germlings would be held up at G2/M checkpoint, and delayed in appressorium maturation.

However, delays in pre-penetration development could not be observed for Δ*rtt109* at the time points tested (Figure 9b). Moreover, the Rtt109c strain, which anomalously showed even greater sensitivity to intracellular stresses caused by methyl viologen but not to external hydrogen peroxide, displayed no delay in appressorium formation either. Overall, these results suggest that the differences in the degree of DNA damage or the extent of cell cycle delay, if any, is too little to be detected with the timepoints and the sample size tested (n=200 for each strain at each time point). Also, the level of endogenous ROS produced does not affect the progression of pre-penetration development in the Δ*rtt109* mutant.

It is possible that DNA damage is more easily bypassed during pre-penetration development because it is crucial that the fungus invades before the host recognises the threat and summons an insurmountable defence response. Another scenario is that mechanisms of prevention and/or repair of DNA damage are more active during pre-penetration development to compensate the lack of Rtt109. Expression analysis revealed significant upregulation of DNA repair-related genes in Δ*rtt109* mutant mycelia (Figure 10 & 11). Although DNA damage levels are still higher in the mutant, it is plausible that the increased DNA repair gene expression

allows normal infectious development in the mutant. It may as well be argued that the slight hindrance in cell cycle progression of Δ*rtt109* is more easily detectable in mycelial growth, where it is amplified through several rounds of cell division, while the single round of cell cycle required for appressorium formation is too short a time frame to observe a delay. However, mutant mycelial growth is disproportionately reduced (55-60% of the wildtype) for this to be the case (Figure 5a).

Another major expectation was that invasive growth of the Δ*rtt109* strain would be restricted if plant-derived ROS is truly toxic to the invading pathogen. Size of the lesions produced by Δ*rtt109* was significantly smaller than those produced by the wildtype and the complementation strain (Figure 8). Sheath inoculation also confirmed delayed colonisation by the mutant (Figure 10). However, it is difficult to distinguish the effect of exogenous stresses *in planta* from the innate growth defect of the mutant. Mutant mycelial growth and lesion size are around 55-60% and 54% of the wildtype, respectively, while the complementation strain grows to 70-80% and produces lesions to 86% of the wildtype (Figure 5 and 8). Lesions are the product of both fungal colonisation and plant responses, so they are not directly comparable to axenic mycelial growth. Nevertheless it is clear that ROS sensitivity of the mutant in axenic culture does not lead to efficient fungal growth restriction by the host plant.

It is highly debatable whether or not the rice blast fungus experiences oxidative

stress during pre-penetration development and invasive growth. On the one hand, deletion of some genes pertinent to antioxidant activity leads to reduced pathogenicity and even induces vigorous host defences. Examples include the mutants of glutathione peroxidase Hyr1 (Huang et al., 2011), redox-regulated transcription factors AP1 (Guo et al., 2011) and Swi6 (Qi et al., 2012) and the pathogenicity factor Des1 (Chi et al., 2009), that demonstrate a correlation between ROS sensitivity in culture and defects in plant colonisation. Furthermore, neutralisation of plant ROS by applying catalase or diphenylene iodonium (DPI), the NADPH oxidase inhibitor, enhances invasive growth and rescue the defects of these mutants (Tanabe et al., 2009, Chi et al., 2009).

On the other hand, a recent study has shown that the intracellular pool of glutathione, a major cytoplasmic antioxidant, remains mostly reduced throughout infectious development and invasive growth, despite transient, localised bursts of ROS (Samalova et al., 2013). Germlings were found to be highly tolerant to ROS *ex planta* and intriguingly, invasive hyphae maintained reduced glutathione pools regardless of host susceptibility or resistance (Samalova et al., 2013). Such evidence suggests that plant ROS is important for defence mobilisation but is not a direct threat to fungal vitality per se.

The results from this thesis also lend support to the notion that ROS sensitivity of the rice blast fungus on medium does not necessarily translate to reduction in pathogenicity and suggests that the fungus is unlikely to experience significant DNA

damage during interaction with the rice plant. The Δ*rtt109* mutant is clearly sensitive to DNA damage potentially caused by ROS, but the expression of ROS scavenging enzymes and antioxidant levels may be sufficient to tolerate the physiological concentrations of ROS during pre-penetration development and invasive growth.

