

**ELUCIDATION OF MOLECULAR AND BIOCHEMICAL
DETERMINANTS IN A NATURAL RICE RHIZOSPHERIC
ISOLATE TO ATTENUATE RICE BLAST PATHOGEN**

Magnaporthe oryzae

by

Carla Aleta Spence

A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biological Sciences

Summer 2014

© 2014 Carla Aleta Spence
All Rights Reserved

UMI Number: 3642361

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



UMI 3642361

Published by ProQuest LLC (2014). Copyright in the Dissertation held by the Author.

Microform Edition © ProQuest LLC.

All rights reserved. This work is protected against unauthorized copying under Title 17, United States Code



ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 - 1346

**ELUCIDATION OF MOLECULAR AND BIOCHEMICAL
DETERMINANTS IN A NATURAL RICE RHIZOSPHERIC
ISOLATE TO ATTENUATE RICE BLAST PATHOGEN**

Magnaporthe oryzae

by

Carla Aleta Spence

Approved:

Randall L. Duncan, Ph.D.
Chair of the Department of Biological Sciences

Approved:

George H. Watson, Ph.D.
Dean of the College of Arts and Sciences

Approved:

James G. Richards, Ph.D.
Vice Provost for Graduate and Professional Education

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed:

Harsh P. Bais, Ph.D.
Professor in charge of dissertation

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed:

Janine Sherrier, Ph.D.
Member of dissertation committee

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed:

Daniel Simmons, Ph.D.
Member of dissertation committee

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed:

Carlton Cooper, Ph.D.
Member of dissertation committee

I certify that I have read this dissertation and that in my opinion it meets the academic and professional standard required by the University as a dissertation for the degree of Doctor of Philosophy.

Signed:

Diane Herson, Ph.D.
Member of dissertation committee

ACKNOWLEDGMENTS

First and foremost, I would like to express sincere gratitude to my advisor, Dr. Harsh Bais, for his continuous support and guidance. Above all, his enthusiasm for science and his optimism have made an otherwise stressful and difficult endeavor exciting and enjoyable. It is his kindness and understanding that earns the deepest respect not only from me but from all of his students and lab members. He is truly a role model in and out of the lab, and I will forever be thankful to him on a professional and personal level. Even when I decided to get pregnant (twice!) during grad school, he was encouraging and understanding. Between his intellect, experience, charisma, and character, I could not have wished for a better advisor. Thank you so much, Harsh.

My gratitude also extends to my committee members who have provided valuable insight and fresh perspectives to this project. Dr. Dan Simmons, who was also my undergraduate research advisor; Dr. Janine Sherrier not only for her guidance, but also for introducing me to Dr. Bais; Dr. Carlton Cooper for his good ideas, encouragement, and positive attitude; Dr. Kirk Czymmek, for his helpful advice in the early stages of this project; and Dr. Diane Herson who kindly stepped in at the last minute, just before my qualifying exam and despite a multitude of other obligations, to help me. Thanks to Dr. Nicole Donofrio for her knowledge and insight about *M. oryzae*. Thanks to Dr. Liz Adams for the experience, guidance, laughs, and tea. Thanks to Doreen Anderson, Betty Cowgill, and Vicki Paisley, for their kindness and helpfulness with administrative tasks. All three have gone above and beyond.

Past and present Bais lab members have also been influential. In particular, thanks to Dr. Meredith Biedrzycki, Dr. Amutha Kumar and Emily Alff. Over the years, the lab has become like family and I'm thankful for current lab members Dr. Gopinath Selvaraj, Amanda Roberson, Samuel Julio-Martins, and particularly Dr.

Venkatachalam Lakshmanan. Venki has truly become a close friend. I'm also thankful to Dr. Vidhyavathi Raman, not just for helping me with *M. oryzae* transformation but also for her friendship. A special thanks to Dr. Brian Whittaker for his thorough and patient mentoring during my rotation in the Boyd lab.

During grad school I formed many new friendships. Thank you to Sarah, Shannon, Lauren, Eric, Beth, Serge, Kevin, Archana, Net, Michelle, Kristin, Sasha, Breonna, Jack, J.B., Padma, Kun, Ross, Rachel, Joan, Cherish and so many others for all the laughs and good times. Thanks to my friends outside of grad school too!

Thank you to the professors who gave me the knowledge and skills needed to complete this project. Thank you to Ms. Judith Thayer, my elementary school teacher and long-time friend. She made a tremendous and lasting impact with her compassion and spirit. In this way, she will never truly be gone. I'm also thankful to my high school biology teacher Ms. Jill Scannell. Her teaching inspired me to pursue an education in this field.

