CHARACTERIZATION OF *LtLysM1* GENE IN *Lasiodiplodia theobromae* AND TAXONOMY AND PHYLOGENY OF SELECTED ASCOMYCETES

DULANJALEE LAKMALI HARISHCHANDRA

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ABSTRACT

The kingdom of fungi is one of the largest and the most diverse groups, with approximately 2.2–3.8 million introduced species and new species are being added every day to its roster. Due to its cosmopolitan distribution, many species are yet to be discovered in the fungal kingdom from their poorly explored habitats and a variety of biodiversity hotspots. The phylum Ascomycota, the largest in the fungal kingdom, consists about 19 accepted classes consisting of over 64000 different species. Among them, Dothideomycetes and Sordariomycetes are the most phylogenetically diverse classes with species from variety of lifestyles such as pathogenic, endophytic, saprobic and many more to discovered from terrestrial and aquatic habitats globally. Even though the past few decades have seen a substantial increase in research related to fungi due to their agricultural, biotechnological and pharmaceutical value, many species in both Dothideomycetes and Sordariomycetes are still very poorly understood and not studied comprehensively. These gaps in knowledge must be filled with more organized and wide-ranging studies to understand their diversity and potential.

The pathogenic potential of the fungi has been an important area of study due to the devastating economic losses that occur in food and crop production. Identification and characterization of these pathogens and understanding their disease causing potential and underlying mechanisms have become important step in disease

management in the recent years. Genomic studies are an important study area in understanding the pathogenic potential of fungi. Genome editing using the CRISPR/Cas9 system is one such approach used in the recent years. Even though this technology is fairly recent, many pathogens of economically important crops have been subjected to in-depth studies using this molecular tool. Herein, I describe the importance of this molecular tool in studying phytopathogenic fungal genomes and how they can be further extended for studies related to pathogenesis process and identification of key genes involved in the process. I also describe how this relatively cheaper, easier to use, and efficient genetic tool has opened many interesting applications for the future of genomic studies and pathogen control.

Botryosphaeriaceae, an important member of the Dothideomycetes, is a geographically diverse fungal group associated with a wide range of economically and environmentally important woody crops. Grape, one of the most economically important crops worldwide, is susceptible to pathogens from Botryosphaeriaceae. The last few decades have seen a substantial increase in comprehensive pathogen identification, but less studies were conducted to understand the molecular aspects related to the pathogenesis. Due to the increase in genomic and transcriptomic studies focused on botryosphaeriaceous pathogens in the recent years, the knowledge related to the genetic basis of pathogenicity has advanced significantly. In China, grape infecting botryosphaeriaceous pathogens such as *Botryosphaeria dothidea*, *Lasiodiplodia theobromae* and *Neofussicoccum parvum* have been reported to cause significant damage and economic losses. Among them, *Lasiodiplodia theobromae* has been recorded as the most virulent. The various comparative genome and transcriptome analyses have provided an insight into what type of genetic background allows these fungi to act as pathogens. Plants have a very sophisticated two-line defense system to face pathogen attacks. Pathogens have the ability to overcome these defenses by secreting proteins known as effectors. LysM motif containing effectors are one such group of secretory proteins that allow the pathogen to overcome plant defense systems and the colonization of the host. In the current study, I am focusing on one of the LysM

effectors, *LtLysM1,* of *Lasiodiplodia theobromae* capable of circumventing the plant defenses and aid in pathogenicity. LtLysM1 was confirmed as a secreted protein using the yeast signal peptide trap assay. The gene was cloned and over-expressed in *Lasiodiplodia theobromae*, resulting in an increase in the pathogenicity on *Vitis vinifera* and reduced pathogenicity was observed when RNA-inference approach. This indicated that the secreted LtLysM1 function as a critical virulence factor during the symptom development in woody plants.

The identification of fungal species has been an interesting and a highly debated topic due to their phenotypic plasticity and polyphyletic nature. Introducing species using both morphological and molecular data has been implemented in the recent years to provide intricate details on a large number of species. In this study, both of these approaches are used to in the investigation of ascomycetes. Fungal species isolated from various hosts from China and Italy were studied.

In addition to studying fungi collected from specific hosts or regions, this study was further expanded to include various new host records and new species from miscellaneous specimens collected from Italy. Species identification with phylogenetic approaches such as Maximum parsimony, Maximum likelihood and Bayesian posterior probabilities using various combinations of gene regions were used in these studies. Appropriate illustrations and descriptions were provided as evidence for accurate identification.

Keywords: LysM, Effectors, Grape, Pathogenicity, Fungal genomes, CRISPR/Cas9

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

Figure **Page**

CHAPTER 1

INTRODUCTION

1.1 Botryosphaeriaceae Pathogens Associated with Grape

Grape is one of the most economically and agriculturally important fruit crops rich in vitamins, minerals and dietary fibers (Food and Agriculture Organization, 2009). Even though grape is largely cultivated for consumption as wines or table grapes, secondary products such as jams, juice, jellies and vinegar are also produced in some areas of the world. European countries contain the highest ground cover for grapes, however, in the recent years, China has emerged as the highest grape producer in the world (International Organization of Vine and Wine, 2018). The loss in grape production can be due to climatic changes, mishandling of harvest or due to diseases (Chethana et al., 2017; Dissanayake et al., 2018; Jayawardena et al., 2018; Manawasinghe et al., 2019). Grapevines are affected with diseases caused by bacteria, fungi, nematodes and viruses (Wilcox, Gubler & Uyemoto, 2015). But among them, fungal diseases are the uppermost concern among grape growers.

The weather conditions in Chinese grape growing areas such as rainy summers and warmer humid climates, provides a suitable habitat for disease causing fungi (Cortesi, 2015). Many devastating fungal affecting grapes such as anthracnose caused by *Elsinoe* diseases *ampelina*, dieback caused by several species in the Botryosphaeriaceae family and the *Diaporthae* species complex, ripe rot, white rot have been reported from Chinese vineyards and has caused a significant economics loss to grape cultivation and production. Correct identification and the vulnerability of the grape cultivar combined with proper agricultural practices are important for the management of these diseases (Wilcox, Gubler & Uyemoto, 2015). In China, the research areas related to the identification and characterization of fungal diseases affecting grape are mostly based

on morphological data. Therefore, studies to identify these diseases in much deeper level would be beneficial for the proper management.

Fungi from the family Botryosphaeriaceae has received attention in the recent years for their ability to cause diseases in many economically important crops including grapes (Denman et al., 2000; Phillips et al., 2013; Yan et al., 2013). Disease symptoms such as cankers and diebacks in grape were prominently identified to be caused by *Botryosphaeria dothidea*, *Lasiodiplodia theobromae* and *Neofussicoccum parvum* in Chinese vineyards (Yan et al., 2013). Even though *Botryosphaeria dothidea* was the most commonly identified pathogen, *Lasiodiplodia theobromae* was identified as the most virulent (Yan et al., 2013, 2018).

The recent decades have shown a significant growth in molecular studies about Botryosphaeriaceae mostly related to the identification and characterization using molecular phylogeny (Denman et al., 2000; Phillips et al., 2013). Understanding the underlying mechanisms related to pathogenicity of these species can be important for implementing control measures. Studies pertaining to pathogenicity related genes and infection mechanisms of these Botryosphaeriaceae pathogens are still not a very common area of research. Thus, they remain poorly understood and contested.

1.1.1 Molecular Studies Related to the Identification and Characterization of Phytopathogenic Fungi

The management of most plant diseases can be relatively easy if their pathogenicity and host resistance is properly understood. Fungi employ a wide array of unique mechanisms governed by pathogenicity genes to colonize and ensure disease establishment in plant hosts (Doehlemann et al., 2017). Pathogenicity genes are defined as genes that are not essential for the completion of the lifecycle but are essential for the disease development (Van De Wouw & Howlett, 2011). These genes cover all aspects of pathogenesis, including structure formation, cell wall degradation, and responding to plant defenses by producing secondary metabolites or toxins.

Functional genomic studies provide the foundation for understanding the genes related to the fungal pathogenesis. Next generation sequencing has advanced the number of fully sequenced pathogenic fungal genomes in the last decade (Hu, 2013; Aylward et al., 2017). Functional annotation of these genomes has provided data on a

plethora of pathogenicity related genes involved in various functions to facilitate host colonization (Morales-Cruz et al., 2015; Paolinelli-Alfonso et al., 2016; Quinlan et al., 2011; Yan et al., 2018). The virulence of the pathogens and the pathways important for their pathogenicity can be understood properly through transcriptomic studies and comparative genome analyses (Morales-Cruz et al., 2015; Paolinelli-Alfonso et al., 2016; Yan et al., 2018). Another generally used method for analyzing pathogenicity genes is proteome and secretome analyses. The protein expressed under certain settings such as different temperatures, pHs and other conditions can be used to evaluate the gene expression inducers and triggers.

These genomic and transcriptomic data can be used to study pathogenesis related genes in both random and targeted manner. Gene knockdowns using RNAi (RNA interference), conventional knockout techniques using homologous recombination, and artificial nucleases with genome modifying or editing ability have been applied in many instances to elucidate the function of a particular gene of interest (Meyer, 2008; Sarkari et al., 2017).

During the microbe-plant interactions, plants have evolved a two-fold defense response. The first layer provides basal responses upon the recognition of pathogen-associated molecular patterns (PAMPs) by plant pattern recognition receptors (PRRs) that activates PAMP-triggered immunity (PTI) (Jones & Dangl, 2006; Macho & Zipfel, 2014; Stergiopoulos & de Wit, 2009). This layer of responses is associated with callose deposition, lignin formation, phenolic compounds deposition, stomata closing, generation of extracellular reactive oxygen species, and a serious of downstream signal transduction events (Han & Kahmann, 2019; Zipfel, 2014). To establish compatible interactions for successful proliferation inside host tissues, microbial pathogens secrete an arsenal of effectors to overcome PTI response. Although pathogens secrete substantial effectors to subvert PTI, plants have evolved a surveillance system to recognize these effector proteins to trigger well known effector-triggered immunity, which includes by hypersensitive cell death and defense-gene activation (Han & Kahmann, 2019; Jones & Dangl, 2006; Selin et al., 2016; Thomma et al., 2011). During past decades, many typical PAMPs including bacterial flagellar peptide flg22, EF-Tu peptide, lipopolysaccharide, peptidoglycan, fungal cell wall chitin, xylanase, and hundreds of effectors such as RxLR, CRN, CFEM, or LysM motif-containing proteins

have been identified and characterized (Han & Kahmann, 2019; Liu et al., 2019; Newman et al., 2013; Thomma et al., 2011; Win et al., 2012).

1.1.2 Identification and Characterization of Pathogenicity Genes in *Lasiodiplodia theobromae*

The comparative genome and transcriptome study conducted by Yan et al. (2018) provided the genomic data on putative pathogenicity genes in the *Lasiodiplodia theobromae* isolated from Chinese grapevines. In this, six LysM domain-containing proteins in *L. theobromae* were identified, among which three were predicted to be functional effector proteins.

The LysM motif is widely found in various proteins such as chitinases, peptidases, receptor-like kinases and effector proteins (Akcapinar et al., 2015; Liu et al., 2019). LysM effector proteins through binding to fungal cell wall chitin, were reported to be able to either protect hyphae from being degraded by plant chitinases, or through binding to the chitinases inhibit the activity of plant chitinase, or prevent plants from recognizing GlcNAc oligomers to avoid plant defense responses (Kombrink & Thomma, 2013; Rovenich et al., 2016). In the current study we provide a comprehensive characterization of LysM effector and its importance in the pathogenicity of *Lasiodiplodia theobromae.* The results obtained in this study can provide a foundation for implementing disease management strategies such as targeted and effective fungicides.

1.2 Importance of Taxonomic Contributions to Ascomycota

Ascomycota is the largest phylum in the Fungal kingdom. In Ascomycota, the highest diversity is observed form class Dothideomycetes and Sordariomycetes. They can be found in almost all ecological niches around the world (Hyde et al., 2013; Maharachchikumbura et al., 2015). With the increase in the available molecular data in the last two decades, these two classes have been extensively studied and has undergone numerous revisions (Hyde et al., 2013; Wijayawardene et al., 2017).

Dothideomycetes are characterized by their bitunicate asci developed in the ascolocular ascoma (Barr, 1979; Barr & Huhndorf, 2001; Eriksson, 1981; Hyde et al.,

2013; Luttrell, 1955). Sordariomycetes are characterized by their perithecial ascomata, paraphysate hamathecium and unitunicate or pseudoprotunicate asci (Eriksson & Winka, 1997). Due the cosmopolitan distribution and their economical importance, it is important to study, identify and characterize these fungi for ecosystem stability and plant, human and animal health.

Accurate identification and the correct taxonomic placement of fungi are the most important foundation in any research aspect related to mycology. This can be achieved only with the combination of both morphological and molecular phylogenetic approaches as employed in the current study of selected micro-fungi.

1.3 Research Objectives

1.3.1 To identify and characterize genes involved in the pathogenesis of *Lasiodiplodia theobromae*.

1.3.2 To resolve the taxonomy and molecular phylogeny of Ascomycetes

1.4 Research Contents

This thesis is divided into eight chapters.

Chapter 1 is the general introduction, which briefly discusses the genomic studies related to Botryosphaeriaceae with emphasis on studies conducted on Lasiodiplodia theobromae. This chapter also provides background information on the taxonomy and phylogeny of Ascomycota, the objectives of this research and the outline of the thesis.

Chapter 2 discusses on the CRISPR/Cas9 system, which is a contemporary molecular tool used for efficient genome editing in phytopathogenic fungi.

Chapter 3 provides details on the LysM domin-containing protein LtLysM1 of *Lasiodiplodia theobromae.* In this study, the gene, *LtLysM1* was cloned and subjected to various functional genomic experiments to determine its role on the pathogenesis of *L. theobromae*.

Chapter 4 provides the contributions to the taxonomy and phylogeny of ascomycetes.

Chapter 5 provides the overall conclusion of above-mentioned studies and suggestions for future work.

CHAPTER 2

CRISPR/Cas9: CONTEMPORARY DESIGNER NUCLEASES FOR EFFICIENT GENOME EDITING IN PHYTOPATHOGENIC FUNGI[1]

Abstract

Plant diseases caused by fungal pathogens are one of the main factors contributing to severe economic losses due to reductions in yield and the quality of crops. Studying the fungal genes related to pathogenicity to reveal their infection mechanism through genome editing can play an important role in the management of these diseases. The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated nuclease 9 (Cas9) system is a versatile tool for genome engineering which has recently been adopted for sequence specific regulation of gene expression in many plant pathogenic fungal genomes. It is the current scientific consensus point of view that this simple RNA guided genome editing tool is cheaper, easier to use, and is higher in gene modification efficiency than any other available gene editing tool. In this mini review, we discuss the molecular mechanisms underlying the CRISPR/Cas9 technique and its recent improvements and applications beyond gene editing. We discuss and summarize a few recent studies targeting phytopathogenic fungal genomes, potential applications, the remaining challenges, and future

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perspectives. Our analysis provides insights into how this method can be more widely applied to combat fungal phytopathogens.

Key words: DNA Repair, Fungal Genomes, Nucleases, Targeted Mutations

2.1 Introduction

Plant pathogenic fungi are among one of the most diverse and economically relevant threats concerning plant diseases (Borrelli et al., 2018). Many agriculturally important staple food crops such as maize, rice, wheat and economically important crops such as grape, tea, and many more face significant losses annually due to the devastating effects of fungal diseases (Gramaje & Armengol, 2011; Yan et al., 2013; Nalley et al., 2016; Thompson & Raizada, 2018). Fungi such as *Blumeria graminis*, *Botrytis cinerea*, *Fusarium graminearum*, and *Pyricularia oryzae* are few examples of fungal pathogens considered to be some of the most important plant pathogens in agriculture (Dean et al., 2012). The emergence of new and more aggressive fungal pathogens has increased substantially since the early 2000s (Fisher et al., 2012), thus developing new control strategies for these diseases have become a relevant issue more than ever.

Management of most plant diseases can be easily done if the pathogenicity of the fungus and the host resistance are understood. Fungi employ a wide array of unique mechanisms to colonize a specific plant host to ensure disease establishment and development (Doehlemann et al., 2017). Plant pathology related research has advanced substantially due to the integration of molecular techniques in understanding pathogenesis (McCartney et al., 2003). The rapid development of techniques such as microscopy, DNA, RNA, and protein sequencing combined with bioinformatics has revolutionized the aspects of pathogen detection, understanding disease progression, and has guided new strategies for improving disease resistance (Soanes et al., 2007; Wang & Jin, 2017). In order to develop new resistance strategies, in depth understanding of the molecular basis of host pathogen interaction can be very important. Regardless of the improvement in molecular research related to the understanding of plant pathogenic interactions, much more crucial and practically utilizable information remains to be discovered. The technological advancements involving genome sequencing have provided a much-needed platform to bridge this knowledge gap.

Owing to the increase in affordable techniques for whole genome sequencing during the past decade, the number of fungal species with complete genome sequences

has significantly increased (Hu, 2013). Many independent studies, as well as combined initiatives, have produced whole genome sequences for many fungal species. More than 1500 fungal genomes have been completely sequenced up to now, which is more than plant and animal genomes combined (SOWF: Leitch et al., 2018). The database of the broad institute alone records more than 100 sequenced genomes of different types of fungi including model organisms, human pathogens, and phytopathogens (Broad institute, 2019). The convergence of this available wealth of genetic information into functionally and clinically relevant knowledge has always been a major challenge faced by researchers in many fields. Thus, establishing efficient and reliable methods to determine the molecular mechanisms of genes responsible for the relevant and specific phenotypes has become a focal point in molecular research. The best approach in determining the function of a gene is to either shut it down or overexpress it within a living organism; however, this can be very tedious and time consuming (Alberts et al., 2002).

Characterization of genotypic and phenotypic relationships using the loss of function approach is considered the best course of action by molecular biologists for decades (Alberts et al., 2002). Gene knockdowns using RNAi (RNA interference), conventional knockout techniques using homologous recombination, and artificial nucleases with genome modifying or editing ability have been applied in many instances to elucidate the function of a particular gene of interest (Meyer, 2008; Sarkari et al., 2017). Conventional knockout methods using homologous recombination and RNAi has been in the frontier of functional genomics as tools that can silence a gene in order to obtain insights into the gene's function (Wang et al., 2017). Homologous recombination was reported to be variably successful and was very cumbersome to perform and gene silencing with RNAi provided a comparatively better alternative (Sen & Blau, 2006). Though these techniques were used to elucidate the gene function, the occurrence of false negative results due to inefficient knockdown of the gene, false positive results obtained due to unintentional gene silencing in off targets, instability of hairpin loops within the target organisms, and the occurrence of hypomorphic phenotypes not mirroring the complete loss of the function of a gene, limited their use (Torres Martínez & Ruiz-Vázquez, 2017). Furthermore, RNAi was reported to be absent in some fungi due to the complete loss of RNAi component related genes during

evolution (Drinnenberg et al., 2011; Nicolás et al., 2013). Therefore, using this system for gene knockdown was not possible for some fungal species. Hence, the search for a better tool remained an issue to overcome these limitations.