III. DNA damage and repair in the rice blast fungus

Tools such as EGFP-Rhm51 (Ndindeng et al., 2010) and YFP-blasticin S deaminase (Arazoe et al., 2013) constructs have been developed for *in vivo* visualisation of DSBs and homologous recombination in *M. oryzae*. The fungus has been found to experience DSBs and undergo homologous recombination throughout its lifecycle (Arazoe et al., 2013). However it is technically challenging to test if DNA damage is suffered more frequently and repaired actively during pre-penetration development and invasive growth compared to when grown in axenic culture.

Of the numerous genes involved in various types of DNA repair, only those involved in DSB repair have been studied to date in the rice blast fungus. Mutants lacking *MoKu80* (Villalba et al., 2008) and *MoLig4* (Kito et al., 2008) of the NHEJ pathway are unaffected in both mycological development and pathogenicity. Loss of the *Rhm* (RAD homolog in *Magnaporthe*) series homologous recombination genes (Ndindeng, 2010), in contrast, leads to decreased mycelial growth, conidiation and formation of smaller lesions, which is reminiscent of Δ*rtt109* phenotypes.

Rtt109 is required for efficient NHEJ in yeast (Jessulat et al., 2008) and homologous recombination in *N. crassa* (Zhang et al., 2014). Despite upregulation of both NHEJ and homologous recombination genes (Figure 11), DNA damage and phenotypic defects resembling that in *Rhm* mutants persist in Δ*rtt109*. This suggests that homologous recombination defects of the Δ*rtt109* mutant may have greater phenotypic consequences than problems in NHEJ. Furthermore, the dispensability of the NHEJ genes (Kito et al., 2008) supports that homologous recombination may be the main repair pathway during the life cycle of *M. oryzae* (Ndindeng, 2010).

IV. Clues from the expression analysis

Deletion of Rtt109 was also found to lead to considerable changes in global gene expression. Among the up-regulated genes were DNA metabolism and repair genes as anticipated, but genes functioning in protein and organic acid transport across the membrane were unexpected (Table 4). Additionally, genes predicted to be involved in secondary metabolism such as the homologs of aflatoxin biosynthesis ketoreductase nor-1, several dehydrogenases and the aflatoxin efflux pump were among the most highly expressed genes. Secondary metabolism genes are usually clustered and heterochromatinised to keep silent until required (Brakhage, 2013). The derepression of these genes in Δ*rtt109* indicates a role of Rtt109 in suppressing their expression. It would be informative to map the chromosomal locations of some of these most highly up-regulated and down-regulated genes to see if there are certain genomic hotspots affected by the absence of Rtt109. Also, secondary

metabolite profiling of the Δ*rtt109* mutant may yield some interesting results. The down-regulated genes showed enrichment of GO terms in amino acid and vitamin biosynthesis as well as mycelial growth (Table 5). This might contribute to the growth defects of the mutant in addition to the elevated DNA damage levels.

V. Conclusion and suggestions for further research

Hypersensitivity of the Δ*rtt109* mutant to genotoxins and the increased level of DSBs make clear that Rtt109 is required for tolerance of DNA damage and genome integrity maintenance in *M. oryzae*. Phenotypic observations suggest that axenic and *in planta* hyphal growth defect of Δ*rtt109* may be due to delay in cell elongation and cell division. The delayed mutant growth *in planta* does not seem proportionate to ROS or genotoxin sensitivity, but rather, representative of the innate decreased growth rate of the mutant, which constitutively suffers a slightly higher level of DNA damage, even in the absence of exogenous stressors. However this hypothesis remains to be tested and it cannot be reconciled with the absence of delay in pre-penetration development.

So, we are left with the following questions: Is *M. oryzae* more prone to DNA damage under certain stages in its life cycle? If so, what are the measures against this? To what extent do the preventative or repair mechanisms contribute to virulence and drive pathogen evolution? It is technically difficult to answer these questions with the current *in vivo* tools available. However, it might still be possible

to check if there is more DSB damage in the Δ*rtt109* germlings either by Rhm51 foci counting (Ndindeng et al., 2010) or by western blotting γH2A levels. Furthermore, testing for homologous recombination and NHEJ frequency in the Δ*rtt109* mutant may also help to clarify the role of Rtt109 in DSB repair and associated recombinational activities that sometimes have far-reaching evolutionary consequences such as the deletion and translocations of Avr-Pita (Sone et al., 2013, Chuma et al., 2011).

In terms of Rtt109 roles in pathogen evolution and escaping R-gene mediated immunity, it would be very interesting to test for any changes in transposon and Avr gene transcript and copy number in the deletion mutant.