Finally, I would like to thank my family. My Mom, Ivy Spence, has shown me the value of hard work as well as the unconditional love of a mother and best friend. My Dad, Barry Spence, has also set an example of hard work, while always maintaining his smile and charm. He is my biggest cheerleader. Both of my parents instilled the value of education and gave me opportunities they did not have. Thanks to my grandmother, Aleta Easter, for her love. I'm thankful to my husband, Sean Fidance, who works so hard to be the perfect husband and father. My life is wonderful because of him. During this project, my life has become so much fuller. I'm thankful for the smiles and exuberance that my son, Trent Fidance, and daughter, Callia Fidance, give me each day. Without them I wouldn't understand the pleasures of motherhood and wouldn't have experienced the immense beauty of pregnancy and natural childbirth. They've given me the vivaciousness and enthusiasm to finish my Ph.D. Also, I'm thankful to my in-laws, Al and Tina Fidance for their love and acceptance. Truly, I appreciate Aleta and Tina for looking after my kids while I am working. I am fortunate to have such a large web of support and love.

To my best friend, Sean Fidance.
All that I am is yours, and always will be.

TABLE OF CONTENTS

LIST OF TABLES	xii
LIST OF FIGURES	xiii
ABSTRACT	xvi

Chapter

1	INTRODUCTION ¹	1
1.1	<i>Magnaporthe oryzae</i> is the Most Devastating Pathogen of Rice	4
1.1.1	<i>M. oryzae</i> 's life cycle	5
1.1.2	<i>M. oryzae</i> 's response to fungicides	6
1.1.2.1	Transcriptional responses to fungicides	8
1.1.2.2	Morphological responses to fungicides	11
1.2	The Microbiome	12
1.2.1	Role of plants in shaping their microbiome	16
1.2.2	Role of microbes in shaping the microbiome	19
1.2.3	Microbiome specificity and evidence for coevolution between microbiome and host	21
1.3	Microbes Can Promote Plant Prowth	24
1.4	Microbes Can Confer Protection Against Phytopathogens	25
1.4.1	Microbial secretion of antibiotics can impede rhizospheric colonization by phytopathogens	26
1.4.2	Microbes can induce systemic resistance in plants against phytopathogens	27
1.5	Multifaceted Functions of Absciscic Acid in Plant Signaling	29
1.6	ABA in Fungi	34
1.7	References	36
2	NATURAL RICE RHIZOSPHERIC MICROBES SUPPRESS RICE BLAST INFECTIONS ²	53

2.1	Abstract.....	53
2.2	Introduction	54
2.3	Materials and Methods	57
2.3.1	DNA extraction from rhizospheric soil and processing for metagenomic analysis.....	57
2.3.2	Isolation and identification of cultured rhizobacteria.....	58
2.3.3	Plant materials and growth conditions	59
2.3.4	<i>In-vitro</i> antibiosis assay	59
2.3.5	Bacterial motility	61
2.3.6	Cyanide measurement	61
2.3.7	Construction of cyanide mutant D5.....	61
2.3.8	Solid-phase microextraction-gas chromatography mass- spectrometry (SPME-GC-MS)	62
2.3.9	Spore germination and appressoria formation.....	63
2.3.10	Evaluation of rhizobacterial-mediated ISR	64
2.3.11	Statistical analysis	66
2.4	Results	66
2.4.1	Isolation and Identification of rhizobacteria.....	66
2.4.2	<i>In-vitro</i> antifungal properties of rice rhizospheric bacterial isolates	71
2.4.3	Characterization of antifungal metabolites from EA105.....	82
2.4.4	EA105 treatment on rice roots primes for against <i>M. oryzae</i>	85
2.5	Discussion.....	89
2.6	Conclusion.....	95
2.7	Acknowledgments	95
2.8	References	96
3	GLOBAL GENE EXPRESION IN RICE BLAST PATHOGEN <i>Magnaporthe oryzae</i> TREATED WITH SOIL ISOLATE EA105 ³	105
3.1	Abstract.....	105
3.2	Introduction	106
3.3	Materials and Methods	110
3.3.1	Fungal and bacterial strains and growth conditions	110
3.3.2	Plant material and growth conditions	111
3.3.3	Sample preparation and on-color microarray	111
3.3.4	Microarray analysis	112
3.3.5	Creating gene knockout mutants in <i>M. oryzae</i>	112
3.3.6	Diffusible and volatile <i>in-vitro</i> inhibition assays	115

3.3.7	Spore germination and appressoria formation assays	115
3.3.8	<i>In-planta</i> infection assays.....	116
3.3.9	Sample preparation and confocal imaging of <i>M. oryzae</i> on rice	116
3.3.10	Statistical analysis	117
3.4	Results	117
3.4.1	Global transcriptional changes in <i>M. oryzae</i> 70-15 following treatment with soil bacteria	117
3.4.2	<i>In-vitro</i> characterization of <i>M. oryzae</i> deletion mutants	125
3.4.3	Ability of rhizobacteria to indirectly protect rice against wild- type and mutant <i>M. oryzae</i> infection	136
3.5	Discussion.....	140
3.6	Acknowledgements	147
3.7	References	148
4	UNDERGROUND SOLUTION TO AN ABOVE GROUND PROBLEM: RICE ISOLATE DISARMS ABA-MEDIATED VIRULENCE OF BLAST PATHOGEN <i>Magnaporthe oryzae</i> TO INDUCE RESISTANCE	155
4.1	Abstract.....	155
4.2	Introduction	156
4.3	Materials and Methods	159
4.3.1	Fungal and bacterial strains and growth conditions	159
4.3.2	Plant materials and growth conditions	160
4.3.3	Rice treatments	160
4.3.4	Spore germination and appressoria formation assays	161
4.3.5	ABA quantification	161
4.3.6	Gene expression.....	162
4.3.7	Statistical analysis	162
4.4	Results	164
4.4.1	Expression of SA- and JA-responsive genes remains elevated in 70-15-infected plants pre-treated with EA105	164
4.4.2	ABA is antagonistic to SA, JA, and ETH signaling.....	169
4.4.3	EA105 may prevent 70-15 spores from increasing ABA biosynthesis and signaling in rice.....	171
4.4.4	ABA may be directly contributing to virulence in <i>M. oryzae</i> ...	180
4.5	Discussion.....	184