To overcome the limitations of conventional gene knockout techniques and RNAi, a more efficient approach known as 'genome editing' came into play (Cox et al,. 2015). This approach used various artificial nuclease systems to induce changes in the target genomes. While RNAi technology was only capable of regulating post-transcriptional gene expression, the artificial nucleases with genome editing capabilities introduce more permanent changes that can be passed onto the next generation (Boettcher & McManus, 2015). Recent studies on functional genomics, gene therapy and transgenic organisms show successful instances in which genome editing was implemented (Urnov et al., 2010; Wood et al., 2011; Reyon et al., 2012; Friedland et al., 2013). By incorporating engineered, programmable and highly specific nucleases, a cellular organism's genome could be edited by introducing a modification into the site of choice at a predetermined locus via insertion, replacement, or by permanent disruption of the gene. The nucleases induce site specific changes in the genome through sequence specific DNA binding domains and nonspecific DNA cleavage domains. Insertions, deletions, and substitutions at the loci of interest are generated by different cellular DNA repair processes (Arazoe et al., 2015a). These changes in genes could lead to modifications of the gene function, creating single nucleotide polymorphisms or disease variants and fusion proteins. For example, through the manipulation of candidate virulence genes in phytopathogenic fungi, the gene's relevance to disease development can be determined using these engineered artificial nucleases.

In genome editing, the reprogrammable recognition site is the most important feature in artificial nucleases (Chandrasegaran & Carroll, 2016). Zinc Finger Nucleases (ZFNs) (Weinthal et al., 2010; Carroll, 2011), transcription activator like effector nucleases (TALENs) (Arazoe et al., 2015b), and RNA guided nucleases (RGNs) in CRISPR/Cas system are being used as genome editing tools (Gaj et al., 2013). Both ZFNs and TALENs function through protein DNA interactions with the use of the modified restriction enzyme Fok I, where the nuclease domain of the enzyme is fused with the DNA binding domains of the transcription factors. Even though *Fok I* is programmable and site specific, targeting a new site requires engineering and cloning

a new protein which limits its use to one time and excludes being used for high throughput applications. The CRISPR/Cas system has gained more favor from researchers (Wu et al., 2014a) because it depends on a small RNA, not DNA, which leads to its specificity (Ran et al., 2013). The CRISPR/Cas9 system can be used to target multiple genes by changing the target single guide RNA (sgRNA) sequences, unlike ZFNs and TALENs that use protein guided DNA cleavage systems and can target only one gene at a time (Gaj et al., 2013).

The presence of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) sequence was first discovered in 1987 by Yoshizumi Ishino while working on DNA ligase of *Escherichia coli* (Ishino et al., 1987). However, the experimental evidence of its function as an adaptive immune system against viruses and phages was not discovered until 2007 by Rodolphe Barrangou (Barrangou et al., 2007). This discovery led to the first biotechnological breakthrough, that naturally occurring CRISPR/Cas systems could be used for immunization against phages (Barrangou & Horvath, 2012). In 2008, the DNA targeting ability of CRISPR/Cas of *Staphylococcus epidermidis* was reported (Marraffini & Sontheimer, 2008), providing evidence that this system can be found in different bacterial species. This gave way to discover different types of Cas enzymes with varying targeting abilities to widen the horizons of potential applications using CRISPR/Cas as a gene editing tool. The guiding ability of Cas9 by mature crRNA (CRISPR RNA) in *E. coli* against virus proliferation was identified in 2012 (Jinek et al., 2012). This discovery opened the gateway for the potential use of CRISPR/Cas9 for gene targeting and genome editing applications. The first report on the use of the CRISPR/Cas9 system for genome editing of fungi was established in 2013 on Saccharomyces cerevisiae (DiCarlo et al., 2013). Subsequently, stable CRISPR/Cas9 systems were established on Trichoderma reesei (Liu et al., 2015) and in several *Aspergillus* species (Nødvig et al., 2015). Since then, the CRISPR/Cas9 system has been successfully implemented for the manipulation of many fungal genomes, and many similar projects are underway for different species of fungi.

Here, we review the molecular mechanism of the CRISPR/Cas9 system, discuss phytopathogenic fungal genomes edited using the CRISPR/Cas9 system and its applications, and finally consider the challenges in manipulating phytopathogenic fungal genomes. The objective of this chapter is to provide a summarized overview of

all up to date information and experiments related to the use of CRISPR/Cas9 for genome editing in plant pathogenic fungi and to provide a one stop information compilation for anyone interested in at a glance understanding of this robust molecular tool.

2.2 The Native CRISPR/Cas System- The Prokaryotic Immune System

The CRISPR/Cas9 system works as an acquired immune system in prokaryotes by acting against any invasive foreign genetic elements from viruses or phages. Cas (CRISPR associated) genes coding for polymerases, nucleases, and helicases are an integral part of the bacterial adaptive immune system (Rath et al., 2015). The CRISPR locus is made of a conserved nucleotide sequence that is periodically repeated throughout the prokaryotic genome. During the initial invasion by the virus or phage, a small nucleotide sequence of the virus is processed by the nucleases of the CRISPR system, and these short nucleotide sequences are integrated to the CRISPR locus as spacers that are positioned between two repeats of the conserved sequence. The spacers act as transcriptional templates and create crRNA. This chimeric CRISPR array interacts with an auxiliary trans activating CRISPR RNA (tracrRNA) (Deltcheva et al., 2011; Chylinski et al., 2013), forming a duplex RNA known as guide RNA (gRNA) that in turn guides the Cas nuclease to cleave the genome of invading viruses or phages. Another small DNA sequence known as the Protospacer Adjacent Motif (PAM), present within the target DNA sequence, facilitates the specific targeting of the Cas nucleases. The PAM sequence is a short DNA sequence about three to five nucleotides in length. The presence of the PAM sequence is a strict requirement for Cas mediated nucleotide cleavage (Karvelis et al., 2015). Cas nucleases contain two domains, RuvC and HNH that cut the PAM containing strand and its complementary strand, respectively, to produce a double stranded break (DSB) (Chen et al., 2014) in the DNA of the invading bacteriophages or plasmids (Figure 1.1). The presence of both domains in the Cas9 enzyme is important for a double stranded break. CRISPR/Cas system can be classified into two major classes based on the components and the mechanism of action (Makarova et al., 2018). The class one system (type I, III and IV) requires several

complex effector proteins for functioning while in the class two system (type II and putative types V and VI); only one RNA guided nuclease is required to cleave the genetic material of the invading pathogen (Chylinski et al., 2014). Through the observations made on the mechanism of the native CRISPR/Cas9, researchers working on different aspects of this system collectively contributed to producing a feasible genome editing tool from the different components (Lander, 2016).

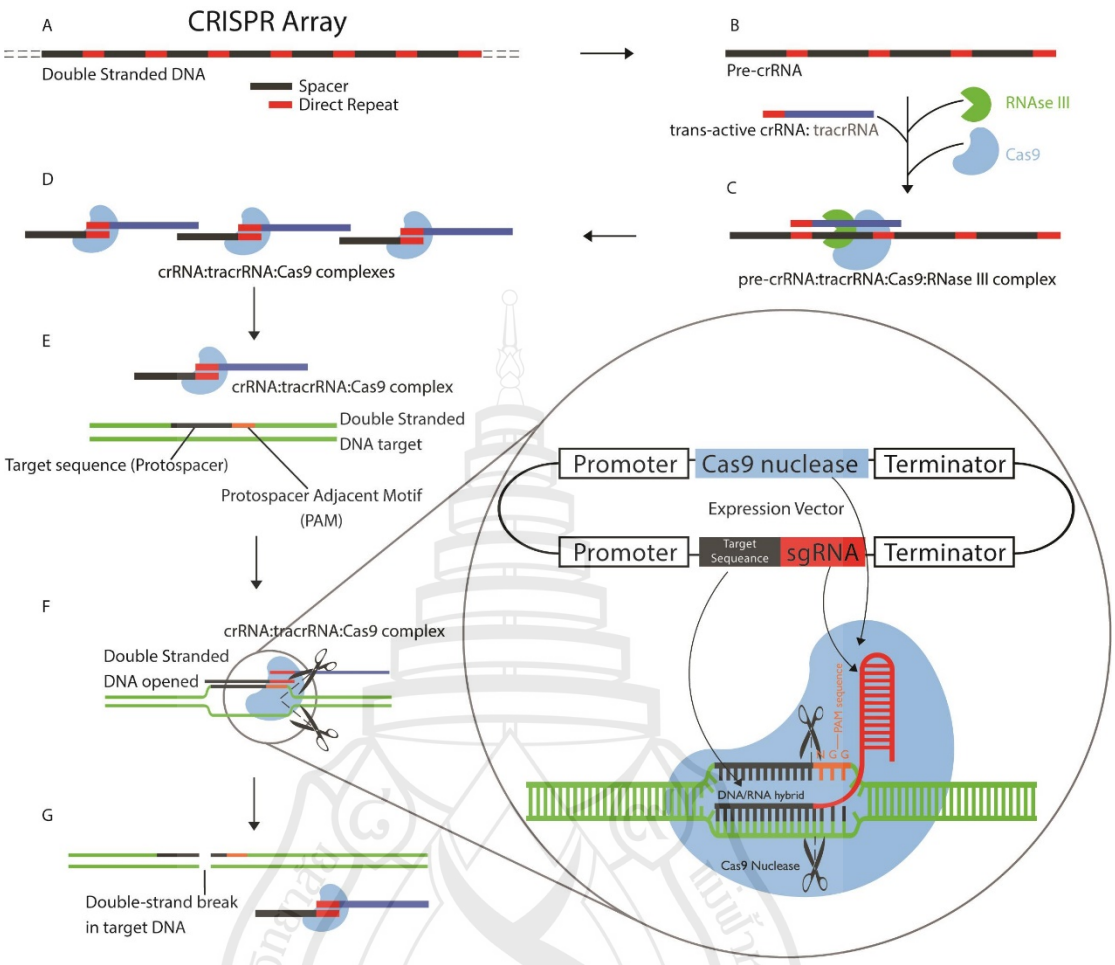
2.3 Repurposing of the CRISPR/Cas9 System for Genome Engineering

Genome manipulation of higher eukaryotic organisms such as mammals, plants, and fungi is reported to be considerably difficult compared to other simpler organisms (Doench et al., 2014). The main reason for this is that many eukaryotic organisms such as filamentous fungi contain complex genomes that are diploid or polypoid (Wang & Coleman, 2019). The edited genomes of these higher eukaryotes in many instances can produce reduced expression of the edited gene rather than producing a complete knockout of the function. Additionally, the transformation of higher eukaryotic cells is considerably more difficult compared with that of prokaryotic organisms due to their complex cellular organization. With the understanding of the biological function and the mechanism of the CRISPR/Cas9 system, the ability to modify the Cas9 to target specific nucleotide sequences in the host paved the way to developing a new method to solve this problem (Sander & Joung, 2014). As a genetic tool, the CRISPR/Cas9 system could introduce heritable changes into the genome via precision insertions and deletions (Wu et al., 2014a, b). This technique has revolutionized the genome editing field; since most of the previous tools did not have the ability to cause stable and heritable changes to the target genomes.

The CRISPR/Cas9 system has been adopted for use in a wide range of eukaryotic organisms including yeast (DiCarlo et al., 2013), plants (Belhaj et al., 2013; Pandey et al., 2019), mammalian cells (Jinek et al., 2013; Cong et al., 2013; Manna et al., 2019) and fish (Hwang et al., 2013).

The aforementioned Class two system only requires a single Cas protein known as Cas9 (Type II) or closely related Cas12a; formerly Cpf1 (Type V) to induce double

stranded breaks (Jinek et al., 2013). Thus, it is much easier to be converted into a genome editing tool. The combination of the tracrRNA with crRNA into a simple guide RNA (gRNA) facilitates the precise identification of the target site by the Cas9 enzyme. This feature is also unique to the type II systemal., 2013). (Cong et al., 2013; Hwang et al., 2013). Furthermore, Cas12a has the explicit ability to catalyze and process the pre-crRNA via innate ribonuclease activity, without requiring tracrRNA and RNase III (Safari et al., 2019). All these factors cumulatively make the type II and type V systems much simpler, more efficient, and more suitable for genome editing (Belhaj et al., 2013). In many stably transformed plant species, whole genome sequencing was performed to detect any off-target mutations caused by Cas9 or Cas12a. Only a low level of off-target mutations could be detected; giving testament to the high specificity of the nucleases (Chen et al., 2019).



Note A Natural CRISPR array is transcribed into pre-CRISPR RNA with the spacers and direct repeats. B The processing of pre-crRNA to crRNAs by tracrRNA and Cas9 and RNase III recruited to the tracrRNA. C Cleavage of the pre-crRNA. D forming a mature crRNA:tracrRNA:Cas9 complex. E crRNA:tracrRNA:Cas9 complex guided by the specific sequence of crRNA attaches to the target sequence. F cleavage of the double stranded DNA. During genome editing, sgRNA (single guide RNA) combination of both crRNA and tracrRNA works as the targeting sequence. G leaving a double stranded break at the target site.

Figure 2.1 Outline of the native Type II bacterial CRISPR system and a blow up of CRISPR/Cas9 function during genome editing

The most significant change when converting naturally occurring CRISPR/Cas9 type II system into a genome editing tool is the fusion of crRNA and tracrRNA into one single RNA strand known as gRNA (guide RNA) or sgRNA (single guide RNA). This provides both the targeting specificity and the scaffolding/ binding ability to Cas9 (Doench et al., 2014). The Cas9:gRNA complex recognizes the PAM sequence and creates a DSB at a specific target site to be repaired using the intrinsic cellular repair mechanisms of the host. The repair mechanism could either be non-homologous end joining (NHEJ) or homology directed repair (HDR) (Jasin & Haber, 2016). The NHEJ mechanism is error prone, which leads to nearly random insertion and deletion mutations (i.e., indels) within the target sequence causing gene knockouts due to dysfunctional open reading frames (ORF), or by mutating a critical region of the protein for which the gene previously encoded. The other repair method, HDR, utilizes homologous recombination guided by a donor DNA template in repairing the break. This leads to precise gene replacement or knock in, mutagenesis, and gene corrections. Other genome editing systems such as ZFN and TALENS also use HDR as the repair mechanism for their genome edits. However, since the CRISPR/Cas9 system uses RNA to specify the editing location, this genome editing system is comparatively less expensive, less time consuming, and much more precise and scalable.

The *Streptococcus pyogenes* Cas9 (SpCas9) is the commonly used nuclease in the type II CRISPR/Cas system. SpCas9 accepts very frequently occurring NGG sequences as the PAM sequence, and thus a wide range of genes can be targeted by this enzyme. The nucleotide arrangement of the PAM sequence can vary depending on the origin of the Cas protein (Table 2.1) (Sander & Joung, 2014).

Table 2.1 Different PAM Sequences

Cas9 species	PAM sequence (5′-3′)
Streptococcus pyogenes (Sp)	NGG or NAG
Staphylococcus aureus (Sa)	NGRRT or NGRRN
Neisseria meningitidis (NM)	NNNNGATT
Streptococcus thermophilus (St)	NNAGAAW
Treponema denticola (Td)	NAAAAC

The presence of both RuvC and HNH domains in the Cas9 is very important, as both domains are required to induce a DSB (Chen et al., 2014). If either one of the domains is mutated, Cas9 retains the ability to bind sgRNA but can only create a single stranded break or a nick. According to the nature of the edit to the genome, Cas9 with a mutated RuvC or HNH domain can be used as Cas9 nickase (Cas9n). When both the domains are mutated (dCas9), it can only bind sgRNA and does not possess the ability to cause double or single stranded breaks. The dCas9 has been used to instigate targeted gene expression without introducing permanent mutation to the gene (Moradpour & Abdulah, 2020). There are several instances where Cas9n and dCas9 have been used for genome editing in mammalian cells (Gao et al., 2016; Hess et al., 2016). In fungi, dCas9 has been used in *Saccharomyces cerevisiae* (Gilbert et al., 2013) and Cas9n has been used in *Aspergillus niger* (Huang et al., 2019) for genome manipulation, but they have not been widely applied for gene editing in other fungal species.

Due to their immense importance in agriculture, phytopathogenic fungi are subjected to in depth molecular studies to identify genes involved in pathogenicity and potential drug target sites to develop efficient control measures. As previously mentioned, establishing a stable genome edit in filamentous fungi can be comparatively tedious. Difficulties in delivering gene editing components through the fungal cell wall, due to the presence of multinucleated cells and scarcity of suitable promoters and plasmids in the target fungi are some major factors contributing to this issue (Donohoue et al., 2018). The CRISPR/Cas9 system does not overcome all these problems but the

editing efficiency is comparatively much higher than any conventional method used to edit the same fungal species previously (Nødvig et al., 2015).

Studying the genomes of plant pathogens to understand disease progression, possible control measures, and identification of genes related to the pathogenicity of the fungi has been attempted by many molecular biologists, with varying degrees of success. Since the dawn of the CRISPR/Cas9 system as a potential genome editing tool, many researchers have begun testing and using this system to edit phytopathogenic fungal genomes.

2.4 CRISPR/Cas9 for Phytopathogenic Fungi

The virulence of fungi is always attributed to several genes working together. Understanding how these genes work towards pathogenesis can be done via insertion, deletion, or replacement of the target genes. Not only does this allow the understanding of the function of a specific gene during pathogenesis, but also establishes target sites that could be later used for pathogen prevention purposes.

Due to the increased availability of whole genome sequences of fungi (Galagan, 2005), the need for efficient genetic tools to exploit and use this information has become highly relevant. Precision genome editing by CRISPR/Cas9 has become an important tool to fill this void (Knott & Doudna, 2018). The last few years have shown great potential for implementing the CRISPR/Cas9 system for genome editing in filamentous fungi. The CRISPR/Cas9 genome editing tool has been successfully used in model fungi such as *Neurospora crassa* (Matsu-ura et al., 2015), several *Aspergillus* species (Nødvig et al., 2015, 2018) including *Aspergillus aculeatus*, *A. brasiliensis*, *A. carbonarius*, *A. fumigatus*, *A. luchunesis*, *A.nidulans*, *A. niger*, *A. oryzae* (Fuller et al., 2015; Zhang et al., 2016; Katayama et al., 2016; Leynaud-Kieffer et al., 2019), *Myceliophthora thermophila* (Liu et al., 2017), Penicillium chrysogenum (Pohl et al., 2016), *Nodulisporium* sp. and *Sporormiella minima* (Zheng et al., 2017), *Talaromyces atroroseus* (Nielsen et al., 2017), *Trichoderma reesei* (Liu et al., 2015) demonstrating that the CRISPR/Cas9 system can be applied to a wide variety of filamentous fungi.

Establishing the CRISPR/Cas9 genome editing system has shown positive outcomes in many phytopathogenic fungi (Table 2.2). The CRISPR/Cas9 system was successful in disrupting genes within phytopathogenic fungi, in which targeted gene disruption was not possible before (Idnurm et al., 2017). Since the CRISPR/Cas9 system is comparatively more efficient and accurate, stable gene edits have been established in phytopathogenic fungi such as *Alternaria alternata* (Wenderoth et al., 2017), *Fusarium graminearum* (Gardiner & Kazan, 2018), *F. fujikuroi* (Shi et al., 2019), *F. oxysporum* (Wang et al., 2018), *Leptosphaeria maculans* (Idnurm et al., 2017; Darma et al., 2019), *Phytophthora capsici* (Miao et al., 2018), *P. palmivora* (Gumtow et al., 2018), *P. sojae* (Fang & Tyler, 2016), *Pyricularia oryzae* (Arazoe et al., 2015a; Foster et al., 2018; Yamato et al., 2019), *Sclerotinia sclerotiorum* (Li et al., 2018), *Shiaria bambusicola* (Deng et al., 2017), *Sporisorium scitamineum* (Lu et al., 2017), *Ustilago maydis* (Schuster et al., 2016) and *Ustilaginoidea virens* (Liang et al., 2018).

Many CRISPR related studies conducted on phytopathogenic fungi were performed in order to establish the first proof of principle of the functionality of the system within the target fungi (Schuster & Kahmann, 2019). In many cases, genes that are not directly related to pathogenicity but that would provide a clear phenotypic change in the mutated state were used as a target to create sgRNA (Wenderoth et al., 2017). Once the system's feasibility is established in this manner, it can be modified to be used on any target gene within that particular organism.