MAGGY is the best studied retrotransposon in *M. oryzae,* and the repressive effect of DNA methylation and RNAi on its retrotransposition has been described previously (Murata et al., 2007). The authors of these studies, however, proposed that DNA methylation and RNAi are not involved in the copy-number dependent suppression of MAGGY. We can speculate the existence of additional mechanisms involving repressors of Ty1 transpositions, known as the Rtt genes (Scholes et al., 2001). Deletion of Rtt109 is known to result in increased Ty1 copy number (Curcio et al., 2007) and overproliferation of repetitive sequences such as rDNA (Ide et al., 2013). Therefore increase in transposition activities could be tested in the Δ*rtt109* background and perhaps in combination with Dcl2 (Murata et al., 2007) or DMT1 deletions (Ikeda et al., 2013) to see the genetic interactions and crosstalk between

the cooperating silencing mechanisms.

Furthermore, there may be epigenetic phenomena such as derepression of infection-specific genes in axenically grown mycelia (Soyer et al., 2014), which could be tested by qRT-PCR. At the telomeres for example, incorporation of H3K56ac is required for the HDAC Sir2 to produce compact, silent heterochromatin by deacetylating lysine-56 (Xu et al., 2007). This suggests that in the absence of Rtt109, inefficient telomeric and subtelomeric heterochromatinisation may lead to derepression of subtelomeric pathogenicity genes.

Rtt109 may also play a role in small RNA biogenesis, as shown in a close relative, *Neurospora crassa* (Zhang et al., 2014). The role of small RNAs in filamentous fungi, let alone plant pathogens, is not well understood. Nevertheless, recent discoveries in filamentous plant pathogens revealed that small RNAs can act as effectors (Weiberg et al., 2013) and sRNA mediated silencing of Avr genes produces epiallelic variation, leading to gain of virulence strains whilst retaining Avr genes (Qutob et al., 2013). These examples show that RNA biology in plant pathogens is a new and exciting field, which should eventually be tied in with chromatin studies perhaps via Rtt109 and the like.

The overall impression is that Rtt109 influences and participates in various interlinked chromatin maintenance processes and genome plasticity mechanisms. There remains much to be discovered on this rather atypical histone

acetyltransferase and chromatin research in the rice blast fungus is still in its infancy. Nonetheless, it is a promising avenue of research and I hope that the leads provided here would contribute in whatever way possible to insightful future endeavours for a richer understanding of the genome information in the chromatin context.

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벼 도열병균 유전체 보전에 관여하는 히스톤 아세틸화 효소 Rtt109의 역할

초 록

곰팡이 특이적 히스톤 아세틸화 효소인 Rtt109는 다양한 크로마틴 기능에 관여하며 유전체 보전과 적응성에 영향을 준다. Rtt109는 특히 DNA 복제와 수선 과정에서 nucleosome 조립에 중요한 역할을 한다. 본 논문에서는 Rtt109를 통한 유전체 보전이 식물 병원체인 벼 도열병균 *Magnaporthe oryzae*의 병원성과 형태발달에 미치는 영향을 연구하였다. Rtt109 유전자 결손은 균사 생장의 감소를 일으키고 이에 따라 식물체내 침입 생장의 감소를 초래한다. Rtt109 삭제변이체에서 DNA 손상 수선 유전자와 스트레스 관련 유전자들의 발현이 증가한 것으로 보아, 균사 생장의 결함은 높게 유지되는 DNA 손상 수준과 관계가 있다고 판단된다. 또한 이 유전자의 결손은 활성 산소를 포함한 다양한 형태의 유전 독성 스트레스에 대한 과민성을 초래한다. 활성산소는 곰팡이에 형태발달과 식물 방어 반응에 필수적이라고 알려져 있다. 하지만 Rtt109에 의한 유전체 보전은 침입 전 발달 단계와 병원성에는 큰 영향을 주지 않는다. 이를 비추어 보아 벼 도열병균은 Rtt109가 없어도 침입과정 중 생성되는 활성

산소와 식물체 내의 스트레스에 영향을 받지 않는다고 추정된다. 결론적으로 Rtt109를 통한 유전체 보전은 벼 도열병균의 정상적인 균사생장과 유전 독성 저항성에 중요한 역할을 한다. Rtt109는 병원성에 직접적으로 관여하지는 않지만 유전체 조절과 진화적으로 의미 있는 기능을 가질 것으로 예상된다.

주요어 : 히스톤 아세틸화효소, 벼 도열병균

학 번 : 2013-21161