4.6	Conclusion	190
4.7	Acknowledgments	191
4.8	References	192
5	FINAL CONSIDERATIONS AND PROSPECTIVE RESEARCH.....	196
	REFERENCES	202
Appendix		
A	Copyright permission for Chapter 1	231
B	Copyright permission for Chapter 2	232
C	Copyright Permission for Chapter 3	233

LIST OF TABLES

Table 2.1.	Primer sequences	65
Table 2.2.	Identification of rice soil isolates	70
Table 2.3.	Comparison of fungal inhibition elicited by EA105	73
Table 2.4.	List of EA105 VOCs	84
Table 2.5.	Volatile metabolite concentrations.	84
Table 2.6.	Spore germination and appressoria formation.....	85
Table 3.1.	List of primers used to create <i>M. oryzae</i> 70-15 deletion mutants	114
Table 3.2.	Gene expression changes in <i>M. oryzae</i>	118
Table 4.1.	Primer sequences.	163

LIST OF FIGURES

Figure 1.1. Schematic of ABA signaling in plants	32
Figure 1.2. Mechanisms by which ABA impedes plant defenses	33
Figure 2.1. Representation of bacteria phyla	67
Figure 2.2. Representation of bacterial genera	68
Figure 2.3. Inhibition of <i>M. oryzae</i> vegetative growth by rice soil isolates	72
Figure 2.4 Swimming and swarming motility of <i>Pseudomonas</i> isolates	73
Figure 2.5. Growth of <i>M. oryzae</i> treated with heat killed cells and growth after inhibition by EA105	74
Figure 2.6. Cyanide production by rice isolates and activity of cyanide mutant D5 against <i>M. oryzae</i>	76
Figure 2.7. Activity of volatile compounds produced by bacteria in the presence of activated charcoal	78
Figure 2.8. Inhibition of <i>M. oryzae</i> appressoria after bacterial treatment	80
Figure 2.9. Activity of EA105 against naturally isolated phytopathogens	81
Figure 2.10. Inhibition of <i>M. oryzae</i> by S methyl thioesters and 1-undecene	83
Figure 2.11. The effect of rhizobacterial priming on rice blast lesion formation ..	87
Figure 2.12. Expression of defense related genes in rice plants treated with rhizobacteria EA105 and EA106	88
Figure 3.1. Differentially regulated <i>M. oryzae</i> genes	119
Figure 3.2. Up-regulated <i>M. oryzae</i> genes	121
Figure 3.3. Down-regulated <i>M. oryzae</i> genes	122
Figure 3.4. Heat maps for functional categories of genes	123

Figure 3.5. RT-PCR validation	124
Figure 3.6. Expression patterns of genes of interest	126
Figure 3.7. Growth rate of mutants	126
Figure 3.8. Morphology of mutants	127
Figure 3.9. Mutant germination and appressoria formation	128
Figure 3.10. Mutant lesions on rice	129
Figure 3.11. Mutant susceptibility to bacterial treatment	131
Figure 3.12. Germination and appressoria formation of mutants with bacterial treatment	133
Figure 3.13. Mutant response to cyanide	135
Figure 3.14. Mutant lesions on primed M-104 rice plants	137
Figure 3.15. Mutant lesions on primed Seraceltik rice plants	138
Figure 3.16. Mutant lesions on primed Nipponbare rice plants	139
Figure 4.1. Expression of SA, JA, and ETH responsive genes in rice plants treated with 70-15 spores, EA105, and both	166
Figure 4.2. Expression of SA, JA, and ETH responsive genes in rice plants treated with 70-15 spores, rhizospheric bacteria, and both	167
Figure 4.3. Gene expression in rice shoots following bacterial treatment	168
Figure 4.4. ABA is antagonistic to JA signaling	170
Figure 4.5. Expression of ABA biosynthesis gene, <i>NCED3</i>	172
Figure 4.6. Quantification of ABA content	173
Figure 4.7. Effect of EA105 and spores on ABA gene expression in rice	176
Figure 4.8. Effect of EA105 and 100 μM exogenous ABA on the expression of ABA-related genes in rice	177