The targeted DSB induced when implementing CRISPR/Cas9 for genome editing is one major contributing factor for the system's higher efficiency. In fungal species where the CRISPR/Cas9 system has been established, when the same donor DNA was introduced without the DSB, the efficiency of HDR was found to be comparatively reduced (Schuster & Kahmann, 2019). In *P. chrysogenum*, when the CRISPR/Cas9 was used, the number of transformants with the desired mutation substantially increased in comparison to traditional HDR (Pohl et al., 2016).

In order to establish a successful CRISPR/Cas9 system, the expression of the sgRNA and the Cas9 should be carefully instigated, as they are the crucial working components of the system. The sgRNA expression should be activated under an efficient strong promoter. Optimized U6 promoters with higher transcriptional efficiency are used for the expression of sgRNA in many filamentous fungi (Schuster

& Kahmann, 2019). For example, in *P. oryzae*, sgRNA expressed under the U6 promoter was shown to have a better genome editing efficiency than that expressed under the TrpC promoter (Arazoe et al., 2015a). Almost all the phytopathogenic fungi edited using the CRISPR/Cas9 system have used the U6 promoter for the transcription of the sgRNA due to its high efficiency. The bacteriophage T7 promoter is also a better alternative when the U6 promoter is not available or when its use is not practical (Fuller et al., 2015). The above-mentioned promoters are some of the frequently used promoters for genome editing in filamentous fungi. Since filamentous fungi possess a plethora of tRNA genes, identifying a more suitable promoter for the specific gene editing purposes should be considered. In vitro synthesis of the sgRNA is the more suitable choice if an efficient promoter could not be established for the expression of sgRNA within the target host. This would help in avoiding any error prone sgRNA being synthesized (Pohl et al., 2016).

The other crucial component of the CRISPR/Cas9 system, Cas9 endonuclease expression in filamentous fungi can be improved through codon optimization. Since Cas9 is of prokaryotic origin, efficient transcription of the endonuclease has been achieved for phytopathogenic fungi through human codon optimized Cas9 in *L. maculans* (Idnurm et al., 2017), *P. sojae* (Fang & Tyler, 2016), *P. palmivora* (Gumtow et al., 2018) and *S. bambusicola* (Deng et al., 2017). Furthermore, the fungal codon optimized Cas9 used in Nødvig et al. (2015) was subsequently used for CRISPR/Cas9 system in many fungal species including the phytopathogen *A. alternata* (Wenderoth et al., 2017) and CRISPR/Cas9 system established in many phytopathogenic fungi has used Cas9 codon optimized for the expression in the host organism (Arazoe et al., 2015a, Foster et al., 2018, Shi et al., 2019). The Cas9 codon optimized for *P. oryzae* used in Arazoe et al. (2015) was subsequently used in *S. sclerotiorum* (Li et al., 2018) and *U. virens* (Liang et al., 2018). The common nuclear localization sequence (NLS) SV40 is also added to both ends of the Cas9 gene for accurate expression (Song et al., 2019). In order to facilitate the efficient expression of the Cas9 enzyme, placing the gene under a strong promoter is important. But, continuous expression of the Cas9 gene would lead to off-target effects and cellular stress. Hence, rather than using constitutive promoters, using an inducible promoter has been implemented as the smart alternative. For example, the promoter of the heat shock protein (hsp70) used for the

expression of Cas9 in *U. maydis* was inducible by temperature changes (Schuster et al., 2016).

The expression of the Cas9 protein could either be done within the cell during transformation or can be added as preassembled RNPs (ribonucleoproteins) (Foster et al., 2018). The use of RNPs can be useful when implementing the CRISPR/Cas9 system across various genetic backgrounds and not only in specific strains engineered to express Cas9 or sgRNA that depend on DNA based expression cassettes for delivery (Al Abdallah et al., 2017).

2.5 The Significance of the CRISPR/Cas9 Mediated Genome Editing in Phytopathogenic Fungi

In the process of management of pathogenic fungi infecting economically important crops, plant disease resistant genes play a very important role. The discovery and identification of these plant genes can be challenging, thus characterizing the effector/avirulence molecules identified by these genes was found to be comparatively simpler (de Jonge et al., 2011). Through targeting these effector genes of phytopathogens using the CRISPR/Cas9 system, the mutated fungi can be used to trigger defense responses to guide the breeding of resistant genes which could potentially be an integral part of developing suitable disease management strategies.

Naturally occurring pathogen populations have the ability to overcome the disease resistance of crops grown in monoculture due to the absence of diversity in resistance against the pathogen (Van de Wouw & Idnurm, 2019). The establishment of new fungal genotypes through targeting conserved, and therefore potentially essential, effectors could lead to the possibility of producing avirulent competitors for the plant pathogenic fungi in the field while also priming the plants against the virulent strains of the fungal pathogen. This could also lead to the identification of the corresponding resistance genes against these phytopathogens, which in turn could provide components in developing more durable resistance in the crops (Vleeshouwers & Oliver, 2014).

For example, the rice blast disease caused by *P. oryzae* is considered as the most devastating rice disease in the world. Even though the disease epidemiology is quite

well understood (Kim, 2001; Greer & Webster, 2001), preventive measures for this disease are still not totally effective. Implementing genome editing to undercut the virulence of this fungus could be potentially very useful. Gene editing using the CRISPR/Cas9 system has proven to be successful in *P. oryzae* in several studies (Arazoe et al., 2015a; Foster et al., 2018; Yamato et al., 2019). If this established system could be used to produce avirulent strains by mutating or completely knocking out important pathogenicity related genes of the native wild type, the mutants could be used as a potential control measure by priming the crop against any aggressive virulent strains of the fungi.

Furthermore, many secondary metabolites produced by fungi have been reported as common virulence factors involved in the pathogenicity process (Darma et al., 2019). Phytohormones such as abscisic acid (ABA) produced by phytopathogenic fungi can manipulate plant immunity to promote disease development. For example, in *P. oryzae*, the deletion of abscisic acid producing gene ABA4 reduced pathogenicity in rice plants and demonstrated that ABA increases spore germination and appressorium formation (Spence et al., 2015). The CRISPR/Cas9 systems established for *Phytophthora palmivora*, *Phytophthora sojae*, *Sclerotinia sclerotiorum*, and *Shiaria bambusicola* used genes related to pathogenicity as the targets and were successful in creating less virulent mutant strains (Fang & Tyler, 2016; Deng et al., 2017; Lu et al., 2017; Gumtow et al., 2018). Through the manipulation of these candidate virulence genes, a mechanistic understanding of pathogenicity could be achieved. The resulting mutant stains can be used to test hypotheses on how these genes are involved in pathogenicity (Li et al., 2018). However, none of the CRISPR/Cas9 phytopathogenic transformants have been tested in the field as potential disease control agents.

Table 2.2 Phytopathogenic fungi and oomycetes edited using the CRISPR/Cas9 system

Species	Disease	Modified gene(s)	Phenotype of the mutant	Significance of the edit	Mutation efficiency %	Reference(s)
Alternaria alternata	Opportunistic plant pathogen causing variety of diseases in a wide host range.	Polyketide-synthase (pksA)	Loss of melanin deposition	Distinguishable phenotypic change in the mutant	-	Wenderoth et al. (2017)
		1,3,8-THN reductase encoding (brm2)	Loss of melanin deposition	Distinguishable phenotypic change in the mutant		
		Orotidine 5′-phosphate decarboxylase	Uracil auxotrophy	Efficient selection of the mutant	-	
		FphA LreA HogA	Reduced conidiation than the wildtype in the dark (reduced by 86% in FphA mutants, 51% in LreA mutants, 48% in HogA mutants) Germination of conidia was delayed in red, blue, green, and far-red light	Demonstrates that germination, sporulation, and secondary metabolism are light regulated in *A. alternata*	-	Igbalajobi et al. (2019)

Table 2.2 (continued)

Species	Disease	Modified gene(s)	Phenotype of the mutant	Significance of the edit	Mutation efficiency %	Reference(s)
Botrytis cinerea	Botrytis bunch rot in grapes Grey mold in many crops	*Bos1* gene	Mutants are resistant against the fungicides iprodione (Ipr) and fludioxonil (Fld)	Efficient selection of the mutant	-	Leisen et al. (2020)
		botrydial (bot2) and botcinic acid (boa6) genes coding for key enzymes important for phytotoxins biosynthesis	-	Reduced pathogenicity in double mutants	-	
Colletotrichum sansevieriae	Anthracnose disease only in plants of the genus *Sansevieria*	Scytalone dehydratase gene (*SCD1*)	loss of melanin deposition	Distinguishable phenotypic change in the mutant (Pink colonies)	97.1	Nakamura et al. (2019)

Table 2.2 (continued)

Species	Disease	Modified gene(s)	Phenotype of the mutant	Significance of the edit	Mutation efficiency %	Reference(s)
Fusarium fujikuroi	"Foolish seedling" disease in rice	Fusarium cyclin C1 (*fcc1*)	Non-accumulation of a specific purple pigment	Distinguishable phenotypic change in the mutant	79.2	Shi et al. (2019)
		Orotidine-5'-phosphate decarboxylase (*ura3*)	Uracil auxotrophy	Efficient selection of the mutant		
		4'-phosphopantetheinyl transferase (*ppt1*)	Lysine auxotrophy	Efficient selection of the mutant		
Fusarium graminearum	Causal organism for wide range of diseases in cereal grain	Osmosensor histidine kinase 1 (FgOs1)	Resistance to the fungicide Fludioxonil	Simple phenotypic assay for mutant selection	1-10	Gardiner and Kazan (2018)
Fusarium oxysporum	Able to cause damage to tomato, banana, legumes cotton.	BIK1 gene encoding a putative polyketide synthase involved in the biosynthesis of bikaverin (Red pigment)	Inability to synthesize bikaverin	Distinguishable phenotypic change in the mutant	~50	Wang et al. (2018)

Table 2.2 (continued)

Species	Disease	Modified gene(s)	Phenotype of the mutant	Significance of the edit	Mutation efficiency %	Reference(s)
		URA5 -orotate phosphoribosyltransferase involved in pyrimidine biosynthesis	Uracil auxotrophy	Efficient selection of the mutant		
Leptosphaeria maculans	Blackleg disease on *Brassica* crops	osmosensing histidine kinase (hos1) gene	Loss of resistance to the fungicide iprodione Reduced growth under high salt conditions	Efficient selection of the mutant	-	Idnurm et al. (2017)
		polyketide synthase gene (*pks5*) abscisic acid-like 7 gene (*abl7*)	Changes in abscisic acid production	Pks1 and Abl1 genes are not involved in the pathogenicity of *Leptosphaeria maculans*		Darma et al. (2019)

Table 2.2 (continued)

Species	Disease	Modified gene(s)	Phenotype of the mutant	Significance of the edit	Mutation efficiency %	Reference(s)
Phytophthora capsici	Blight and fruit rot of peppers and other important commercial crops	Oxysterol binding protein-related protein-1 (ORP1)	High levels of resistance to the fungicide oxathiapiprolin	Efficient selection of the mutant	-	Miao et al. (2018)
Phytophthora palmivora	Bud rot and fruit rot in a wide range of crops	Cystatin-like extracellular protease inhibitors (PpalEPICs)	Increased papain sensitivity of in vitro growth and reduced pathogenicity during infection of papaya fruits	Efficient selection of the mutant and reduced virulence on the target host	-	Gumtow et al. (2018)
Phytophthora sojae	Soybean stem and root rot	The RXLR effector gene Avr4/6	Reduction in pathogenicity	Efficient selection of the mutant	-	Fang and Tyler (2016)
		Oxysterol binding protein-related protein-1 (ORP1)	High levels of resistance to the fungicide oxathiapiprolin	Efficient selection of the mutant	-	Miao et al. (2018)

Table 2.2 (continued)

Species	Disease	Modified gene(s)	Phenotype of the mutant	Significance of the edit	Mutation efficiency %	Reference(s)
Pyricularia oryzae	Rice blast disease	Scytalone dehydrogenase (SDH) gene	loss of melanin deposition	Distinguishable phenotypic change in the mutant	36.1–80.5	Arazoe et al. (2015a)
		Polyketide synthase encoding ALB1 and RSY1	Albino or orange-red colonies formed	Distinguishable phenotypic change in the mutant	-	Foster et al. (2018)
		Scytalone dehydrogenase (SDH) gene	loss of melanin deposition	Distinguishable phenotypic change in the mutant	20-43	Yamato et al. (2019)
Sclerotinia sclerotiorum	Cottony rot, Watery soft rot, Stem rot, Drop, crown rot and Blossom blight	Oxalate biosynthesis gene Ssoah1	Overproduction of compound appressoria, Decreased pigmentation on the surface of sclerotia, Diffuse pattern of sclerotium development in culture	Distinguishable phenotypic change in the mutant Reduced virulence across a wide range of hosts	38-45	Li et al. (2018)

Table 2.2 (continued)

Species	Disease	Modified gene(s)	Phenotype of the mutant	Significance of the edit	Mutation efficiency %	Reference(s)
		Polyketide synthase gene for melanin biosynthesis Sspks13	Lack of pigmentation in compound appressoria	Distinguishable phenotypic change in the mutant		
Shiaria bambusicola	Bamboo blight	Major facilitator superfamily (MFS) gene in the hypocrellin gene cluster	No production of hypocrellin	Attenuated virulence on bamboo leaves	-	Deng et al. (2017)
Sporisorium scitamineum	Sugarcane smut	Mating related Mfa2 gene	Inability to get into filamentous growth after mating with a sex-opposite strain	Efficient selection of the mutant	21.7-39.1	Lu et al. (2017)
Ustilago maydis	Corn smut	bW2 and bE1	Loss of filament formation in charcoal containing agar plates	Distinguishable phenotypic change in the mutant	50–90	Schuster et al. (2016, 2017)
Ustilaginoidea virens	False smut disease in rice	*USTA* ustiloxin and *UvSLT2* MAP kinase genes	Increased sensitivity to cell wall stresses but tolerance to hyperosmotic or oxidative stresses	Efficient selection of the mutant	-	Liang et al. (2018)

Furthermore, many secondary metabolites produced by fungi have been reported as common virulence factors involved in the pathogenicity process (Darma et al., 2019). Phytohormones such as abscisic acid (ABA) produced by phytopathogenic fungi can manipulate plant immunity to promote disease development. For example, in *P. oryzae*, the deletion of abscisic acid producing gene ABA4 reduced pathogenicity in rice plants and demonstrated that ABA increases spore germination and appressorium formation (Spence et al., 2015). The CRISPR/Cas9 systems established for *Phytophthora palmivora*, *Phytophthora sojae*, *Sclerotinia sclerotiorum*, and *Shiaria bambusicola* used genes related to pathogenicity as the targets and were successful in creating less virulent mutant strains (Fang & Tyler, 2016; Deng et al., 2017; Lu et al., 2017; Gumtow et al., 2018). Through the manipulation of these candidate virulence genes, a mechanistic understanding of pathogenicity could be achieved. The resulting mutant stains can be used to test hypotheses on how these genes are involved in pathogenicity (Li et al., 2018). However, none of the CRISPR/Cas9 phytopathogenic transformants have been tested in the field as potential disease control agents.

Fungal phytopathogens spontaneously developing resistance towards chemical fungicides is a major concern in crop production. The CRISPR/Cas9 system established for *L. maculans* (Idnurm et al., 2017) and *P. capsici* (Miao et al., 2018) disrupted genes that were characterized previously as genes inferring fungicide resistance in the mutated state. *L. maculans* mutants previously discovered resistant to iprodione were used for gene disruption and *P. capsici* mutants resistant to oxathiapiprolin were produced using the CRISPR/Cas9 system. Characterization of these genes can be used to understand and develop novel fungicides like oxysterol binding protein homolog inhibitor fungicides for the control of *Phytophthora* spp., (Miao et al., 2018).

The use of chemical fungicides and reluctance to incorporate transgenic crops into their diets are important concerns for consumers. Thus, developing more resilient disease control strategies is an important issue. Implementing the CRISPR/Cas9 system to identify fungal genes that are required for growth and viability or effectors that are essential for virulence can provide crucial information to develop inspired disease control strategies in the future. Spray induced gene silencing (SIGS) is one such approach in which double stranded RNAs (dsRNAs) that target essential pathogen genes are sprayed onto plant surfaces, which leads to disease control (Wang & Jin,

2017). These could be used as species specific “fungicides” due to specific targeting of genes.

Enhancing the biocontrol aptitudes of well-known fungal antagonists using the CRISPR/Cas9 system could also be a potential control method (Muñoz et al., 2019). The genetically enhanced antagonists with efficient metabolic pathways that trigger the biosynthesis of secreted proteins and secondary compounds could act as a frontline of defense against the invading fungal phytopathogens (Syed Ab Rahman et al., 2018). Through the programmable silencing of genes contributing to the competing reactions, the genes involved in the production of antagonistic substances such as diffusible antibiotics, toxins, extracellular cell wall degrading enzymes, and other volatile organic compounds could be enhanced (Van de Wouw & Idnurm, 2019).

Though implementing CRISPR/Cas9 system for fungal genome editing is still in its infancy, the simplicity and efficiency of this system have shown to be much more beneficial and user friendly than any other genome editing tool currently available. This is due to the easy adaptability when used across different species of fungi. With the insights gained from the already existing systems of CRISPR/Cas9 for phytopathogenic fungi, the question of how this gene editing system can be used for other fungal species can be readily answered.

2.6 Challenges in CRISPR/Cas9

Although there are considerable merits with regards to the efficiency and specificity of the CRISPR/Cas9 system, when practically implementing the system, some drawbacks are also present (Zhang et al., 2014). These problems also need to be addressed to increase the efficiency and to obtain the full potential of this system.

One major concern with the CRISPR/Cas9 system is the off-target mutations. When using SpCas9 for genome editing, the NGG sequence acts as the PAM region. Utilizing only NGG as the single acceptable PAM sequence might lead to off-target mutations in instances where the target sequence has high homology elsewhere in the genome (Lin et al., 2014). This can be overcome by using novel *S. pyogenes* Cas9 variants with different PAM sequences or by using Cas9 homologs derived from

species other than S. pyogenes (Table 2.1). Adapting Cpf1 (Cas12a) instead of Cas9 can also help in avoiding off-target mutations as the PAM sequence for Cas12a (TTTV) can avoid Cas9 PAM, if it frequently occurs throughout the target genome (Ungerer & Pakrasi, 2016). Recently, genome editing using Cpf1 has successfully been implemented in filamentous fungi (Vanegas et al., 2019). On the other hand, the CRISPR/Cas9 system specifically designed for transient expression (Nagy et al., 2017) and systems with the ability to remove the Cas9 gene after genome editing (Wang et al., 2016) have also been implemented to avoid off-target mutations. Furthermore, using RNPs as the delivery method for the CRISPR/Cas9 components can also help in reducing off-target mutations. For example, in one study on *A. fumigatus*, where RNPs were used as the Cas9 delivery system, no off-target mutations could be detected (Al Abdallah et al., 2017).

Through the designing of highly specific sgRNAs, the low level of off targeting by the Cas nucleases could be avoided. Databases and software tools such as CasOT (Xiao et al., 2014), sgRNAcas9 (Xiao et al., 2014), E-CRISP (Heigwer et al., 2014), CHOPCHOP (Montague et al., 2014, Labun et al., 2016), CRISPRdirect (Naito et al., 2015), CRISPRscan (Moreno-Mateos et al., 2015) and CRISPOR (Haeussler et al., 2016) have been developed for easy search for potential off targets within the genome and for the optimized production of the components of the CRIPSR/Cas9 system. The continuous expression of the Cas9 gene could be toxic to the host cells. The solution for this is the transient expression of CRISPR machinery to limit the strain to the cells. In addition, careful design of the sgRNA and limiting the longevity of the Cas9 sgRNA complex in a cell could increase the editing specificity of the CRISPR/Cas9 system.