Figure 4.9. Root expression of a putative rice beta glucosidase is highly induced by 70-15 spore treatment.	178
Figure 4.10. Visualization of ABA-related gene expression data in rice	179
Figure 4.11. <i>M. oryzae</i> 70-15 spores treated with 0 to 100 μM ABA.	182
Figure 4.12. <i>M. oryzae</i> 70-15 spores treated with growth regulators GA, IAA, IBA, and kinetin.	183
Figure 4.13. <i>M. oryzae</i> 70-15 spores treated with EA105 and/or exogenous ABA.	184
Figure 5.1. Overview of the mechanisms by which EA105 confers protection against rice blast	198

ABSTRACT

Rice, a staple food crop world-wide, suffers devastating yield losses as a result of blast disease caused by fungal pathogen *Magnaporthe oryzae*. Currently there are no methods for eradicating blast, and strategies for controlling the disease are flawed; their efficacy is limited, resistance develops rapidly, and many pose environmental and health hazards. A relatively new concept for increasing crop yields is the use of beneficial microbes to enhance growth or reduce disease. Microbial crop solutions are often safer and longer-lasting alternatives to chemical treatments, with considerable results. In an effort to find candidates for controlling fungal blast in rice, microbes were isolated from rhizospheric soil of California field-grown rice plants. Eleven of these isolates were identified through 16S rRNA sequencing and Fatty Acid Methyl Ester (FAME) analysis. One isolate in particular, *Pseudomonas chlororaphis* EA105 (hereafter EA105), was striking in its antifungal activity, drastically reducing vegetative growth of *M. oryzae* and almost completely halting the formation of *M. oryzae*'s appressoria, a structure which is required for penetration into the host. Although the identity of compounds involved in EA105's antifungal activity still remain unknown, the antagonistic effects seem to be largely independent of cyanide, a compound which is essential to the biocontrol capabilities of most pseudomonads. When rice plants were root-treated with EA105 prior to *M. oryzae* infection, there were fewer lesions and the size of lesions was reduced. Plant defense mechanisms are typically mediated through salicylic acid (SA), jasmonic acid (JA), and/or ethylene (ETH). In plants which were treated with EA105, there was induction of genes

involved in JA and ETH signaling while the expression of genes involved in SA signaling were largely unaffected.

In developing and understanding biocontrol strategies, an important factor is the response of the pathogen to the treatment. Global transcriptional analysis was done on *M. oryzae* treated with EA105, revealing multiple genes of interest as potential targets of EA105's antifungal activity. Knockout mutants were created in three genes of interest in *M. oryzae* and studied for their response to EA105 treatment and their pathogenicity *in planta*. Two of the mutants, one in a trichothecene biosynthesis gene (MGG_08440) and the other in a stress inducible gene (MGG_03098), showed enhanced pathogenicity *in vitro*, by forming more appressoria than the wild type in the presence of EA105. Interestingly, the deletion mutant of the stress-inducible gene was impaired more than the wild-type in forming lesions on plants which were primed with EA105, suggesting a role for this gene in the initiation of induced systemic resistance (ISR). Narrowing in on the precise mechanisms of biocontrol is an important step in development of biocontrol strategies, and can be facilitated through global examination of the pathogen's response to treatment.

In addition to examining the role of defense signals SA, JA, and ETH, another critical plant hormone, abscisic acid (ABA), which is mainly implicated in response to abiotic stress, was also investigated. ABA is an agonist of the plant defense hormones SA, JA, and ETH, and as a result, high levels of ABA reduce plant resistance to pathogens. Some phytopathogens, including *M. oryzae*, have evolved mechanisms to trigger increased ABA biosynthesis in plants as part of the virulence process. EA105 prevents *M. oryzae* from up-regulating *NCED3*, the key enzyme involved in ABA biosynthesis. Similarly, EA105 prevented *M. oryzae* from up-regulating a putative

rice beta glucosidase that is likely involved in activating conjugated inactive forms of ABA. The total concentrations of ABA in roots and shoots remained unchanged, which, along with previous research, suggests that ABA perception and signaling events may be more critical than actual ABA levels. Further, ABA appears to function not only as a suppressor of plant defense, but also a promoter of pathogenesis in *M. oryzae* through the acceleration of spore germination and appressoria formation. Spores and mycelia of *M. oryzae* produced ABA, though at levels lower than in plants, further indicating that ABA also plays an important role in fungi. However, even with the addition of exogenous ABA, EA105 was able to counter the virulence-promoting effects of this compound. In summary, EA105 can directly antagonize fungal growth and pathogenesis as well as increase host resistance to blast, mediated through JA and ETH signaling, and through the suppression of ABA-related susceptibility. Therefore, EA105 shows promise as a biocontrol solution which may reduce the severity of blast, a disease which threatens global food security.