Given that the specific targeting activity of the Cas9 enzyme depends on the presence of the PAM sequence downstream of the target sequence (Jinek et al., 2012), genes without the PAM sequence could not be targeted and edited using the CRISPR/Cas9 system. One way to overcome this PAM dependence is by using Cas variants with frequently occurring PAM sequences. However, rationally engineered SpCas9 variants with relaxed PAM recognition have also been developed to reduce the PAM dependency of the CRISPR/Cas9 system (Nishimasu et al., 2018; Ren et al., 2019).

Retaining the required specificity of the designed sgRNA is another challenge in the CRISPR/Cas9 system. Post transcriptional modifications by RNA polymerase II within the host cell make it difficult for the synthesis of sgRNA, as such modifications would hinder the specificity. Without the specificity of the sgRNA, the targeting of the correct gene cannot be achieved.

Through the understanding of the mechanism of action, despite the challenges this method presents, the CRISPR/Cas9 for genome editing is now used in many different scientific fields for a variety of different purposes because of its abundant potential and the new possibilities it presents in the field of genome editing. Filamentous fungi play a very significant role in a variety of contexts. They can be consumed as a food source in the form of mushrooms, they can be an important plant, human and animal pathogens and they are used to produce natural compounds with medicinal properties, pigments used in textile industries and enzymes that can be used in biodegradation, biofuel production and fermentation. The CRISPR/Cas9 system with reverse genetics approach has been used in several studies in order to obtain certain desirable traits or to increase the production efficiency of certain chemicals or enzymes. In one study, white button mushroom *Agaricus bisporus* was modified to resist browning, and since no foreign DNA was introduced through CRIPSR/Cas9 system, it is exempt from GMO regulations in the United States (Waltz, 2016). In *Myceliophthora thermophila* the CRISPR/Cas9 system allowed simultaneous deletion of four genes involved in cellulose production pathway, in which the resulting strain produced three-fold more lignocellulose in comparison to the parental strain (Liu et al., 2017). Another example is that through the silencing of genes that hinder the production of desired metabolites, the metabolic flux can be redirected to favor the production of the chemical or enzyme of our choice to increase the production efficiency and yield (Donohoue et al., 2018). Many *Aspergillus* species like *A. aculeatus*, *A. brasiliensis*, *A. carbonarius*, *A. luchuensis*, *A. nidulans*, and *A. niger*, which are a source of and producer of enzymes, were few of the first filamentous fungal species edited using the CRISPR/Cas9 system (Nødvig et al., 2015).

2.7 Conclusions

It is very important to consider which genome editing tool is more suitable for providing the answer to the relevant biological question pertaining to a research interest. There are many options now available for performing loss of function of genes of phytopathogens related to disease progression. In the past few years, the CRISPR/Cas9 system for genome editing has been successfully implemented in many fields and has advanced greatly. The simplicity, high efficiency, low cost, and the versatility of this system show great potential. There are many successful examples providing evidence that the CRISPR/Cas9 system is one of the best tools in the genome editing arsenal for studying fungal genomes, and the system will be implemented in fungal genetic research for years to come. Functional genomics has played a very important role in understanding the pathogenicity mechanism of many phytopathogenic fungi. Although many CRISPR/Cas9 related studies done up to now have only been to provide proof of function within the selected target organisms, the results gained by these studies can be further developed in order to combat plant diseases caused by many pathogenic fungi. As mentioned above, the CRISPR/Cas9 system is able to produce heritable changes in the genomes of the target phytopathogens. By understanding the involvement of a particular gene in the progression of a fungal disease, further studies can be conducted to provide suitable prevention strategies to reduce the devastating effects of phytopathogens like *P. oryzae* through precise editing to silence the pathogenicity related genes understand virulence related characteristics. Moreover, with the ability of CRISPR/Cas9 system to produce simultaneous gene edits throughout multiple locations, several pathogenicity related genes could be silenced in one go.

The safety of food crops is a very important factor to be considered when genetics related control measures are utilized. Since the CRISPR/Cas9 system does not introduce any foreign genes into the mix of the existing genes, CRISPR/Cas9 edited phytopathogens can be considered relatively safe compared with phytopathogens modified with other gene editing tools. It should also be considered that the release of an avirulent mutant strain in the field could produce highly virulent strains after mating with the wild type. Thus, before considering the introduction of a mutated strain as a

potential control measure in the field, the target organism should be selected from its native environment to avoid any genetic differences that would potentially give rise to novel, highly virulent combinations. Thus, the potential should be carefully examined and explored before any genome modified strain is released into the field as a control measure.

Resequencing the whole genome of the fungal host edited via the CRISPR/Cas9 system could be used to understand issues such as off-target effects that have not been addressed properly. With finding solutions to overcome the pitfalls faced by this impressive new toolbox, it is highly likely that future researchers will benefit massively by overcoming the challenges faced by CRISPR/Cas9 gene editing today, and future research will improve the ability to elucidate gene function, thus advancing the field for the further growth of science and technology.

CHAPTER 3

A LysM DOMAIN-CONTAINING PROTEIN LtLysM1 IS IMPORTANT FOR VEGETATIVE GROWTH AND PATHOGENESIS IN WOODY PLANT PATHOGEN *Lasiodiplodia theobromae*[2]

Abstract

Lysin motif (LysM) proteins are reported to be necessary for the virulence and immune response suppression in many herbaceous plant pathogens, while far less is documented in woody plant pathogens. In this study, we preliminarily characterized the molecular function of a LysM protein LtLysM1 in woody plant pathogen *Lasiodiplodia theobromae*. Transcriptional profiles revealed that LtLysM1 is highly expressed at infectious stages, especially at 36 and 48 hours post inoculation. Amino acid sequence analyses revealed that LtLysM1 was a putative glycoprotein with 10 predicted N-glycosylation sites and one LysM domain. Pathogenicity tests showed that overexpressed transformants of LtLysM1 displayed increased virulence on grapevine shoots in comparison with that of wild type CSS-01s, and RNAi transformants of LtLysM1 exhibited significantly decreased lesion length when compared with that of wild type CSS-01s. Moreover, LtLysM1 was confirmed to be a secreted protein by a yeast signal peptide trap assay. Transient expression in *Nicotiana benthamiana* together with protein immunoblotting confirmed that LtLysM1 was an N-glycosylated protein. In contrast to previously reported LysM protein Slp1 and OsCEBiP, LtLysM1 molecule did not interact with itself based on yeast two hybrid and co-immunoprecipitation

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assays. These results indicate that LtLysM1 is a secreted protein and functions as a critical virulence factor during the disease symptom development in woody plants.

Keywords: Grapevine, *N*-glycosylation, Pathogenicity, Secreted Protein

3.1 Introduction

During microbe-plant interactions, plants have evolved two tier of defense responses in the face of pathogen attacks. The first layer provides basal responses based on the recognition of pathogen-associated molecular patterns (PAMPs) by plant pattern recognition receptors (PRRs) that activates PAMP-triggered immunity (PTI) (Jones & Dangl, 2006; Macho & Zipfel, 2014; Stergiopoulos & de Wit, 2009). This layer of responses are associated with callose deposition, lignin formation, phenolic compounds deposition, stomata closing, generation of extracellular reactive oxygen species, and a serious of downstream signal transduction events (Han & Kahmann, 2019; Zipfel, 2014). To establish compatible interaction for successful proliferation inside host tissues, microbial pathogens secrete an arsenal of effectors to overcome PTI response. Although pathogens secrete substantial effectors to subvert PTI, plants have evolved a surveilence system to recognize these effector proteins to trigger wellknown effector-triggered immunity, which includes by hypersensitive cell death and defense-gene activation (Han & Kahmann, 2019; Jones & Dangl, 2006; Selin et al., 2016; Thomma et al., 2011). During past decades, many typical PAMPs including bacterial flagellar peptide flg22, EF-Tu peptide, lipopolysaccharide, peptidoglycan, fungal cell wall chitin, and xylanase, and hundreds of effectors such as RxLR, CRN, CFEM, or LysM motif-containing proteins have been identified and characterized (Han & Kahmann, 2019; Liu et al., 2019; Newman et al., 2013; Thomma et al., 2011; Win et al., 2012).

The LysM motif is widely found in various proteins such as chitinases, peptidases, and receptor-like kinases and effector proteins (Akcapinar et al., 2015; Liu et al., 2019). LysM effector proteins were reported to be able to either protect hyphae from being degraded by plant chitinases through binding to fungal cell wall chitin and inhibit the activity of plant chitinase through binding to the chitinases or prevent plant recognizing GlcNAc oligomers to avoid plant defense responses (Kombrink & Thomma, 2013; Rovenich et al., 2016). For example, Slp1 in *Magnaporthe oryzae* functions as a competitive inhibitor of PRR protein CEBiP and suppresses chitin-induced immune responses in rice (Mentlak et al., 2012). Ecp6 in *Cladosporium fulvum* was found to be involved in chitin oligosaccharide sequestration through collective

actions of LysM1 and LysM3 domains, and also involved in perturbation of host immune receptor dimerization via LysM2 domain (Liu et al., 2012; Sánchez-Vallet et al., 2013). Also, Mg1LysM and Mg3LysM in *Mycosphaerella graminicola* were reported to be able to protect fungal hyphae from hydrolysis by plant hydrolytic enzymes (Marshall et al., 2011). Another two LysM proteins ChELP1 and ChELP2 in *Colletotrichum higginsianum* could also bind to chitin and suppresses chitin-triggered activation of immunerelated plant mitogen-activated protein kinases (Takahara et al., 2016). Another documented chitin-binding protein Vd2LysM in *Verticillium dahlia* suppresses chitininduced immune responses and protected fungal hyphae against hydrolysis (Kombrink et al., 2017). Moreover, LysM effectors have also been identified to contribute to circumvent plant defense responses and establish arbuscular mycorrhizal symbiosis (Schmitz et al., 2019; Zeng et al., 2020). Furthermore, LysM effectors are involved in mycoparasitism in pathogenic fungi (Romero-Contreras et al., 2019). Even though the biological functions and regulatory mechanisms of LysM proteins have been widely characterized in various fungi, but little is documented in opportunistic plant pathogen *Lasiodiplodia theobromae*.

Lasiodiplodia theobromae, a member of Botryosphaeriaceae family, can change its lifestyle from endophytic to pathogenic when host defense is weakened by internal and external factors (Chethana et al., 2016; Paolinelli-Alfonso et al., 2016). The fungus is widely distributed in tropical and subtropical regions and is able to infect a wide range of hosts (Correia et al., 2016). *Lasiodiplodia theobromae* is emerging as one of the most aggressive pathogens that causes severe grapevine canker disease in vineyards, resulting in considerable yield losses to global grape industry (Rodríguez-Gálvez et al., 2015; Úrbez-Torres, 2011; Yan et al., 2013). So far, numerous pathogenicity-related genes have been identified in *L. theobromae* based on genome sequencing and bioinformatic analyses (Félix et al., 2019; Yan et al., 2018), functional mechanisms of these genes, however, is barely documented.

Based on genome sequence (Yan et al., 2018), we identified six LysM domain-containing proteins in *L. theobromae* CSS-01s, of which three were predicted to be functional effector proteins. Here, one LysM protein LtLysM1 (*Lasiodiplodia theobromae* LysM domain effector 1) was preliminarily characterized. Relative transcription analyses and pathogenicity tests revealed that LtLysM1 contributes to the

virulence of *L. theobromae* during the infection process. Additionally, LtLysM1 also displayed an effect on the vegetative development of *L. theobromae*. Moreover, LtLysM1 was confirmed to be a secretory protein by a yeast signal peptide trap system. Surprisingly, LtLysM1 was N-glycosylated when expressed in *N. benthamiana*. Collectively, these results indicate LtLysM1 functions as a secretory protein and play an important role in pathogenesis and vegetative development of *L. theobromae.*

3.2 Materials and Methods

3.2.1 Fungal and Plant Materials

The *L. theobromae* wild type isolate CSS-01s and fungal transformants were cultured on potato dextrose agar (PDA 1 l: 200 g potato, 20 g dextrose, and 20 g agar) and maintained at 28°C at equal light and dark cycles. The fungi and bacteria used in the study were stored in PDA slants at 4°C and 50% glycerol at –80°C, respectively. Mycelium plugs from 2-day-old cultures and healthy green shoots of *Vitis vinifera* cv. Summar Black from Yanqing, China were used for pathogenicity assays. Mycelia cultured in complete medium (0.6% [w/v] yeast extract, 0.3% [w/v] casein acid hydrolysate, 0.3% [w/v] casein enzymatic hydrolysate, and 1% [w/v] sucrose) were collected for RNA extraction. Bacteria were cultured in Luria-Bertani (1% [w/v] tryptone, 0.5% [w/v] yeast extract, and 1% [w/v] NaCl at pH 7.5). *Nicotiana benthamiana* used for transient expression was grown in a controlled growth chamber and maintained at 25°C.

3.2.2 Amino Acid Sequence Analyses

The LtLysM1 signal peptide was predicted with Signal P 5.0 Server with server default parameter settings (http://www.cbs.dtu.dk/services/SignalP/). The LysM domain of LtLysM1 was identified through BLASTP program against Pfam database using LtLysM1 amino acid sequence as a query (El-Gebali et al., 2019). The N-glycosylation sites were predicted using the N-GlycoSite tool.

3.2.3 RNA Extractions and Quantitative Real-Time Reverse Transcription PCR Analyses

Wounds of green grapevine shoot were inoculated with mycelium plugs cut with a cork-borer and then maintained under an alternating dark/ light cycle in a growth chamber with 70% humidity and 25°C. Inoculated grapevine tissues were harvested at different time points (12, 24, 36, 48, 60 and 72 hours post inoculation [hpi]) for RNA extraction. Fungal mycelia cultured in complete medium broth on a shaking incubator at 160 rpm and 25°C for 36 h were used for RNA extraction. Total RNA was isolated using an EASYspin Plus Complex Plant RNA Kit (Aidlab, Beijing, China) according to the manufacturer's instructions. Isolated RNA was reverse transcribed into cDNA using the transScript OneStep gDNA Removal and cDNA Synthesis SuperMix kit (TransGen Biotech, Beijing, China) by following the manufacturer's instructions. The transcriptional profiles of LtLysM1 were assessed via quantitative real-time polymerase chain reaction (qRT-PCR). The qRT-PCR assays were conducted using 7500 real-time PCR system (Applied Biosystems, Singapore) with 2× RealStar Green Fast Mixture with ROX II (GenStar, Beijing, China). The qRT-PCR was performed in 16 µl final volumes consist of 1.0 µl cDNA, 0.3 µl primer, 6.4 µl sterile water, and 8 µl RealStar Green Fast Mixture with ROX II. The amplification program was as follows: 2 min for denaturation at 95°C, followed by 40 cycles of 95°C for 15 s, and 60°C for 30 s. The actin gene was used as the internal reference. Relative transcript levels were calculated using the 2-ΔΔCT method (Livak & Schmittgen, 2001). The experiment was repeated at least twice with three independent replicates for each sample. Primers used in the experiments were listed in Table 3.1.

Table 3.1 Primers used in this study

Primer name	Sequence (5′-3′)	Use of primer
LysM1AB-f	CGGAATTCATGTCATCCTGCAATACC	Used for the
LysM1AB-r	CGGGATCCTCAGTCCAAAACCGTGAT	construction of LtLysM1 prey and bait vectors
LysM1EX-f	TCGGGTACCCGGGGATCCATGTTCAAG TCTTGTCATAT	Used for the construction of
LysM1EX-r	GTCGACTCTAGAGGATCCGTCCAAAA CCGTGATATTTC	LtLysM1 transient expression vector
LysM1SP-f	CGGAATTCATGTTCAAGTCTTGTCA	Used for the
LysM1SP-r	TATCTCGAGGTTGAGTGCGGTGGT	construction of *pSUC2:LtLysM1* fusion vector
LysM1OE-f	CGGAATTCATGTCATCCTGCAATACC	Used for the
LysM1OE-r	CGGGATCCTCAGTCCAAAACCGTGAT	construction of LtLysM1 over expression vector
LysM1RNAi-Sf	TATAAGCTTGGACAACGAAACCTGCCT	Used for the
LysM1RNAi-Sr	CGGAATTCATTGTCCTTGGCGTGCTC	construction of LtLysM1 RNAi vector
LysM1RNAi-ASf	CGGGATCCATTGTCCTTGGCGTGCTC	
LysM1RNAi-ASr	AACTGCAGGGACAACGAAACCTGCCT	
AcqRT-f	CCAAGTCCAACCGTGAGAAG	Used for the internal
AcqRT-r	GAAGCGTACAGCGACAGAAC	control of qRT-PCR detection
LysM1qRT-f	CCAATGTCGGGCAGGAAATC	Used for the
LysM1qRT-r	GCGGTAGTAGCGGTCTCAT	transcript level detection of *LtLysM1* gene

3.2.4 Pathogenicity Tests of Overexpressed and RNAi Transformants of LtLysM1 Gene

The LtLysM1 open reading frame (ORF) was amplified with primer pairs LysM1OEf/LysM1OE-r (Table 1) and then subcloned downstream of the PtrpC promoter in the modified pKSNTP vector. Afterwards, the fusion construct, referred to as pKSNTP:LtLysM1, was transformed into *L. theobromae* protoplast using polyethylene glycol (PEG)-mediated transformation method as described by Yan et al. (2018). Resultant transformants were screened against neomycin resistance and confirmed by qRT-PCR analysis. Two LtLysM1 overexpressed transformants were selected for subsequent pathogenicity tests on detached *Vitis vinifera* cv. 'Summer Black'. Wounds of green grapevine shoots were inoculated with mycelium plugs cut with a cork-borer and then maintained under an alternating dark/light cycle in a growth chamber with 70% humidity and 25°C. Lesion lengths of inoculated shoots were measured at 72 hpi. At least six biological replicates of per LtLysM1 overexpression transformant were performed.

For RNAi transformation, we amplified sense fragment with primer pairs LysM1RNAi-Sf/LysM1RNAi-Sr and antisense fragments with primer pairs LysM1RNAiASf/LysM1RNAi-ASr, respectively, and then ligated both fragments into the pRTN vector in the given order. Subsequently, the fusion vector, named as pRTN:LtLysM1, was transformed into the *L. theobromae* protoplast with methods similar to overexpression vector transformation. The protocols used for pathogenicity tests of RNAi transformants were also similar to that of the overexpressed transformants.

3.2.5 Functional Validation of Signal Peptide of LtLysM1 using a Yeast Signal Peptide Sequence Trap System.

To validate the function of LtLysM1 signal peptide, a yeast signal peptide trap assay was performed (Jacobs et al., 1997; Klein et al., 1996; Lee & Rose, 2012). We engineered a pSUC2:LtLysM1 fusion construct, in which the signal peptide of LtLysM1 was in frame upstream the truncated yeast invertase which lacks its signal peptide. Subsequently, the fusion construct pSUC2:LtLysM1 was introduced into invertase secretion-defective yeast YTK12 using the Yeastmaker Yeast Transformation

System 2 kit (TaKaRa, Tokyo, Japan). The resulting transformants were tested for their growth CMD-W (0.67% yeast nitrogen base without amino acids, 0.075% tryptophan dropout supplement, 0.1% glucose, 2% sucrose and 2% agar) and YPRAA (1% yeast extract, 2% peptone, 2% raffinose, 2 μg/ml antimycin A) media, respectively. Transformants expressing pSUC2:Avr1b and pSUC2:Mg87 were used as positive control and negative control, respectively (Fang et al., 2016; Jacobs et al., 1997). All the yeast transformants were incubated at 28°C for 4 days to observe their growth and for then photographed.