Chapter 1

INTRODUCTION¹

With the human population increasing exponentially, the availability of nutritious food sources is of primary importance. By 2050 the world's population is estimated by the Food and Agriculture Organization of the United Nations (FAO) to exceed 9 billion. Rice production will need to increase by about 10 million tonnes per year to prevent not only hunger, but also rising costs that would devastate the way of life for millions who are already close to poverty (International Rice Research Institute). As of 2012, 870 million people, constituting an eighth of the world population, were undernourished and more than 3 million children succumb to malnutrition each year. For many Asian nations, the cost of rice is directly linked to food security (Farooq *et al.*, 2009; Sanogo and Amadou, 2010) and shortages of rice, driving increases in prices, will increase hunger. The United States also has a multi-billion dollar rice industry, primarily in the Mississippi delta region, that would benefit from increased rice yield (Pimbucha and Kaiser, 2009). Rice is one of the most important food crops for humans, along with corn and wheat. Consumption of rice accounts for 20% of worldwide human calorie intake, followed closely by wheat at 19% and maize at 5% (FAO). A fundamental constraint is the amount of land which is available and amenable to farming. Thus, it will be necessary to maximize crop yields by making each plant more productive and by reducing the amount of crop loss due to disease and abiotic stress.

Fertilizers provide essential nutrients to plants, increasing growth and production; however, they are largely overused and contribute significantly to environmental pollution. Excessive nitrogen and phosphorous in particular have led to mass eutrophication of water bodies, particularly in China, destroying the surrounding ecosystems (Li *et al.*, 2001, Yang *et al.*, 2008). The effects of excess fertilizers are widespread and long lasting, leading not only to eutrophication but also to soil acidification, calcium and potassium depletion, and emission of greenhouse gases such as nitrous oxide (Vitousek *et al.*, 1997).

Similarly, chemical pesticides and fungicides are also overused, leading to unintended environmental consequences. Endocrine-disrupting chemicals in pesticides impact wildlife and humans, and effects can persist for generations as these chemicals are stored in fat tissue and mobilized during pregnancy and lactation at which time they can be passed to progeny (Colborn *et al.*, 1993, Diamanti-Kandarakis *et al.*, 2009). Environmental xenoestrogens are linked to multiple male reproductive disorders, seen in humans and wildlife, as well as precocious puberty and cancers (Toppari *et al.*, 1996). In the case of fungicides, effectiveness is also an issue with many pathogens quickly developing resistance.

Genetically modified plants are also being developed to increase yield or nutritional content. One of the first examples was Golden rice which produces beta-carotene, a precursor to vitamin A, in the rice grain. In regions where rice consumption is high, vitamin A deficiencies are common (Ye *et al.*, 2000). Ventria bioscience developed a proprietary system which was used to express the human breastmilk proteins lactoferrin and lysozyme in rice, which are useful not only in infant formula but also facilitate rehydration, particularly after gastrointestinal

illnesses such as cholera (Nandi *et al.*, 2002). In terms of reducing crop loss, transgenic rice plants have been created that are resistant to infections by rice blast pathogen *M. oryzae* (Imamura *et al.*, 2010; Li *et al.*, 2009a; Li *et al.*, 2009b; Shinjo *et al.*, 2011), but there are several issues that prevent their use as a primary solution to blast disease. First, these plants can be riskier to grow and may have reduced yields due to reallocation of resources to support constitutive overexpression of the transgene (Jung *et al.*, 2010). So, although they are more resistant to disease, the yields may be lower. In addition, these crops are monocultures that lack the genetic diversity to survive changing environmental conditions and resist other pathogens. About 90% of rice is grown in Asia on small farms where yields are crucial for income and community food supply. Thus, farmers are reluctant to use transgenic rice plants due to the higher risk of losing the entire crop. A further complication is the public controversy and stigma associated with the use of genetically modified organisms (GMOs).

For thousands of years, farmers have realized the effect of soil on plant growth. Aristotle's successor, Theophrastus, noticed enhanced plant growth when geographically diverse soils were mixed, which was likely due to increased microbial diversity in the soil (Tisdale and Nelson 1975). Within the past few decades, the term 'disease suppressive soil' was coined to describe soils in which plants were less likely to be affected by pathogens (Schroth and Hancock, 1982) as we now realize is due to the presence of microbes with the ability to protect plants against disease. These microbes are referred to as biological control, or biocontrol, organisms. Exploiting the roles of natural rhizospheric microbes may provide an effective and sustainable approach to increase crop yields and reduce crop loss due to disease, an

accomplishment which will save lives and improve the quality of life for millions across the globe.

1.1 *Magnaporthe oryzae* is the Most Devastating Pathogen of Rice

Rice blast, the disease caused by the fungal pathogen *M. oryzae*, is the most devastating disease of rice, ruining enough crops each year to feed about 60 million people (IRRI). It is a global problem, encountered by 85 countries, and currently no solution is in place which can eradicate the disease from an infected field (Kato, 2001). Adding further to the threat on food security, *M. oryzae* has more recently been found to infect other staple food crops including wheat, oats, barley, and pearl millet. In a poll of plant pathologists, *M. oryzae* was ranked number 1 as the most scientific and economically important plant pathogen (Dean *et al.*, 2012).