3.2.6 *Agrobacterium tumefaciens* Mediated Transient Expression of LtLysM1 in *N. benthamiana*

The LtLysM1 ORF was cloned with primers listed in Table 1 and ligated to the transient expression vector p35S-GFP. The fusion vector p35S-GFP:LtLysM1 was transformed into A. tumefaciens strain GV3101 using the freeze-thaw method, respectively (Fang et al., 2016). The recombinant A. tumefaciens strains were cultured in a shaking incubator at 28°C overnight followed by centrifugation at 5,000 rpm for 10 min. The precipitates were washed three times with sterile water and then resuspended in infiltration buffer (10 mM MES, pH 5.7, 10 mM MgCl2, and 150 μM acetosyringone) to get an optical density value OD600 = 0.5. Then, A. tumefaciens transformants were infiltrated into 4-week-old *N. benthamiana* leaves with a sterile syringe.

3.2.7 Protein Extraction and Immunoblotting Analysis

For protein extraction, N. benthamiana leaves harvested at 48 hours post infiltration with *A. tumefaciens* were ground into powder in liquid nitrogen and then incubated with RIPA lysis buffer (Beyotime Biotechnology, Nanjing, China) on ice for 10 min followed by centrifugation at 12,000 rpm for 20 min at 4°C. Subsequently, the supernatant solution was mixed with 5× loading buffer and then boiled for 10 min. Next, protein extracts were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically blotted onto a PVDF membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% skim milk in TBST buffer (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20) for 1h at room temperature and further incubated with anti-GFP antibody (1:2,000, Cell

Signaling Technology, Danvers, MA, USA) for 1 h at room temperature, followed by three washes (10 min each) with TBST buffer. After rinsing thoroughly, the membranes were incubated with a luminolbased enhanced chemiluminescence horseradish peroxidase substrate (Thermo Fisher Scientific, Waltham, MA, USA) followed by exposure with X-film.

3.2.8 *N*-glycosylation Analyses

LtLysM1 protein was transiently expressed in *N. benthamiana* via the p35S-GFP vector, respectively, and then extracted at 48 h post infiltration with the methods described above. Total proteins were treated with PNGase F and endoglycosidase H (Endo H; New England Biolabs, Beverly, MA, USA) according to the manufacturer's directions followed by immunoblotting analyses.

3.2.9 Yeast two Hybrid Assay

The ORF of *LtLysM1* without signal peptide sequence was amplified using the primer pairs listed in Table 1 and subcloned into pGBKT7 as the bait vector *pGBKT7:LtLysM1*. Additionally, the same fragment was ligated to pGADT7 as the prey vector *pGADT7:LtLysM1*. Subsequently, both *pGBKT7:LtLysM1* and *pGADT7: LtLysM1* were co-transformed into yeast strain AH109 using the Yeastmaker Yeast Transformation System 2 kit (TaKaRa). The resulting transformants were tested for their growth on synthetic dropout SD/-Leu/-Trp/-His medium.

3.2.10 Pull Down Assay

The LtLysM1 ORF was cloned with primers listed in Table 3.1 and ligated to the transient expression vectors pGWB414 and p35S-GFP, respectively. The fusion vector *pGWB414:LtLysM1* and *p35S-GFP:LtLysM1* were transformed into *A. tumefaciens* strain GV3101 using the freeze-thaw method, respectively (Fang et al., 2016). The recombinant *A. tumefaciens* strains were infiltrated into *N. benthamiana* leaves for further protein expression. The *A. tumefaciens* infiltration and protein extraction were performed using the methods described above. Subsequently, the isolated proteins were incubated with GFP-Trap MA beads for 2 h at 4oC. Subsequently, the GFP-Trap MA beads were magnetically separated and repeatedly washed twice with washing buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM

EDTA). Next, the GFP-Trap MA beads were resuspended in 80 µl 2× SDS-sample buffer and then boiled for 10 min at 95oC to dissociate immunocomplex from GFP-Trap MA beads. The beads were magnetically separated and immunoblotting analyses were performed with the supernatant according to the methods described above.

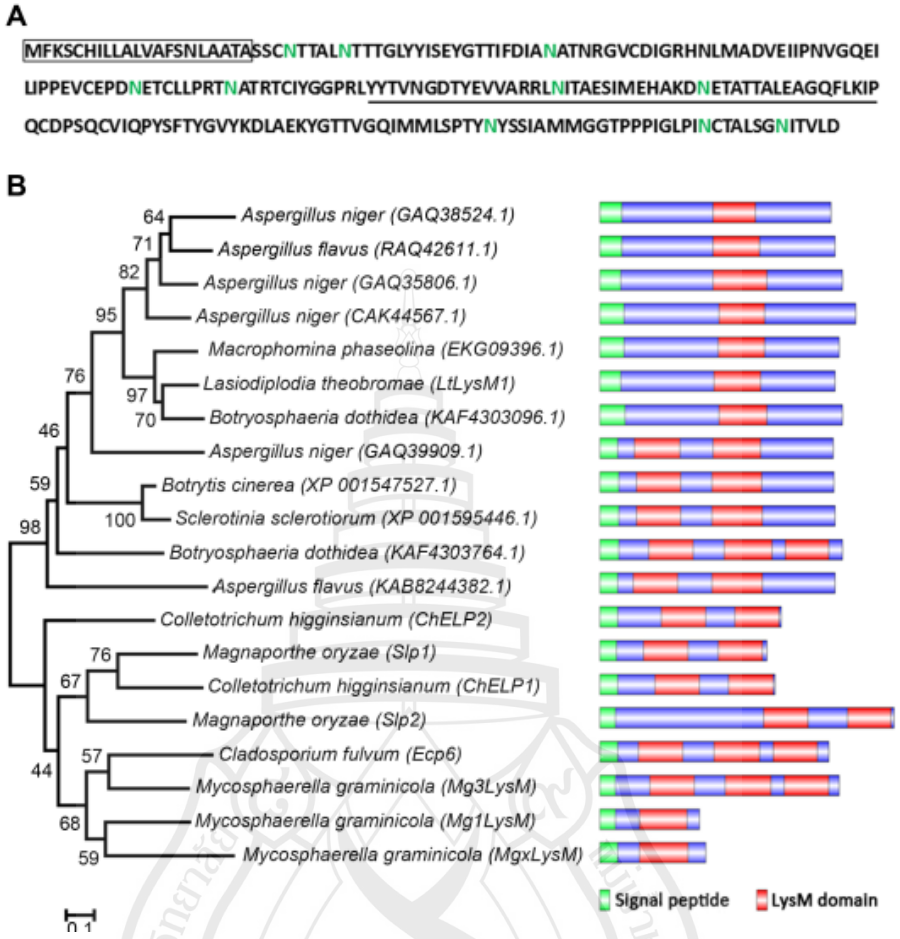
3.3 Results

3.3.1 Structural Features of LtLysM1

Based on SignalP 5.0 program, LtLysM1 was predicted to contain a signal peptide with 21 amino acids, suggesting that LtLysM1 may be a secreted protein. BLASTP search against Pfam database reveals that LtLysM1 contains a typical LysM domain (PF01476, amino acid residues 111-156). Using *N*-GlycoSite tool (https:// www.hiv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html), *LtLysM1* was annotated to contain ten putative N-glycosylation sites, located at Asn25 (NTT), Asn^{30} (NTT), Asn^{50} (NAT), Asn^{89} (NET), Asn^{98} (NAT), Asn^{140} (NET), Asn^{197} (NYS), Asn^{216} (NCT), and Asn^{223} (NIT), suggesting that LtLysM1 may be a glycoprotein (Figure 3.1A). Moreover, phylogenetic analyses of LtLysM1 with other reported LysM proteins including Slp1 and Slp2 in *M. oryzae*, Ecp6 in *C. fulvum*), ChELP1 and ChELP2 in *C. higginsianum*, and Mg1LysM, Mg3LysM, and MgxLysM in *M. graminicola*, were carried out to reveal their relatedness to each other (Figure 3.1B). The phylogenetic tree shows LtLysM1 shares certain homology with other LysM proteins and LysM proteins are distributed widely across fungal pathogens.

3.3.2 Expression Profile of LtLysM1 during *L. theobromae* Infection

As LtLysM1 is predicted to be a secreted protein based on bioinformatics analyses, we attempt to examine the relative transcript level of LtLysM1 at different infection stages by qRT-PCR assays. Results showed that expression levels of *LtLysM1*were highly induced at 36 and 48 hpi, equal to about thirty and forty times of that at mycelial stage (Figure 3.2), indicating LtLysM1 may be involved in the virulence of *L. theobromae* during the infection process.



Note (A) Amino acid sequence analyses using different programs. Signal peptide prediction was performed with SignalP 5.0 Server. Amino acids with black box indicate the putative LtLysM1 signal peptide. N-glycosylation sites were predicted using the N-GlycoSite tool. Amino acids in green color denote predicted N-glycosylation sites. Amino acids underlined mark the putative LysM domain. (B) The LysM proteins are distributed widely across fungal pathogen. The amino acid sequences of LysM proteins from different fungi species were sourced from NCBI database and then used to generate the phylogenetic tree through MEGA7 with the neighbor-joining method, 1,000 replicates. Bootstrap percentage support for each branch is indicated at the nodes. The number and location of LysM domain were predicted using BLASTP analyses against Pfam database with each amino acid sequence as the query.

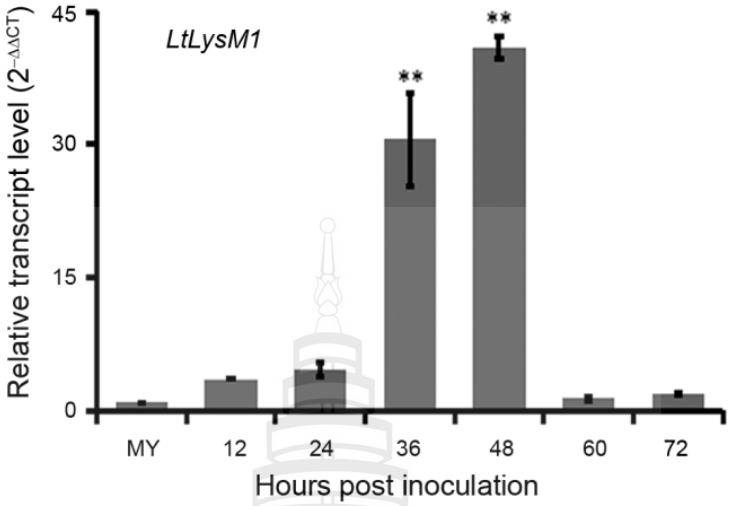
Figure 3.1 Structural and phylogenic analyses of *LtLysM1* in *Lasiodiplodia theobromae*

3.3.3 The Importance of LtLysM1 for the Virulence of *L. theobromae*

To characterize the contribution of LtLysM1 to the virulence of *L. theobromae*, *LtLysM1* gene was overexpressed and silenced in vivo via PEG-mediated protoplast transformation, respectively. The resultant transformants were screened for neomycin resistance and confirmed by qRT-PCR analysis. Two of each overexpressed and RNAi transformants of *LtLysM1* were selected for further phenotypic analyses. Colony diameter comparisons between overexpressed transformants (LtLysM1-OE1 and LtLysM1-OE2), RNAi transformants (LtLysM1-RNAi1 and LtLysM1-RNAi2), and wild type CSS-01s showed that overexpression of *LtLysM1* did not affect the vegetative hyphae growth of *L. theobromae* on PDA media, and RNAi of *LtLysM1*, however, has shown a significantly effect on the hyphal growth (Figure 3.3A), suggesting that a certain expression level of *LtLysM1* is necessary for the vegetative hyphae growth of *L. theobromae*.

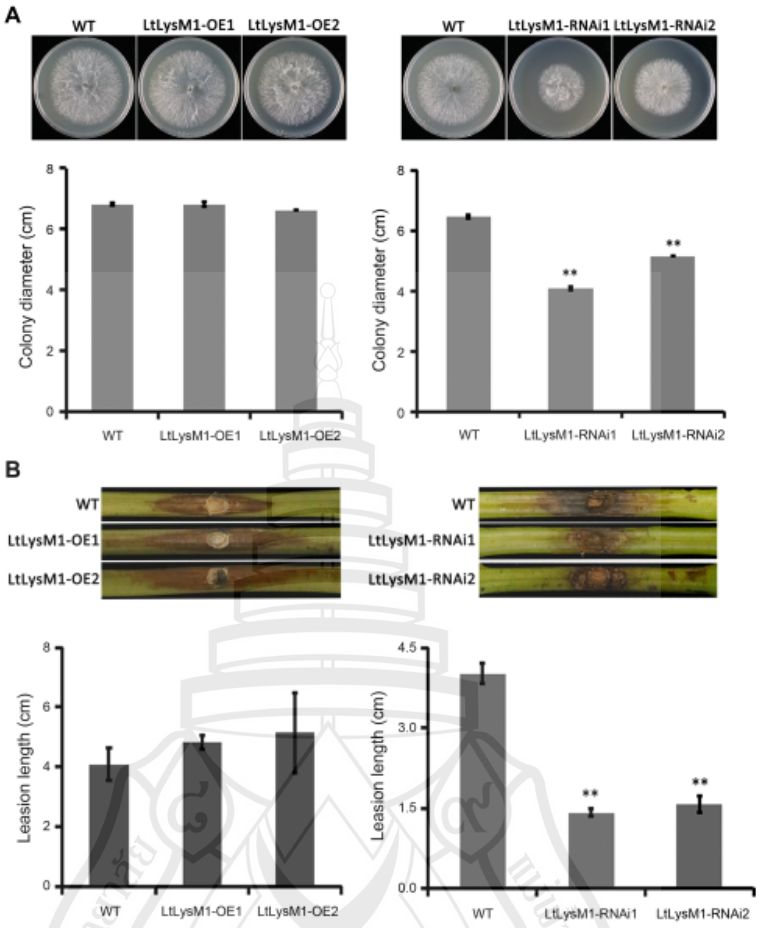
3.3.4 Functional Validation of LtLysM1 Signal Peptide

To verify the function of *LtLysM1* signal peptide, a yeast signal sequence trap system was adopted. We constructed a *pSUC2:LtLysM1* fusion vector, in which the signal peptide of *LtLysM1* was in frame to yeast invertase which lacks its own signal peptide. Subsequently, the fusion vector *pSUC2:LtLysM1* was transformed into yeast YTK12 strain which was defective in invertase secretion. Yeast transformants expressing N-terminal sequences of *Phytophthora sojae Avr1b* and *M. oryzae Mg87* were used as positive and negative controls, respectively. Yeast transformants carrying pSUC2 vector grew on CMD-W media. However, only transformants expressing *pSUC2:LtLysM1* or the positive control grew on YPRAA medium with raffinose as the sole carbon source (Figure 3.4). These results indicate that *LtLysM1* signal peptide guides the raffinose secretion in yeast cells and that LtLysM1 is a secreted protein with a functional signal peptide.



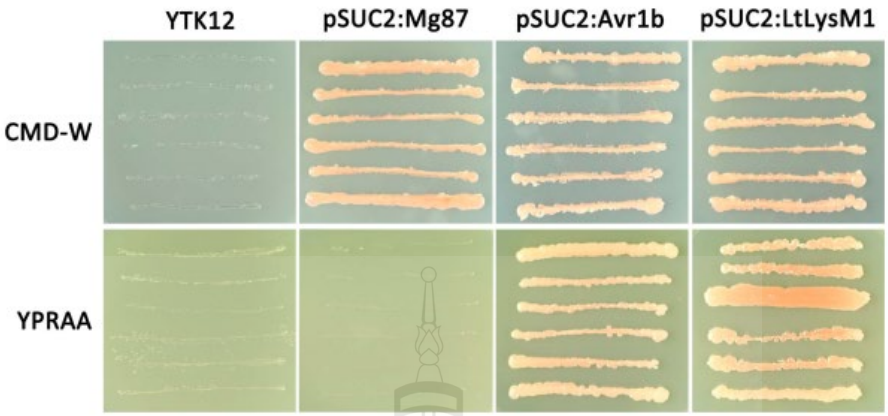
Note The mycelia (MY) of wild type CSS-01s cultured in liquid complete medium for 36 h and grapevine tissues infected by wild type CSS-01s were harvested at 12, 24, 36, 48, 60, and 72 hours post inoculation (hpi) for RNA extractions. The isolated RNA was reverse transcribed into cDNA for LtLysM1 gene expression analyses. Relative transcript levels of *LtLysM1* at different time points post inoculation were normalized by actin gene and calibrated against that of mycelia. Relative transcript level of *LtLysM1* was calculated using the 2-ΔΔCT method. The assays were performed with three independent biological repetitions and three replicates each. A representative set of data are presented. Data are means ± standard error. Asterisks represent significant difference (LSD test, $P < 0.01$).

Figure 3.2 Relative transcript levels of LtLysM1 at differently infectious stages



Note (A) Colonial morphology and diameter comparison of wild type CSS-01s, overexpressed transformants (LtLysM1-OE1 and LtLysM1-OE2), and RNAi transformants (LtLysM1-RNAi1 and LtLysM1-RNAi2) on potato dextrose agar media. Colony diameter was measured and Images were photographed at 36 h post inoculation. Asterisks represent statistically significant difference (least significant difference [LSD] test, **P < 0.01). (B) Pathogenicity assays of the strains mentioned in A. One-year-old grapevine shoots were inoculated with mycelial plugs (5 mm in diameter), and then contained in a chamber at 28ºC. The inoculated shoots were photographed at 3 days post inoculation. At least six biological replicates of per LtLysM1 transformant were performed. Error bars represent standard error of six replicates. Asterisks denote statistically significant difference (LSD test, **P < 0.01).

Figure 3.3 *LtLysM1* is important for the virulence and vegetative growth of *Lasiodiplodia theobromae*



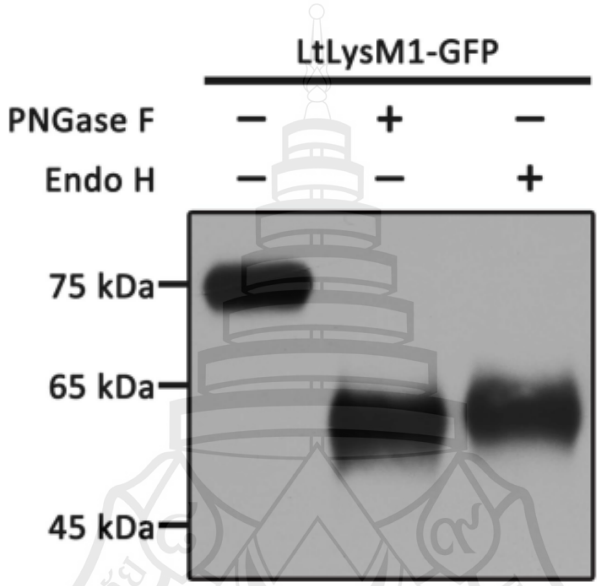
Note Yeast YTK12 transformed with *pSUC2:LtLysM1* fusion vector were plated on CMD-W and YPRAA media, respectively. The signal peptide of LtLysM1 was able to guide the secretion of yeast invertase and therefore yeast transformants expressing *pSUC2:LtLysM1* grew on YPRAA plate with raffinose as sole carbon source. The untransformed strain YTK12 grew neither on CMD-W medium nor on YPRAA medium. Transformants expressing *pSUC2:Avr1b* and *pSUC2:Mg87* were used as positive and negative controls, respectively.