M. oryzae is part of a cryptic species complex (CSC) and is sometimes also referred to as *M. grisea*. A CSC is a group of organisms that appear to be closely related though their exact relationship has not yet been determined. It was found that isolates of this CSC from the grass *Digitaria* appear to be a different species and do not interbreed with isolates from other plants including rice. Since then, isolates from *Digitaria* are referred to as *M. grisea* while other members of the CSC which were isolated from rice and other crops are termed *M. oryzae*, though both names are often used interchangeably in the literature. Prior to January 2013, the International Code for Botanical Nomenclature permitted different names for different life stages of a fungus, and therefore *M. oryzae* and *M. grisea* are also sometimes referred to as *Pyricularia oryzae* or *P. grisea* to describe their asexual reproductive stage, which in the case of this fungus is predominant. For consistency and clarity, the name *M. oryzae* will be used throughout.

1.1.1 *M. oryzae*'s life cycle

M. oryzae typically infects aboveground plant tissue and is transmitted from plant to plant by wind, dew droplets, or rain. Infections spread rapidly from plant to plant and the infection begins when the initial fungal conidiospore lands on a hydrophobic plant surface, such as the waxy cuticle covering rice leaves. The spore germinates quickly, within 2 to 3 hours, and the end of the germ tube then begins to swell to form a specialized infection structure called an appressoria (Talbot 2003). Within the appressoria, compatible solutes accumulate to increase water potential and turgor pressure (Wilson and Talbot 2009). Glycerol is the most abundant compatible solute which accumulates to concentrations of approximately 3.2 molar (deJong *et al.*, 1997). The wall of the appressoria is rich in chitin and lined with a layer of melanin which provides strength and reduces pore size to contain solutes and withstand turgor pressure of up to 8 MPa (Howard and Valent 1996). The pressure allows for the formation of a penetration peg which can break through the cuticle and enter the plant. Although *M. oryzae* physically breaches the plant cell wall, it only invaginates the cell membrane and begins growing narrow filamentous invasive hyphae throughout epidermal cells. As the disease quickly progresses, the hyphae begin to swell and enlarge, forming bulbous invasive hyphae. At the tips of hyphae, a biotrophic interfacial complex (BIC) eventually forms, where effector molecules are accumulated and released into the host cells (Giraldo *et al.*, 2013).

M. oryzae is a hemibiotrophic fungi, starting its disease cycle as a biotrophic fungus invading living tissue before eventually switching to a necrotrophic lifestyle and causing cell death in the host. Before disease symptoms begin to appear, host cells will be filled with invasive hyphae. Characteristic diamond shaped necrotic lesions first appear on leaves and spread until the entire leaf becomes necrotic. The

lesions can also appear on other structures such as nodes, which can weaken the plant and cause it to break and fall over. After the lesion forms, aerial hyphae give rise to a structure known as the conidiophore. Mitotic divisions of a progenitor cell in the tip of the conidiophore give rise to three conidiospores which are released to initiate a new disease cycle (Talbot 2003). All aerial plant parts can be affected, and the disease prevents maturation of the rice grain, reducing yield even before the plant dies (Howard and Valent, 1996; Talbot, 2003). As many as 20 to 60 thousand spores can be produced in one night by a single lesion (Kato et al, 2001). The disease cycle can be completed in as little as 7 days, though lesions typically continue to produce spores for 20 days (Kato *et al.*, 2001). *M. oryzae* thrives in hot humid environments and plants are more susceptible to infection when they are under stress from drought (during drain-off after the flooding period) or from excess nitrogen fertilizers. Essentially, the most favorable conditions for *M. oryzae* infections are commonly found where rice is grown.

1.1.2 *M. oryzae*'s response to fungicides

Fungicides have been used as a strategy to control blast infections, but many factors including effectiveness prevent them from becoming a viable solution to rice blast. *M. oryzae* is naturally resistant to many fungicides and quickly develops resistance to new antifungal compounds. Some examples include Benomyl, a product of DuPont, kasugamycin, an antibiotic produced by *Streptomyces*, and carpropamide, all of which became ineffective in as little as two growing seasons due to the emergence and dominance of resistant *M. oryzae* strains (Hamada *et al.*, 1967; Sakurai *et al.*, 1976; Takagaki *et al.*, 2004). Furthermore, the use of fungicides has been associated with other unintended consequences such as direct impact on human health,

contamination of food products, soil, air, water, and impact on non-target organisms. The toxicity of some fungicides is unknown (isoprothiolane, probenazole), while others have been associated with cancer (benomyl, phthalide, coumarins) and birth defects such as anophthalmia and blindness (benomyl) (Nomoto and Mori, 1997; Yoon *et al.*, 2011). One study showed that 15 different pesticides that are used on rice were all detected at various levels in harvested brown rice, sometimes exceeding the recommended consumption limits (Pareja *et al.*, 2012). Additionally, sub-lethal concentrations of azoles, which themselves are moderately toxic to humans, elicit fungi to produce trichothecenes, which are extremely toxic and pose a food safety issue (Kulik *et al.*, 2012). Yet another consequence of pesticides is reduced soil fertility due to a reduction of beneficial microorganisms inhabiting the soil (Aktar *et al.*, 2009).

Compounds with fungicidal activity against *M. oryzae*, either synthetically produced or naturally produced by soil microbes, fall into several categories. Respiratory inhibitors include coumarins (Yoon *et al.*, 2011), cyanide, antimycin A, and SSF-126 (Yukioka *et al.*, 1997). Several fungicides have been developed which are melanin biosynthesis inhibitors (MBI), with melanin being crucial to pathogenicity and survival on plants. Melanin biosynthesis depends on the fusion of five isoprenyl units, two reduction reactions, two dehydration reactions, and finally a polymerization. MBIs are divided into two categories based on their specific activity, MBI-R which inhibit reductases and MBI-D which inhibit dehydratases involved in melanin biosynthesis. MBI-D includes carpropamide, diclocymet, and fenoxanil while MBI-R includes azoles (such as tricyclazole), pyroquilon, and phthalide (Takagaki *et al.*, 2004). Strains of *M. oryzae* which develop resistance to one fungicide (ex.

carpropamide) also become resistant to other fungicides in the same class (such as diclocymet and fenoxanil). In the case of carpropamide, resistant *M. oryzae* strains became predominant after two growing seasons and contained a single amino acid substitution that drastically reduced the affinity of MBI-D fungicides for the dehydratase enzyme. This resulted in the inability of the fungicide to competitively inhibit the binding site and melanin biosynthesis was no longer impaired in the presence of any of the MBI-D fungicides (Suzuki *et al.*, 2007; Takagaki *et al.*, 2004; Yamada *et al.*, 2004). In addition to respiratory inhibitors and MBIs, there are also toxic compounds which interfere with the fungi's ability to tolerate reactive oxygen species (ROS). ROS is a natural defense response that results in the accumulation of compounds such as H₂O₂, but will lead to apoptosis if the response persists or ROS accumulates beyond a threshold level. Phthalide is not only an MBI, but it also inhibits superoxide dismutase and catalase (Nikolaev and Averyanov, 1991), which are involved in ROS detoxification. Lastly, there are compounds such as probenazole which have no direct effect against the fungi, but rather trigger ISR in plants, which results in lessened disease symptoms from *M. oryzae* (Iwata, 2001). Each class of fungicides differs in the mechanism by which inhibition is achieved. Often, the response of *M. oryzae* also differs, as seen by morphological differences as well as gene expression changes.

1.1.2.1 Transcriptional responses to fungicides

M. oryzae mounts well defined responses to toxins which result in both gene expression and physical changes. These transcriptional and morphological changes can be a general response to stress, or specific to the type of compound to which *M. oryzae* was exposed. One of *M. oryzae*'s most well studied transcriptional responses

is the up-regulation of genes involved in trichothecene biosynthesis. As previously mentioned, trichothecene is extremely toxic to eukaryotes and functions to inhibit protein synthesis at the ribosome. It also impedes plant defense responses by interfering with cell wall fortification and callose deposition (Jansen *et al.*, 2005). Certain environmental conditions, such as the presence of sodium bicarbonate, can reduce the low basal expression of trichothecene synthesis (*tri*) genes (Roinestad *et al.*, 1994), while the high levels of ROS or the presence of azole fungicides at sub-lethal concentrations drastically increase the expression of *tri* genes (Audenaert *et al.*, 2012; Kulik *et al.*, 2012). When respiration is blocked by compounds such as cyanide, antimycin A, or SSF-126, genes involved in alternative respiration are induced. This alternative pathway diverges from the conventional cytochrome pathway at ubiquinone and ends with an alternative oxidase that can directly reduce oxygen. This system is widespread across plants, bacteria, and fungi, including *M. oryzae*, but many allelic variations exist in these genes which make the alternative pathway less effective or ineffective (Yukioka *et al.*, 1997). The alternate oxidase (*aox*) gene was one of the first of these genes to be identified in *M. oryzae* and was shown to be induced by SSF-136 and H₂O₂ (Yukioka *et al.*, 1998). Most *M. oryzae* strains which are sensitive to respiratory inhibitors lack two cysteine residues in the *aox* gene which are highly conserved in plants (Yukioka *et al.*, 1997). The detoxification of ROS is also associated with specific transcriptional events. In yeasts and filamentous fungi there is a conserved mechanism for coping with ROS which is mediated through the induction of specific genes to either detoxify ROS (catalase, superoxidismutase) or to repair ROS damage and maintain cellular homeostasis. The process is coordinated by proteins in the AP-1 family of bZIP activating proteins (Liu *et al.*, 2005).

Transporters also play a role in fungicide response/tolerance by promoting the efflux of toxic compounds out of fungal cells. There are two main classes of transporters, the ATP-binding cassette (ABC) transporters which use energy generated by ATP hydrolysis, and the major facilitator superfamily (MFS) which use the proton motive force to move substances across membranes. Both classes contain many transporters which differ in their specificities. For example, there are at least 30 ABC transporters belonging to 6 sub families and there are 17 MFS families, only two of which function as drug antiporters, DHA12 and DHA14. Some transporters have a broader range of substrates, such as Pdr5p which is induced by multiple fungicides, whereas others are more specific (Del Sorbo *et al.*, 2000). For example, Pdr12p transports only C1-C7 organic acids, such as benzoic, sorbic, or propionic acid. Likewise, the transporter encoded by *abc2* is induced by some but not all fungicides. Azoles (including tricyclazole), pyroquilon, carpropamide, and benomyl all induce transcription of *abc2* but little to no transcriptional effect is seen after treatment with phthalide, isoprothiolane, or kasugamycin (Lee *et al.*, 2005). Additionally, *abc1* transcription is also strongly induced in *M. oryzae* in response to azoles.