Figure 3.4 Functional validation of the predicted signal peptide of LtLysM1

3.3.5 *N*-glycosylation Analyses of LtLysM1

In previous studies, many secreted pathogenic effector proteins were reported to be *N*-glycosylated in vivo (Chen et al., 2014; Fang et al., 2016). Therefore, we set out to investigate whether LtLysM1 shared similar molecular feature. During the preliminary studies, we found that LtLysM1 contains ten putative *N*-glycosylation sites based on NGlycoSite prediction. To substantiate the posttranslational modifications, proteins transiently expressed in *N. benthamiana* were initially digested by PNGase F, an amidase that cleaves between the innermost GlcNAc and asparagine residues of high mannose and complex oligosaccharides from N-linked glycoproteins, and Endo H, a recombinant glycosidase which cleaves within the chitobiose core of high mannose and some hybrid oligosaccharides from N-linked glycoproteins (Chen et al., 2014), respectively. Subsequently, each digestion was further examined by immunoblotting

analyses with anti-GFP antibody. Results showed that proteins digested with PNGase F and Endo H displayed smaller band size compared to that of untreated mature LtLysM1 protein (Figure 3.5), indicating that LtLysM1 was an *N*-glycosylated protein in plant cell.



Note LtLysM1 was transiently expressed in *Nicotiana benthamiana* leaves and then extracted for deglycosylation analyses. Total protein extractions were digested with or without PNGase F or endoglycosidase H (Endo H) at 37ºC for 1 h and then examined by immunoblotting analyses with an anti-GFP antibody.

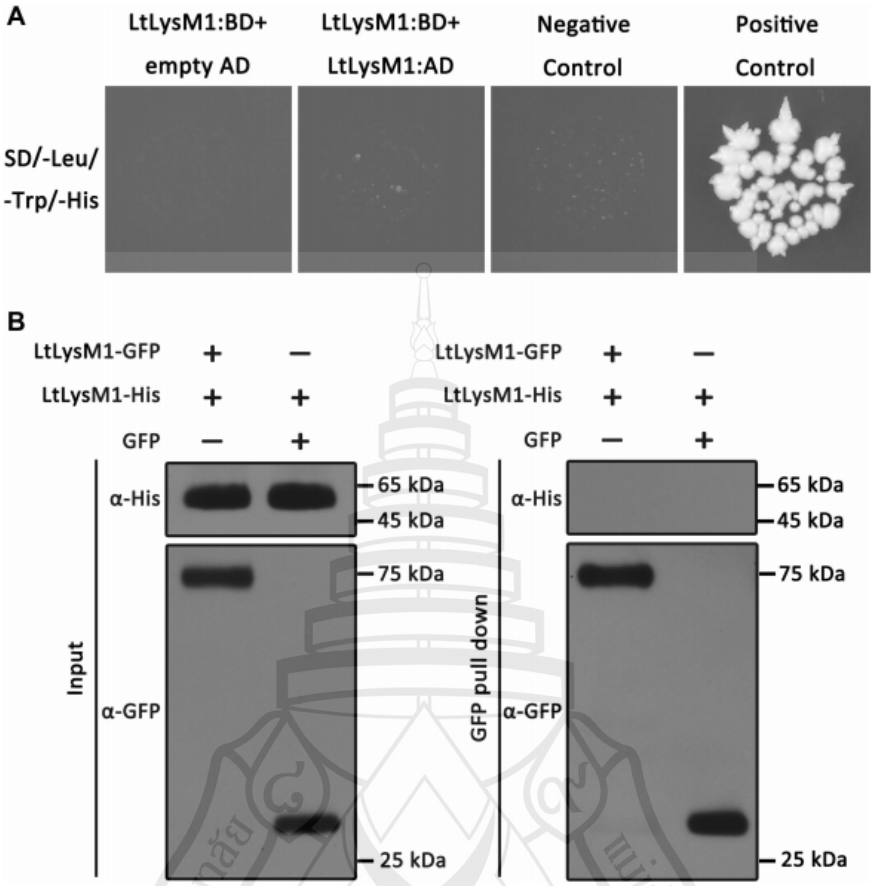
Figure 3.5 LtLysM1 is an *N*-glycosylated protein in plant cells

3.3.6 Self-interaction Analyses of LtLysM1 Protein

The LysM proteins including Slp1 in *M. oryzae* (Mentlak et al., 2012) and OsCEBiP in rice were characterized to be able to form dimer complexes for their molecular functions (Shimizu et al., 2010). To investigate whether the dimerization occurs in LtLysM1 molecules, we constructed the prey vector *pGADT7:LtLysM1* and bait vector *pGBKT7:LtLysM1*, in which the encoding sequences of LtLysM1 signal peptide were removed, and then transformed both the vectors into yeast strain AH109

simultaneously. Resultant yeast transformants were detected for their growth on synthetic dropout media. Results showed that yeast transformants expressing LtLysM1 did not grow on SD/-Leu/-Trp/-His media (Fig. 3.6A), suggesting that LtLysM1 molecule did not have the capacity to interact with itself.

To supply more cogent evidences, further GFP pull down experiments were conducted. The LtLysM1-GFP proteins isolated from *N. benthamiana* leaves were incubated with LtLysM1-His proteins extracted from *N. benthamiana leaves.* Protein immunoblotting analyses showed that LtLysM1 was not pulled down by itself (Fig. 3.6B), which was consistent with results demonstrated above. These results strongly supported that LtLysM1 molecule did not interact with itself physically and homodimerization complexes were not formed among LtLysM1 molecules.



Note (A) Yeast cells expressing prey vector *LtLysM1:AD* and bait construct *LtLysM1:BD* were tested for their growth on SD/-Leu/-Trp/-His media. In both vectors, the encoding sequence of LtLysM1 signal peptide were removed off. Yeast transformant carrying empty prey vector *pGADT7* with empty bait vector *pGBKT7* was used as negative control. Yeast transformant carrying *pGADT7-T* and *pGBKT7-53* vectors was used as positive control. Plates were photographed at 3 days post inoculation. (B) LtLysM1 did not interact with itself via GFP pull down experiments. Recombinant proteins LtLysM1-GFP and LtLysM1-His from Nicotiana benthamiana were subjected to GFP pull down analyses. Interactions between LtLysM1 molecules were detected through immunoblotting tests with anti-GFP and anti-His antibodies

Figure 3.6 LtLysM1 molecule does not interact with itself

3.4 Discussion

Lasiodiplodia theobromae, a woody plant pathogen belonging to Botryosphaeriaceae family, has received much attention recently for the considerable damage caused by this fungus in fruiter's industry. Research on this fungus have made great progress during the past two decades including strain isolation, diseases symptoms characterization, enzymatic activity analyses, and genome and transcription analyses (Cao et al., 2020; Félix et al., 2019; Gonçalves et al., 2019; Paolinelli-Alfonso et al., 2016; Úrbez-Torres et al., 2008; Yan et al., 2018). However, investigation on the pathogenic mechanism are still at a preliminary stage and remain stagnant because of the multinuclear cell structure of this fungus, which seriously constrains the genetic manipulations in molecular studies. Here, we set out to expand the molecular research on this fungus and perform an investigation on the biological functions and molecular features of a LysM domain containing protein LtLysM1 in *L. theobromae*, and therefore, this study means the first step in molecular research of this fungus.

LysM proteins have been documented in a wide range of organisms including animals, plants, fungi, and bacteria (Buist et al., 2008; Kombrink et al., 2011; Sánchez-Vallet et al., 2013). Some LysM proteins were characterized as virulence factors in plant pathogenic fungi (de Jonge et al., 2010; Kombrink et al., 2017; Marshall et al., 2011; Mentalk et al., 2012; Takahara et al., 2016). Here, we found LtLysM1 was a pathogenicity-related protein during infection process upon qRT-PCR analyses. Because of the lack of effective and efficient gene deletion strategy on multinuclear protoplast, we performed overexpression and gene-silencing of LtLysM1 gene. The pathogenicity assays revealed that LtLysM1 is contributed to the full virulence of *L. theobromae*, confirming the results of qRT-PCR tests. Moreover, *LtLysM1* was proven to be important for the mycelial growth in RNAi transformants of *LtLysM1*, but overexpressed transformants of *LtLysM1* did not exhibit increased mycelial growth. It is plausible that a certain expression level of *LtLysM1* is sufficient for maintaining mycelial growth under normal growth condition. Therefore, *LtLysM1* is an important regulator that mediates the mycelial development in *L. theobromae*. Moreover, LtLysM1 was proven to be a small secreted protein based on the yeast signal peptide

trap system which has also been widely used to identify the signal peptide in other fungi (Fang et al., 2016; Gu et al., 2011; Oh et al., 2009). These data indicate that LtLysM1 can be secreted to the outside cellular space during plant-pathogen interaction and plays an important role in disease symptom development.

N-Glycosylation has been reported to be an important feature for the function of effector proteins. For example, the *N*-glycosylation of Slp1 in *M. oryzae* was required to suppress host immunity and additionally regulate the chitin-binding ability of Slp1 (Chen et al., 2014). Also, the *N*-glycosylation of UV_6205 and UV_1423 in *Ustilaginoidea virens* were involved in regulating host cell death (Fang et al., 2016). In this study, we transiently expressed LtLysM1 protein in *N. benthamiana* via two expression systems and treated the protein extracts with two enzymes PNGase F and Endo H. Proteins digested with two enzymes exhibited smaller band size when separated on 12% SDS-PAGE, revealing LtLysM1 was an *N*-glycosylated protein. Additionally, a total of ten *N*-glycosylation sites were predicted based on our predicted, whereas we did not examine the actual *N*-glycosylation sites of this protein. More questions such as the biological functions and molecular roles of *N*-glycosylation in this protein also need further investigation.

Molecular dimerization has been reported in many effector proteins. *Slp1* in *M. oryzae* was reported to be capable of forming protein aggregates based on protein-protein interaction (Mentlak et al., 2012). Another two LysM receptor molecules OsCERK1 with CEBiP were able to form a hetero-oligomer complex to induce downstream immune responses (Shimizu et al., 2010). Plant pathogenic effectors form complexes to overcome plant immunity to promote disease by interacting with themselves or with other effector proteins (Alcântara et al., 2019). *PsCRN63* in *P. sojae* was capable of suppressing PCD associated with PTI via self-dimerization (Li et al., 2016). However, it was found that LtLysM1 molecule did not interact with itself based on yeast two hybrid assay. This may be due to structural and functional differences in comparison to other LysM proteins.

Many published LysM effectors were characterized with multiple LysM domains. For example, Ecp6 (SánchezVallet et al., 2013), Mg3LysM (Marshall et al., 2011), and ChELP7 (Takahara et al., 2016) were identified to contain three LysM domains. Slp1 (Mentlak et al., 2012), ChELP1, ChELP2 (Takahara et al., 2016), and

Vd2LysM (Kombrink et al., 2017) were reported to contain two LysM domains. Moreover, structural analyses of Ecp6 revealed that composite LysM1-LysM3 binding site provides a single binding event with ultra-high affinity for chitin binding, whereas the LysM2 may be involved in suppression of chitin-triggered immunity by preventing the immune receptor dimerization (Sánchez-Vallet et al., 2013). Unlike these effectors, LtLysM1 was predicted to contain a single LysM domain, implying that LtLysM1 may function in a different manner. Thus, further structural analyses will be conducive to reveal the molecular functions of LtLysM1.

CHAPTER 4

CONTRIBUTONS TO THE TAXONOMY OF ASCOMYCETES

4.1 Taxonomic Contributions to Family *Didymellaceae*

Didymellaceae Gruyter, Aveskamp & Verkley

Neodidymelliopsis Qian Chen & L. Cai

Notes: *Neodidymelliopsis* was introduced by Chen et al. (2015), with *Neodidymelliopsis cannabis* (G. Winter) Q. Chen & L. Cai as the type species (Chen et al., 2015; Hyde et al., 2016)

Neodidymelliopsis farokhinejadii S.A. Ahmadpour & M. Mehrabi-Koushki, Sydowia 69: 175 (2017)

Facesoffungi number: FoF04975; (Figure 4.1)

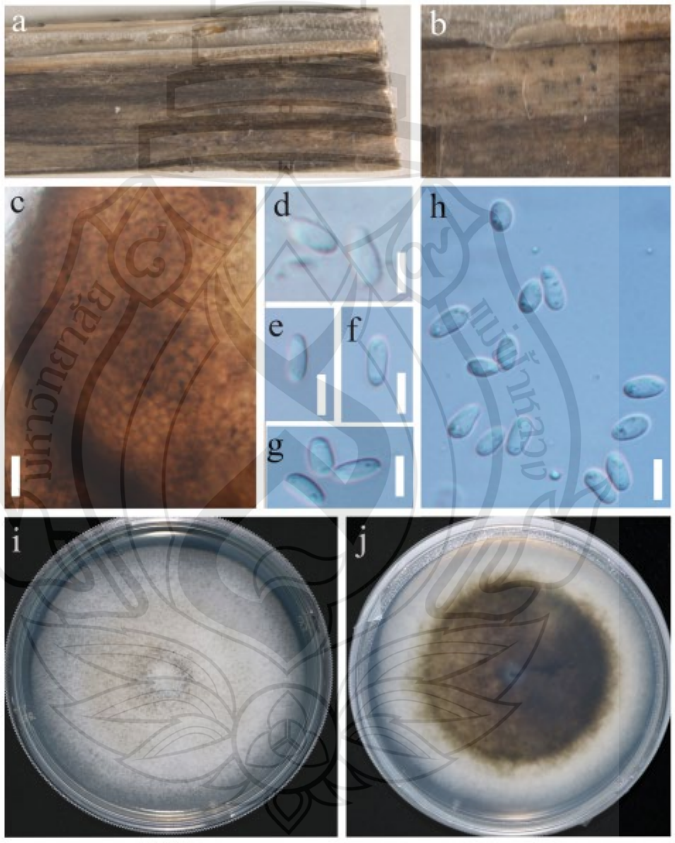
Saprobic or *necrotrophic* on dead arial stem of *Cirsium* sp. *Sexual morph* Undetermined. *Asexual morph* Coelomycetous. *Conidiomata* on host 75–105 µm diam. (x = 74 µm, n = 10), pycnidial, solitary, scattered, globose to subglobose, black, semi-immersed to immersed; pycnidial wall pseudoparenchymatous, textura angularis dark brown to hyline cells. *Chlamydospores* not observed. *Conidiophores* not observed. *Conidiogenous cells* not observed. *Conidia* on host 4.5–7.2 9 2.3–3.6 µm diam. (x = 6 9 3.2 µm, n = 40), oblong to ellipsoid, hyaline, aseptate, smooth-walled; in culture, 4.6–7.5 9 2.4–3.9 µm diam. (x = 6 9 3.4 µm, n = 40), ellipsoidal to cylindrical, sometimes allantoid, hyaline, smooth- and thin-walled, aseptate, with small polar guttules.

Culture characteristics: Colonies on PDA reach 25–30 mm diam. after 7 days at 25 °C, with white aerial mycelium, surface floccose to wooly, margin regular, pale greenish olivaceous; reverse dull green.

Material examined: ITALY, Forlı `-Cesena Province, near Castrocaro Terme, on dead aerial stem of *Cirsium* sp. (Asteraceae), 25 February 2018, Erio Camporesi, living culture (MFLUCC 18-1569).

Genbank numbers: ITS: MK084580, LSU: MK084581.

Notes: *Neodidymelliopsis farokhinejadii* was first reported on dead branches of *Eucalyptus* sp. and was subsequently found on *Citrus limon*, *Conocarpus erectus*, *Ziziphus* sp., *Juglans regia* and *Cupressus* sp. (Ahmadpour et al., 2017) Our new isolate of *N. farokhinejadii* was collected from Forlı `-Cesena Province in Italy. This is the first report of *N. farokhinejadii* on *Cirsium* species (Farr & Rossman, 2019). Morphological observations such as spore characteristics support the inclusion of our taxa into *Neodidymelliopsis* (Chen et al., 2015). DNA sequence analyses from the LSU, ITS, TUB2 and RPB2 genes confirms that our taxon clades together with *N. farokhinejadii*.



Note a, b Conidiomata on the host tissue. c Section of the pycnidial wall.d–h Conidia. i Upper view of 7-day old culture. j Reverse view of 7-day old culture. Scale bar: c–h = 10 µm

Figure 4.1 *Neodidymelliopsis farokhinejadii* (JZB–H380023, new host record)

Nothophoma quercina (Syd. & P. Syd.) Q. Chen & L. Cai, Stud. Mycol. 82: 213. 2015.

Facesoffungi number: FoF04974; Figure 4.2

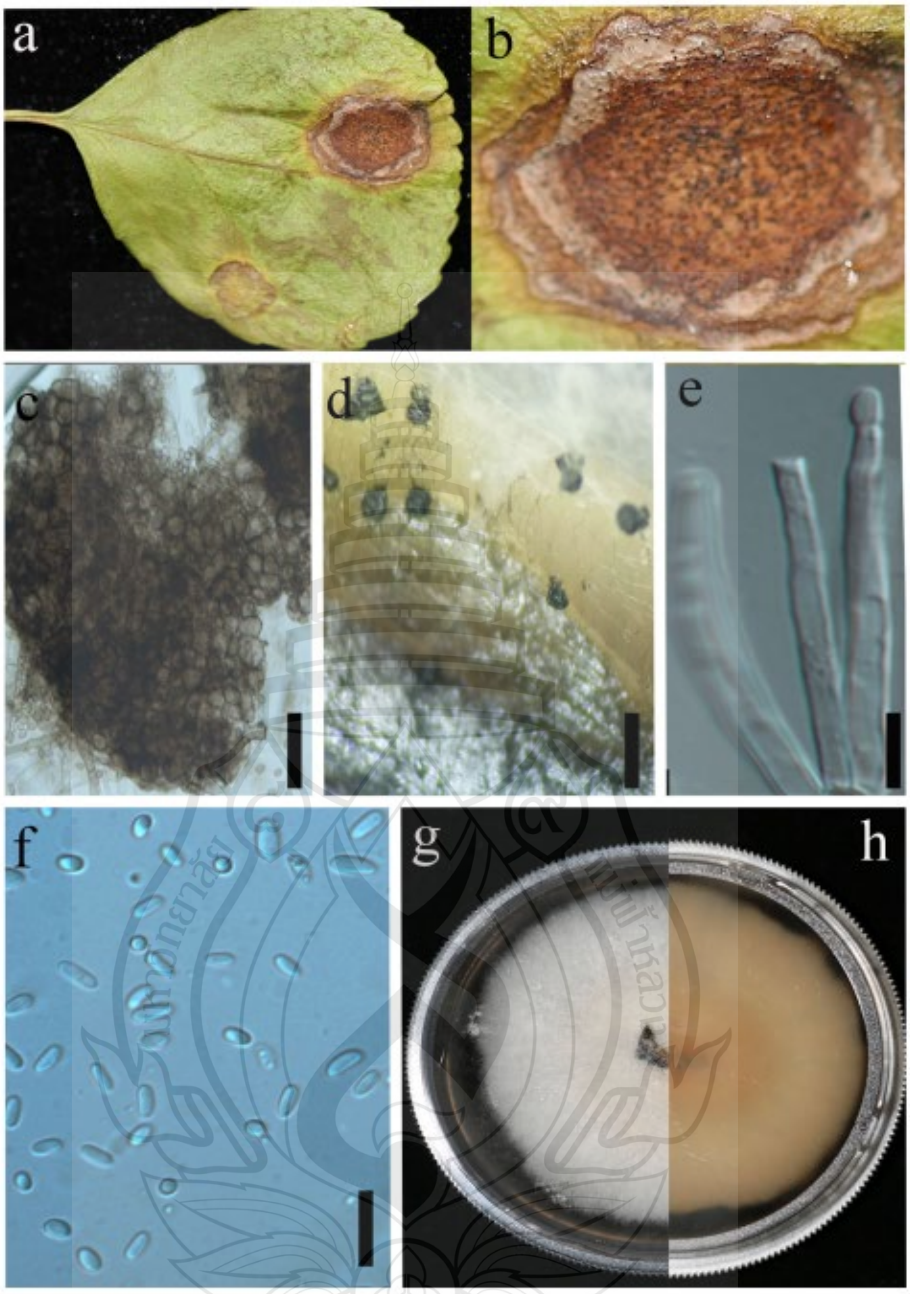
Pathogenic on leaves of *Buxus megistophylla* H.Le´v. *Sexual morph* Undetermined. *Asexual morph Conidiomata* produced on the PDA surface, 0.19–0.47 mm (x = 0.25 mm, n = 10) diam., pycnidial, solitary, scattered, globose to irregularly shaped, black, ostiolate. Pycnidial wall multi-layered, composed of pale brown, pseudoparenchymatous cells. *Conidiogenous cells* phialidic, hyaline, simple, doliiform to ampulliform, variable in size. *Conidia* 4–5.6 9 1.4–3.3 µm (x = 5.2 9 2.3 µm, n = 40), variable in size and shape, subglobose to oval or obtuse, initially hyaline, but brown at maturity, aseptate, smooth-walled. Conidial exudates not recorded.

Culture characteristics: Colonies on PDA reach 80 mm diam. after 7 days at 28 °C, with regular margin, dull white aerial mycelium surface floccose to wooly, greenish olivaceous to olivaceous near the centre and reverse dark ochreous in the centre and white in the margin.

Material examined: CHINA, Haidian District, Beijing, on leaves of *Buxus megistophylla* (Buxaceae), November 2017, D. Harishchandra, living culture (JZB380024, MFLUCC 18-1568)

GenBank numbers: ITS: MK070136, LSU: MK070139.

Notes: *Nothophoma quercina* has been reported as a pathogen on *Pistacia vera* (Chen et al., 2013), *Chaenomeles sinensis* (Yun & Oh, 2016), *Olea europaea* (Moral et al., 2017) and was reported as a saprobe on *Quercus* sp. In Ukraine (Chen et al., 2015). This is the first record of *N. quercina* on *Buxus* sp. Phylogenetic analysis with the combined sequence data of ITS, TEF-1, TUB2 and RPB2 of our strain (MFLUCC 18-1568) clusters together with the ex-type strain of *Nothophoma quercina* (CBS 633.92) with relatively high bootstrap and Bayesian probabilities (99% MP/1.00 PP).



Note a, b Conidiomata on the host tissue. c Section of the pycnidial wall. d Pycnidia on PDA. e Conidiogenous cells on culture. f Conidia on culture. g Upper view on colonies on PDA. h Lower view on colonies on PDA. Scale bar: c = 1 mm, d–f = 20 µm

Figure 4.2 *Nothophoma quercina* (JZB380024, new host record)

Diplodia Fr., Annales des Sciences Naturelles Botanique 1: 302 (1834)

The genus *Diplodia* was introduced by Montagne in 1834 with *Diplodia mutila* (Fr.) as the type species. It is a large genus with ca. 1000 species names recorded in Index Fungorum. 31 of which are known from culture (Phillips et al., 2013; Dissanayake et al., 2016; Gonzalez-Domínguez et al., 2017; Yang et al., 2017). Species in this genus can be pathogens, endophytes or saprobes on a wide range of woody hosts with some being important pathogens causing severe damage to many important plant species (Phillips et al., 2012).

4.2 Taxonomic Contributions to Family Botryosphaeriaceae

Diplodia Fr., Annales des Sciences Naturelles Botanique 1: 302 (1834)

The genus *Diplodia* was introduced by Montagne in 1834 with *Diplodia mutila* (Fr.) as the type species. It is a large genus with ca. 1000 species names recorded in Index Fungorum. 31 of which are known from culture (Phillips et al., 2013; Dissanayake et al., 2016; Gonzalez-Domínguez et al., 2017; Yang et al., 2017). Species in this genus can be pathogens, endophytes or saprobes on a wide range of woody hosts with some being important pathogens causing severe damage to many important plant species (Phillips et al., 2012).

Diplodia torilicola Harishchandra, Camporesi, A.J.L. Phillips & K.D. Hyde, sp. nov.

Index Fungorum number: IF 556364; Facesoffungi number: FoF 05980; Figure 4.3

Etymology: Name reflects the host genus *Torlilis*.

Holotype: JZBH3140012.

Saprobic on dead aerial stem of *Torilis arvensis*. Sexual morph: Undetermined. Asexual morph: Coelomycetous. Conidiomata on host 99–160 µm diam ($\bar{x}$ = 128 µm, n = 10) pycnidial, solitary, scattered, globose, black, semiimmersed to immersed. Ostiole single, centrally located. Conidiomata wall 30–40 µm wide ($\bar{x}$ = 34 µm, n = 10) at both sides, multi-layered, brown to black cells of textura angularis. Conidiophores not observed. Conidiogenous cells cylindrical, thin-walled, hyaline 16–

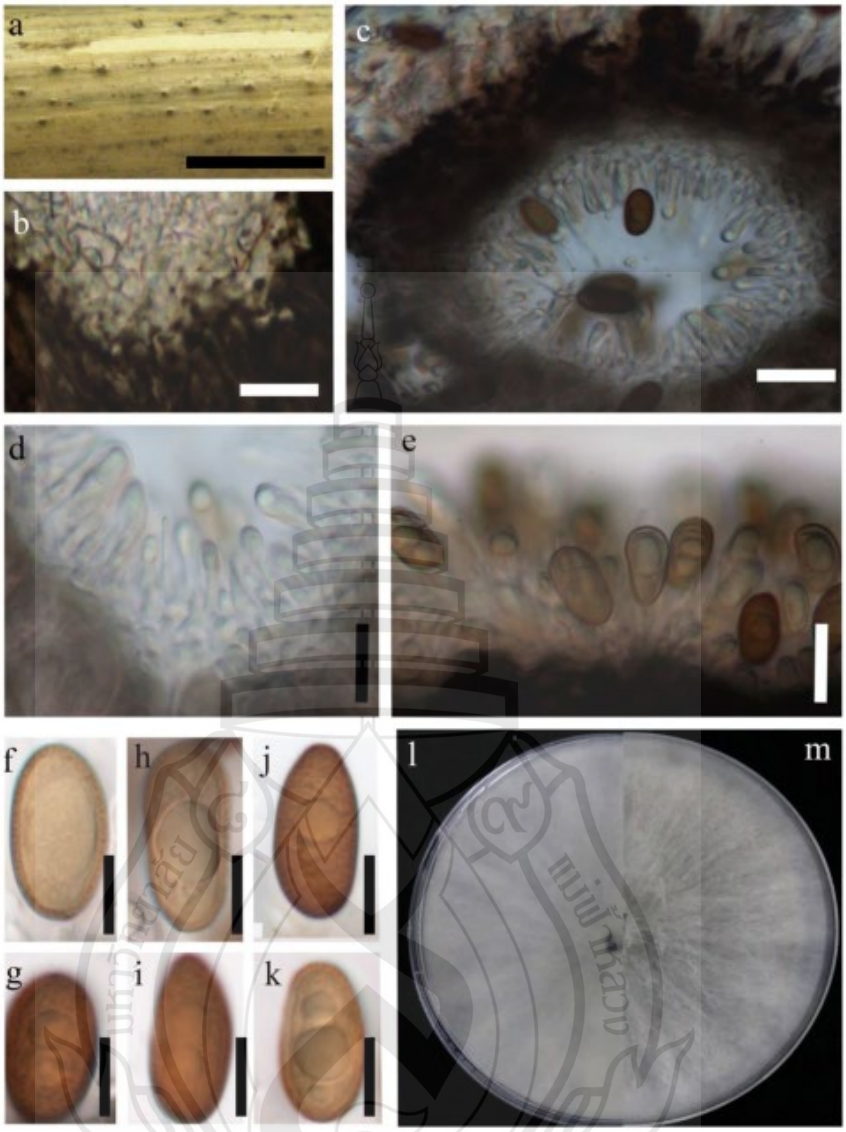
25 µm high × 3–9 µm wide ($\bar{x}$ = 16.8 × 4.6 µm, n = 20) producing a single conidium at the apex. Conidia 17–22 µm long × 8–11 µm wide ($\bar{x}$ =21×8.8 µm, n=40), initially hyaline soon becoming pigmented, dark brown when mature, aseptate, ellipsoid to ovoid with central guttules.

Culture characteristics: Colonies on PDA attaining 90 mm diam within 3 days at 25 °C, with white moderately aerial mycelium and white in reverse. Mycelia becoming dense, cottony and black with age.

Material examined: ITALY, near Castrocaro Terme in Forlì-Cesena Province, on dead aerial stem of *Torilis arvensis*, 11 December 2017, Erio Camporesi, JZBH3140012 (holotype).

GenBank numbers: ITS: MK625223; TEF1: MK640502.

Notes: *Diplodia torilicola* is most closely related to *D. crataegicola* but forms a well-supported independent lineage from it. It differs by possessing larger conidia that are ellipsoid to ovoid (21×8.8 µm) whereas *D. crataegicola* has smaller (14 × 9 µm) globose to subglobose conidia (Ariyawansa et al., 2015). This is the first report of a *Diplodia* species on *Torilis arvensis* (Farr & Rossman, 2019).



Note a Conidiomata on the host tissue. b Section of the pycnidial wall. c Cross section of conidiomata on host. d–e Conidiogenous cells. f–k Conidia. l Lower view of 7-day old culture. m Upper view of 7-day old culture. Scale bars: a= 1000 µm, b–e= 20 µm, f–k= 10 µm

Figure 4.3 *Diplodia torilicola* (JZB3140012, holotype)

4.3 Other Miscellaneous Contributions

4.3.1 Halotthiaceae

Halotthiaceae Ying Zhang, J. Fourn. & K. D. Hyde, Mycologia 105(3): 604 (2013).

Index Fungorum number: IF563123; Facesoffungi number: FoF 08247, 7 species.

Saprobic or pathogenic on terrestrial, freshwater and marine hosts. Sexual morph: Ascomata medium to large in size, immersed, semi-immersed, erumpent or superficial, sometimes present under a pseudoclypeus, mostly ostiolate. Peridium multi-layered, outer layer of small, irregular brown to dark brown, thick walled, pseudoparanchymatous cells, inner layer black to dark brown, sometimes with large lumina or pseudoparenchymatous cells arranged in textura angularis, sometimes textura prismatica. Hamathecium comprising dense or narrowly cellular, septate, simple or branched pseudoparaphyses. Asci 8-spored, bitunicate, fissitunicate, cylindrical, clavate, subclavate to fusiform, long or short pedicellate, with or withour ocular chamber. Ascospores 1–3- seriate, fusiform, clavate, ellipsoid or subellipsoid, sometimes initially hyaline becoming pale brown, dark brown to blackish brown at maturity, 1-septate, phragmosporous, distoseptate or dictyosporous, constricted or not at the septum, with or without gelatinous sheath, without appendages. Asexual morph: in Halotthia pycnidial. Conidiophores simple obclavate. Conidia (or spermatia) subglobose, ovoid or ellipsoidal, hyaline.

Type – *Halotthia* Kohlm

Notes – *Halotthiaceae* was introduced by Zhang et al. (2013c) with the type species Halotthia, and comprised the genera Mauritiana, Phaeoseptum and Pontoporeia (Hyde et al., 2013; Wijayawardene et al.; 2014b). Ariyawansa et al. (2015a) introduced three new genera into this family, *Brunneoclavispora*, *Neolophiostoma* and *Sulcosporium*. Hyde et al. (2018) removed *Phaeoseptum* from *Halotthiaceae* and introduced it to a new family *Phaeoseptaceae*. Prominent and thick septa in the ascospores can be observed in all members of Halotthiaceae, and can be used as a diagnostic characteristic for this family (Zhang et al., 2013c). All the genera included

in this family except *Pontoporeia* are monotypic. Most of the previously introduced genera were identified in freshwater and marine habitats (Suetrong et al., 2009; Zhang et al., 2013c) but *Brunneoclavispora* and *Sulcosporium* were identified from terrestrial habitats (Ariyawansa et al., 2015a). Based on multi-gene phylogenetic analyses, the family formed a monophyletic clade close to *Sporormiaceae, Roussoellaceae, Lophiostomataceae* and *Phaeoseptaceae* in Pleosporales (Suetrong et al., 2009; Zhang et al., 2013c; Ariyawansa et al., 2015a; Hyde et al., 2018).

Halotthia posidoniae (Durieu & Mont.) Kohlm., Nova Hedwigia 6: 9 (1963). Fig. 85

Index Fungorum number: IF 331652; Facesoffungi number: FoF 08848.

Material examined – France, PyrénéeOrientales, dredged near Banyuls-sur-Mer, on *Posidonia oceanica*, 19 May 1962, J. Kohlmeyer No. 808c, (NY 01389657, type).

Other genera included

Brunneoclavispora Phookamsak & K.D. Hyde, Fungal Divers. 75: 71 (2015).

Index Fungorum number: IF 551326; Facesoffungi number: FoF 00893; 1 morphological species (Species Fungorum 2020), 1 species with molecular data.

Type species – *Brunneoclavispora bambusae* Phookamsak & K. D. Hyde, Fungal Divers. 75: 71 (2015).

Notes – *Brunneoclavispora* was reported as saprobic on bamboo. The clavate ascospores are a unique characteristic of *Brunneoclavispora* which differentiates it from other members of *Halotthiaceae*. This genus shows similar morphological characteristics to *Phaeoseptum* but differs in having ascospores with tail-like basal appendages (Zhang et al., 2013c; Ariyawansa et al., 2015a) (see detailed morphology Ariyawansa et al., 2015a).

Mauritiana Poonyth, K.D. Hyde, Aptroot & Peerally, Fungal Diversity 4: 102 (2000).

Index Fungorum number: IF 337503; Facesoffungi number: FoF 08249; 1 morphological species (Species Fungorum 2020), 1 species with molecular data.

Type species – Mauritiana rhizophorae Poonyth, K.D. Hyde, Aptroot & Peerally, Fungal Diversity 4: 102 (2000).

Notes – *Mauritiana* can be distinguished from others of this family by the asci with comparatively shorter pedicel. The genus was reported on *Rhizophora mucronata* (see detailed morphology Poonyth et al., 2000).

Neolophiostoma S. Boonmee & K.D. Hyde, Fungal Divers. 75: 74 (2015).

Index Fungorum number: IF 551404; Facesoffungi number: FoF 00961; 1 morphological species (Species Fungorum 2020), 1 species with molecular data.

Type species – *Neolophiostoma pigmentatum* Boonmee & K.D. Hyde, Fungal Divers. 75: 74 (2015).

Notes – *Neolophiostoma* can be characterized by immersed ascomata with carbonaceous peridium, and hyaline, fusiform ascospores. The genus is saprobic on dead wood (see morphology Ariyawansa et al., 2015a).

Pontoporeia (Durieu & Mont.) Kohlm., Nova Hedwigia 6: 5 (1963).

Index Fungorum number: IF 337503; Facesoffungi number: FoF 08250; 2 morphological species (Species Fungorum 2020), 1 species with molecular data.

Type species – *Pontoporeia biturbinata* (Durieu & Mont.) Kohlm., Nova Hedwigia 6: 5 (1963).

Sphaeria biturbinata Durieu & Mont., in Durieu, Expl. Sci. Alg., Fl. Algér. 1(livr. 13): 497 (1848) [1846-49].

Notes – *Pontoporeia* is saprobic on *Posidonia oceanica*, *Suaeda monoica* and *Avicennia marina*. This is the only genus in this family to contain more than one species. *Pontoporeia* is characterized by irregular peridium, filiform septate pseudoparaphyses, broadly clavate, ovate or ellipsoidal asci and ascospores with germ pore at both ends and a 2-layered wall. It is also the only member of Halotthiaceae with asci lacking an ocular chamber (see morphology Kohlmeyer & Kohlmeyer, 1979; Suetrong et al., 2009; Devadatha & Sarma, 2018, www/marinefungi.org).

Sulcosporium Phookamsak & K.D. Hyde, in Ariyawansa et al., Fungal Divers. 75: 77 (2015).

Index Fungorum number: IF 551328; Facesoffungi number: FoF 00894; 1 morphological species (Species Fungorum 2020), 1 species with molecular data.

Type species – *Sulcosporium thailandicum* Phookamsak & K.D. Hyde [as 'thailandica'], in Ariyawansa et al., Fungal Divers. 75: 77 (2015).

Notes – The striate, thick walled ascospores are a distinct characteristic of *Sulcosporium*. It is distinguished from other members of Halotthiaceae by the hamathecium embedded in mucilaginous matrix. The genus was reported as a pathogen on grasses, and causing necrotic leaf spots (see morphology Ariyawansa et al., 2015a).

Ecological and economic significance

Halotthiaceae is a small family mainly consisting of monotypic genera. They are found as saprobes in marine and terrestrial environments. Halotthia posidoniae, predominantly found in marine environment is pathogenic to Posidonia oceanica. Sulcosporium thailandicum causes necrotic leaf spots on grass blades.

4.3.2 *Pseudocoleodictyosporaceae*

Pseudocoleodictyosporaceae Doilom & K.D. Hyde, Fungal Divers. 82: 107–182(2017).

Index Fungorum number: IF 551979; Facesoffungi number: FoF 01856, 4 species.

Saprobic on bark of dead and living Tectona grandis. Asexual morph: Colonies on natural substrate, punctiform, sporodochial, superficial, gregarious, scattered, numerous, black. Hyphae sometimes superficial and sometimes immersed, pale brown to brown, septate, slightly constricted at the septa. Conidiophores wide, micronematous, erect to slightly curved, constricted at the septa, arising from hyphae. Conidiogenous cells blastic, integrated, terminal, determinate. Conidia dictyosporous to bulbil-like, very variable in size and shape; globose to ellipsoidal to irregular, with a protruding basal cell; truncate at the base, initially pale brown, becoming brown to dark brown, muriform, horizontal on conidiogenous cell. Sexual morph: Undetermined.

Type – *Pseudocoleodictyospora* Doilom & K.D. Hyde.

Notes – Pseudocoleodictyosporaceae was established by Doilom et al. (2017) to accommodate two genera, Pseudocoleodictyospora and Subglobosporium. In combined multi-gene phylogenetic analysis with LSU, rpb-2 and SSU, Pseudocoleodictyosporaceae constituted a wellsupported clade adjacent to Roussoellaceae and Torulaceae (Doilom et al., 2017). The species in Pseudocoleodictyosporaceae were distinct from its sister clades, supporting its

establishment as a new family. All the species from this family are recorded as saprobes on the bark of living or dead teak (Doilom et al., 2017).

Pseudocoleodictyospora Doilom & K.D. Hyde. Fungal Divers. 82: 107–182(2017).

Index Fungorum number: IF 551980; Facesoffungi number: FoF 01857; 3 morphological species (Species Fungorum, 2020), 3 species with molecular data.

Type species – *Pseudocoleodictyospora tectonae* Doilom & K.D. Hyde.

Notes – Genus *Pseudocoleodictyospora* was named for its similarities with *Coleodictyospora* in dark sporodochia, dictyosporous, to bulbil-like, muriform, horizontal conidia produced on conidiogenous cell, but Pseudocoleodictyospora lacks a hyaline sheath. Due to the lack of sequence data for *Coleodictyospora*, no strain of *Coleodictyospora* was used in the phylogenetic analysis used for the introduction of this genus (Doilom et al., 2017). Morphology of type species see Doilom et al. (2017).

Pseudocoleodictyospora tectonae Doilom, Bhat & K.D. Hyde, in Doilom et al., Fungal Divers. 82: 107–182(2017).

Index Fungorum number: IF 551969; Facesoffungi number: FoF 01859.

Description – see Doilom et al. (2017).

Material examined – Thailand, Chiang Rai Province, Mae Chan District, on dead bark of *Tectona grandis*, 1 July 2012, M. Doilom, (MFLU 15-3527, holotype).

Other genus included

Subglobosporium Doilom & K.D. Hyde, Fungal Divers. 82: 138 (2016).

Index Fungorum number: IF 551982; Facesoffungi number: FoF 01861; 1 species (Species Fungorum, 2020), 1 species with molecular data.

Type species – *Subglobosporium tectonae* Doilom & K.D. Hyde, in Doilom et al., Fungal Divers, Fungal Divers. 82: 107–182 (2017).

Notes – Conidia of *Subglobosporium* are in punctiform, superficial colonies in pits or cracks on bark, black, globose to subglobose on natural substrates. In combined genes of LSU, rpb-2 and SSU phylogenetic analysis, Subglobosporium forms a distinct clade within *Pseudocoleodictyosporaceae* basal to the *Pseudocoleodictyospora* clade.

Ecological and economic significance

Most species from *Pseudocoleodictyosporaceae* were identified and reported from teak (Tectona grandis L.f.) as saprobes. They have been only reported from Thailand (Farr & Rossman, 2019).

4.3.3 Cylindrocladiella

Cylindrocladiella Boesew., Canadian Journal of Botany 60 (11): 2289 (1982)

Nectricladiella Crous & C.L. Schoch, Studies in Mycology 45: 54 (2000)

Background

Boeswinkel (1982) established *Cylindrocladiella* to accommodate five Cylindrocladium-like species producing small, cylindrical conidia. Even though the generic status of *Cylindrocladiella* was initially opposed by Crous and Wingfield (1993), later studies on morphological comparisons by Crous et al. (1994) and molecular data (Victor et al., 1998; Schoch et al., 2000) supported the establishment of *Cylindrocladiella* as a genus. This genus is commonly confused with the asexual morph of *Calonectria* but can be distinguished by clear morphological differences, such as aseptate stipe extensions, different branching patterns of the conidiophores and comparatively small, aseptate conidia. Although species are generally not regarded as important plant pathogens, correct identification is essential for disease control and biosecurity implications.

Classification—Ascomycota, Sordariomycetes, Hypocreomycetidae, Hypocreales, Nectriaceae

Type species—*Cylindrocladiella parva* (P.J. Anderson) Boesew.

Distribution—as a soil-borne fungus, the species in *Cylindrocladiella* have a cosmopolitan distribution in various geographically and climatically distinct regions around the world (Farr & Rossman, 2020).

Disease symptoms—black-foot disease, damping-off, leaf spot, root rot and shoot die-back Many species belonging to *Cylindrocladiella* are opportunistic plant pathogens but they are not considered as primary pathogens. They can be isolated associated with disease symptoms such as leaf spot, damping off and shoot die-back (Scattolin & Montecchio, 2007; Pham, 2018). Chocolate brown lesions around the shoots spread primarily to be followed by wilting of the shoot tip, reddish

discolouration, dropping of leaves, and finally plant death (Brielmaier-Liebetanz et al., 2013). Characteristic symptoms of the black-foot disease include a reduction in root biomass and root hairs with sunken and necrotic root lesions (Agustí-Brisach & Armengol, 2013). Symptoms of Cylindrocladiella root rot are black lesions on the tap and lateral roots, wilting and foliar necrosis, and the outer bark of the seedlings will crack and become loose (Sinclair & Lyon 2005).

Hosts—Species are soil-borne, weak pathogens of forestry, agricultural and horticultural crops. There are 270 records of *Cylindrocladiella* associated with different plant species (Farr & Rossman 2020). Among them, different *Vitis* species and *Eucalyptus* species are common hosts associated with different species of *Cylindrocladiella*.

Morphological based identification and diversity

Cylindrocladiella can be distinguished from related species by penicillate and/or subverticillate symmetrically branched conidiophores which produce small, cylindrical, 1-septate conidia and aseptate stipe extensions (Lombard et al., 2012). The generic status of *Cylindrocladiella* was earlier strongly contested (Sharma & Mohanan, 1991), however, based on morphological evaluation and comparisons by Crous and Wingfield (1993) and Crous et al. (2017) confirmed its generic status. Victor et al. (1998) and Schoch et al. (2000) provided molecular data to support generic status. Lombard et al. (2012) in his revision of *Cylindrocladiella* mentioned that only two species have been recognized with their respective *Nectricladiella* sexual morph. Rossman et al. (2013) proposed that the generic name *Cylindrocladiella* be used rather than Nectricladiella. Lombard et al. (2015) showed that Cylindrocladiella formed a monophyletic group in Nectriaceae (Wijayawardene et al., 2020).

Molecular based identification and diversity

Using RFLPs and AT-DNA data, Victor et al. (1998) recognised seven species in the genus. Schoch et al. (2000) added another species based on ITS and partial tub2. Van Coller et al. (2005) introduced the use of his3 sequence data for this group. A combined multilocus phylogeny of his, TEF-1, TUB2and ITS was used by Lombard et al. (2012) which resulted in 18 new *Cylindrocladiella* species and several unresolved species complexes. Lombard et al. (2017) introduced six new species based on a combined ITS, TEF-1 and TUB2 dataset. Pham (2018) introduced five new species

based on his, TEF-1, TUB2 and ITS sequence data and Marin-Felix et al. (2019) introduced two new species based on ITS, TEF-1 and sequence data.

4.3.4 Wojnowiciella

Wojnowiciella Crous, Hern.-Restr.& M.J. Wingf., Persoonia 34, 201 (2015)

Background *Wojnowiciella* was introduced by Crous et al. (2015) to include *Wojnowiciella eucalypti* which exhibited somewhat similar morphological characteristics to *Wojnowicia*, such as setose pycnidia, with ampulliform, enteroblastic, phialidic conidiogenous cells, but differed with apapillate conidiomata lacking setae and having dark brown conidia.

Classification—Ascomycota, Pezizomycota, Dothideomycetes, Pleosporales, Phaeosphaeriaceae

Type species—*Wojnowiciella eucalypti* Crous, Hern.-Restr. & M.J. Wingf

Distribution—Australia (Hernandez-Restrepo et al., 2016), China (Crous et al., 2015; Giraldo et al., 2017), Colombia (Crous et al., 2015; Giraldo et al., 2017), New Zealand (Crous et al., 2019), South Africa and Western Cape (Crous et al., 2016)

Disease symptoms—Leaf spots

Most species are reported as saprobes with the exception of *Wojnowiciella cissampeli*, *W. eucalypti* and *W. vibruni* which were isolated from leaves and twigs of *Cissampelos capensis*, *Eucalyptus* and *Viburnum utile* respectively (Hernandez-Restrepo et al., 2016). Their pathogenicity or disease symptoms are not indicated clearly and there is a need to establish pathogenicity of these species.

Hosts—*Cissampelos capensis*, *Dactylis* sp., *Eucalyptus grandi*s, Rosa sp., *Leptocarpus* sp., *Lonicera* sp., *Spartium* sp. and *Viburnum utile* (Farr & Rossman, 2020).

Morphological based identification and diversity

Wojnowiciella was introduced to include species that were phylogenetically distinct but morphologically similar to *Wojnowicia* (Crous et al., 2015). *Wojnowiciella* is characterized by apapillate conidiomata without setae and dark brown conidia. Some species of *Wojnowiciella* also produce hyaline microconidia. Karunarathna et al. (2017) first reported the sexual morph of *W. dactylidis*. Phookamsak et al. (2019) transferred

Wojnowicia rosicola to *Wojnowiciella rosicola* based on morphology and phylogenetic analyses.

Molecular based identification and diversity

Wojnowiciella is a well-supported genus in the family Phaeosphaeriaceae (Phookamsak et al., 2019). A combined multiloci phylogeny of LSU, SSU, TEF-1 and ITS is used in placing species of *Wojnowiciella* within Phaeosphaeriaceae. To identify species within the genus ITS, LSU, RPB2 and TEF-1 are used (Marin-Felix et al., 2019; Phookamsak et al., 2019).

CHAPTER 5

CONCLUSIONS

5.1 Overall Conclusions

The present study focusses on the understanding the molecular mechanism related to pathogenicity via functional characterization of virulence genes of Botryosphaeriaceae, specifically LysM domain containing effector LtLysM1 of *Lasiodiplodia theobromae* and taxonomy of various fungal taxa in the phylum Ascomycota.

LysM proteins have been documented in a wide range of organisms including animals, plants, fungi, and bacteria. The current study was conducted to expand the molecular research on *L. theobromae* and to investigate on biological functions and molecular features of a LysM domain containing protein LtLysM1. This study is the first step in molecular research pertaining to the LysM domain containing genes of *L. theobromae*. The ability of *LtLysM1* to induce pathogenesis was confirmed by overexpressing and silencing the gene. In addition to its ability to induce pathogenicity, its importance in inducing the vegetative growth of *L. theobromae* is also explored.

As the second section of the current dissertation, This study focused on taxonomic identification of various fungal species both pathogenic and non-pathogenic. As the first part in this section a study was conducted on post-harvest grape pathogen *Trichothecium roseum*. In this study, *T. roseum* was identified as the cause of post-harvest pink rot in grapes with morpho-molecular analyses. The previous studies conducted on *T. roseum* were solely based on the morphological characteristics to identify the species or used ITS sequences to compare with the data available in the NCBI database. These studies lack phylogenetic analyses. Due to the lack of sequence data available for this genus, a single-gene (ITS region) for the phylogenetic analysis was used to confirm the placement of our isolates for better clarification. Furthermore, the pathogenicity of *T. roseum* was confirmed on wounded and non-wounded grape

berries. According to the assay, the appearance of symptoms accelerated on the wounded berries compared to the non-wounded berries. Furthermore, even in the non-wounded berries, if the fungi reached any natural openings, the berry degradation rate vastly increased. These results indicate that *T. roseum* uses natural openings or wounds as entry points. Therefore, mishandling the grape harvest could make them more susceptible to infection by *T. roseum*. The taxonomy and pathogenicity of *T. roseum* have not been comprehensively studied to date. This study is the first to provide morphological, cultural, and phylogenetic analysis combined with pathogenicity assays for better characterization of *T. roseum* as a grape post-harvest pathogen. This study also provides the first disease report for *T. roseum* on grapes in China.

Pestaloid species are commonly isolated from various hosts from many geographical locations. In the current study, one new species of *Pestalotiopsis* along with eight new host records (same species isolated from different hosts), reported from diseased leaves of ornamental and forest plants from Shandong Province, China was characterized with morphological and phylogenetic data. This study is the first that explored the diversity of *Pestalotiopsis* and *Neopestalotiopsis* species associated with ornamental and forest plants in Shandong Yellow River peninsula. In this study, we used combined analyses of ITS, TUB2 and TEF1 datasets to characterize *Neopestalotiopsis* and *Pestalotiopsis* species associated with ornamental and forest plants in China and the analysis provided evidence to describe the species introduced in this study to be phylogenetically distinct from the other species of their respective genera. Morphology descriptions, photographic illustrations and reasonings for these novelties are provided for each taxon.

Fungi from the family Diatrypaceae are important pathogens and decomposers. In the current study, five new species from three different genera of Diatrypaceae with morphological and molecular data is introduced. As molecular data are lacking in many genera of Diatrypaceae, leading to unstable phylogenetic placements, the data obtained in this study would contribute towards proper resolving of spices in future studies whilst adding to the diversity of fungal species from Diatrypaceae collected from China. Morphology descriptions with photographic illustrations and evidence for these novelties are provided for each taxon introduced in this study.

Furthermore, the present study was extended to provide the contributions to the taxonomy and phylogeny of fungal taxa belonging to other various genera. This was done using the materials obtained from China and Italy. Complete species descriptions and their taxonomic placements were provided for fungal taxa which includes novel species and host records.

5.2 Research Advantages

This study was the first to focus on the characterization of LysM domaining containing genes in a Botryosphaeriaceae pathogen. Even though many studies have been conducted on other pathogenic fungi, to the best of my knowledge this study is the first to study on an opportunistic pathogen with LysM genes.

The characterization of *Trichothecium roseum* as a post-harvest pathogen in grapes with molecular taxonomy has not been conducted prior to this study. The results of this study would provide a baseline for correct identification of this pathogen in the future.

Furthermore, the current study has introduced new species and new host records and has contributed to the fungal taxonomy with DNA sequence data. The data collected in these studies are important to understand the fungal taxonomy and biodiversity all over the world. Additionally, DNA sequence data from different gene regions were created, annotated properly and deposited in GenBank to be used for phylogenetic studies in the future.

5.3 Future Work

In the current study only one LysM gene was functionally characterized. The comparative genome and transcriptome analyses of *L. theobromae* has established the presence of two more LysM effector proteins. It would be interesting to study if the same functions and the behaviors observed by the LtLysM1 is present for the other effectors of this pathogen. Since the LysM domain containing effectors of other fungal pathogens has the chitin binding ability to shield the pathogen from host detection, it

would be interesting to study the presence of the same ability in the other LysM effectors of *L. theobromae*.

5.4 Publications

5.4.1 First Author Publications

Harishchandra, D. L., Zhang, W., Li, X, et al. (2020). A LysM domain-containing protein LtLysM1 is important for vegetative growth and pathogenesis in woody plant pathogen *Lasiodiplodia theobromae*. *Plant Pathol J, 36*, 323–334. https://doi.org/10.5423/PPJ.OA.05.2020.0084

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5.4.2 Co-author Publications

Chen, Y. J., Jayawardena, R. S., Bhunjun, C. S., Harishchandra, D. L. & Hyde, K. D. (2020). *Pseudocercospora dypsidis* sp. Nov. (Mycosphaerellaceae) on *Dypsis lutescens* leaves in Thailand. *Phytotaxa, 474*, 218–234. https://doi.org/10.11646/phytotaxa.474.3.2

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Jayawardena, R. S., Hyde, K. D., Chen, Y. J., et al. (2020). One stop shop IV: taxonomic update with molecular phylogeny for important phytopathogenic genera. *Fungal Diversity, 103*, 87–218.

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APPENDICES

APPENDIX A

CHEMICAL REAGENTS AND MEDIA

1. **Potato dextrose agar (PDA)** used for fungal cultivation

   Potato 200 g

   Dextrose 20 g

   Agar 15 g

   Boil 200 g of Potato in distilled water and mix thoroughly. Then filter the potato extract and 20 g of dextrose were added, boil for 1 minute to completely dissolve the powder and bring volume to 1000 ml. Autoclave at 121 °C for 15 minutes.

2. **Water agar (WA)** used to culture fungi prior to culture them on PDA

   Agar 15 g

   Suspend agar in distilled water and mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder and bring volume to 1000 ml. Autoclave at 121 °C for 15 minutes.

3. **Complete media**

   Yeast extract 0.6% [w/v]

   Casein acid hydrolysate 0.3% [w/v]

   Casein enzymatic hydrolysate 0.3% [w/v]

   Sucrose 1% [w/v]

   Completely dissolve and autoclave at 121 °C for 15 minutes

4. **Luria-Bertani media (LB)**

   Tryptone 1% [w/v]

   Yeast extract 0.5% [w/v]

   NaCl 1% [w/v]

APPENDIX B

ABSTRACTS OF PUBLICATIONS

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CRISPR/Cas9: Contemporary designer nucleases for efficient genome editing in phytopathogenic fungi

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Abstract

Plant diseases caused by fungal pathogens are one of the main factors contributing to severe economic losses due to reductions in yield and the quality of crops. Studying the fungal genes related to pathogenicity to reveal their infection mechanism through genome editing can play an important role in the management of these diseases. The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated nuclease 9 (Cas9) system is a versatile tool for genome engineering which has recently been adopted for sequence specific regulation of gene expression in many plant pathogenic fungal genomes. It is the current scientific consensus point of view that this simple RNA guided genome editing tool is cheaper, easier to use, and is higher in gene modification efficiency than any other available gene editing tool. In this mini review, we discuss the molecular mechanisms underlying the CRISPR/Cas9 technique and its recent improvements and applications beyond gene editing. We discuss and summarize a few recent studies targeting phytopathogenic fungal genomes, potential applications, the remaining challenges, and future perspectives. Our analysis provides insights into how this method can be more widely applied to combat fungal phytopathogens.

Keywords – DNA repair – Fungal genomes – Nucleases – Targeted mutations

Introduction

Plant pathogenic fungi are among one of the most diverse and economically relevant threats concerning plant diseases (Borrelli et al. 2018). Many agriculturally important staple food crops such as maize, rice, wheat and economically important crops such as grape, tea, and many more face significant losses annually due to the devastating effects of fungal diseases (Gramaje & Armengol 2011, Yan et al. 2013, Nalley et al. 2016, Thompson & Raizada 2018). Fungi such as *Blumeria graminis*, *Botrytis cinerea*, *Fusarium graminearum*, and *Pyricularia oryzae* are few examples of fungal pathogens considered to be some of the most important plant pathogens in agriculture (Dean et al. 2012). The emergence of new and more aggressive fungal pathogens has increased substantially since the early 2000s (Fisher et al. 2012), thus developing new control

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The Plant Pathology Journal

A LysM Domain-Containing Protein LtLysM1 Is Important for Vegetative Growth and Pathogenesis in Woody Plant Pathogen *Lasiodiplodia theobromae*

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Lysin motif (LysM) proteins are reported to be necessary for the virulence and immune response suppression in many herbaceous plant pathogens, while far less is documented in woody plant pathogens. In this study, we preliminarily characterized the molecular function of a LysM protein LtLysM1 in woody plant pathogen *Lasiodiplodia theobromae*. Transcriptional profiles revealed that LtLysM1 is highly expressed at infectious stages, especially at 36 and 48 hours post inoculation. Amino acid sequence analyses revealed that LtLysM1 was a putative glycoprotein with 10 predicted *N*-glycosylation sites and one LysM domain. Pathogenicity tests showed that overexpressed transformants of *LtLysM1* displayed increased virulence on grapevine shoots in comparison with that of wild type CSS-01s, and RNAi transformants of *LtLysM1* exhibited significantly decreased lesion length when compared with that of wild type CSS-01s. Moreover, LtLysM1 was confirmed to be a secreted protein by a yeast signal peptide trap assay. Transient expression in *Nicotiana benthamiana* together with protein immunoblotting confirmed that LtLysM1 was an *N*-glycosylated protein. In contrast to previously reported LysM protein Slp1 and OsCEBiP, LtLysM1 molecule did not interact with itself based on yeast two hybrid and co-immunoprecipitation assays. These results indicate that LtLysM1 is a secreted protein and functions as a critical virulence factor during the disease symptom development in woody plants.

Keywords : grapevine, *N*-glycosylation, pathogenicity, secreted protein

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During microbe-plant interactions, plants have evolved two tier of defense responses in the face of pathogen attacks. The first layer provides basal responses based on the recognition of pathogen-associated molecular patterns (PAMPs) by plant pattern recognition receptors (PRRs) that activates PAMP-triggered immunity (PTI) (Jones and Dangl, 2006; Macho and Zipfel, 2014; Stergiopoulos and de Wit, 2009). This layer of responses are associated with callose deposition, lignin formation, phenolic compounds deposition, stomata closing, generation of extracellular reactive oxygen species, and a serious of downstream signal transduction events (Han and Kahmann, 2019; Zipfel, 2014). To establish compatible interaction for successful proliferation inside host tissues, microbial pathogens secrete an arsenal of effectors to overcome PTI response. Although pathogens secrete substantial effectors to subvert PTI, plants have evolved a surveilence system to recognize these effector proteins to trigger well-known effector-triggered immunity, which includes by hypersensitive cell death and defense-gene activation (Han and Kahmann, 2019; Jones and Dangl, 2006; Selin et al., 2016; Thomma et al., 2011). During past decades, many typical PAMPs including bacterial flagellar peptide flg22,

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