Fungicides also typically induce genes in the *hog* pathway, responsible for general and osmotic stress responses. This pathway is not present in mammals, which makes it a prime target for development of fungicides (Jiang *et al.*, 2011). At least one of the genes in the *hog* pathway also plays a role in trichothecene biosynthesis, which is induced by ROS as well as fungicides such as tricyclazole. Interestingly, when one of the genes in the *hog* pathway was knocked out in *Fusarium*, the mutant showed increased sensitivity to high osmolarity of salts and metal ions, but showed increased

resistance to some fungicides (Jiang *et al.*, 2011). Genes within the *hog* pathway play important roles in fungal stress response.

1.1.2.2 Morphological responses to fungicides

In addition to the transcriptional changes that occur in the presence of a toxin, morphological changes also occur, and some are characteristic of particular toxins. For example coumarins have no effect on mycelial growth, but drastically reduce spore germination and alter the appearance of germinating spores (Yoon *et al.*, 2011). Another example is the protein AFP which is synthesized by some fungi and plants, and reduces spore germination and alters hyphal morphology. AFP interacts with glucopyranosylceramide in the plasma membrane of hyphae, causing a K⁺ efflux and a Ca²⁺ influx which results in hyper-branching of hyphae and reduction in growth at the hyphal tips (Coca *et al.*, 2004; Jha and Chattoo, 2010). Benomyl specifically targets fungal microtubules, causing characteristic disturbances in the cytoskeleton (Hess and Nakai, 2000; Li *et al.*, 2005; Singh *et al.*, 2008). An obvious effect from the MBIs is the lack of pigmentation from inhibition of melanin biosynthesis, but they also result in other diverse phenotypes. Tricyclazole for example, has no effect on young hyphae, but leads to aggregation and curling of older hyphae into ring structures. Additionally, it causes swelling and rupture of hyphal tips and leads to the production of membranous sacs which accumulate at the periphery of the cytoplasm and nucleus. Additionally, azoles cause breakages in the nuclear envelope and lead to disordered membranes but do not affect cell wall integrity (Mares *et al.*, 2006). Overall, there are general and more distinct changes in gene expression and morphology when *M. oryzae* is treated with fungicides, as a result of different mechanisms of action.

However, there are many flaws which prevent fungicides from effectively controlling blast infections.

1.2 The Microbiome

Overall fitness of higher eukaryotes depends not only on the organism's genes and ability to adapt, but also on the microbes that live in and on the organism. These microbes can be beneficial, harmful, or neutral but as a whole they have a large impact on the ability of the host to survive in its environment (Rosenberg *et al.*, 2010). The microbes living in, on, or surrounding an organism are called microbiota and comprise the microbiome. This encompasses bacteria, fungi, archaea, and viruses (Dridi *et al.*, 2011, Mendes *et al.*, 2011), including both epiphytic and endophytic microbes (Bordiec *et al.*, 2011, Porras-Alfaro and Bayman, 2011). The study of the human microbiome has revealed the extent to which these microbes impact health and disease. The Human Microbiome Project, similar to the Human Genome Project, sought to fully understand the complement of bacteria, archaea, and fungi associated with humans (Turnbaugh *et al.*, 2007). The skin, nasal, and particularly the gut microbiome are well studied, with many prebiotics and probiotics now available for maintaining healthful microbiomes (Kong, 2011, Gibson and Roberfroid, 1995, Fuller, 1989). The microbiome is also involved in the development and regulation of mammalian immune systems (Kaplan *et al.*, 2011), and has been implicated in obesity and metabolic diseases (Tilg and Kaser 2011) as well as regulating the severity of genetic diseases such as cystic fibrosis (Zemanick, Sagel and Harris 2011). This has led to the development of medical treatments that aim to maintain a balance of beneficial microbes in the human microbiome and such treatments can be highly successful (Cani and Delzenne, 2011, Zemanick *et al.*, 2011).

It is clear that microbes play a vital role in human fitness, and the same holds true for plants. Thus far, the evidence suggests that plant microbiomes are just as vital as human microbiomes, and possibly more so due to the stationary nature of plants. Plants are in constant contact with their surrounding soil, making the communications and associations with microbes integral to their survival. There are many parallels that can be drawn between the function of the microbiome in animals and plants. For one, just as the immune system in animals is regulated by microbes, an analogous processes in plants, known as induced systemic resistance (ISR), is also modulated by microbes. Another parallel is the seemingly dichotomous relationship between the ability of the microbiome to both cause and prevent disease. In addition to those commonalities, plant microbiomes are also important for nutrient cycling and growth promotion which impact the ecosystem through processes such as carbon sequestration (Podila *et al.*, 2009). From the beginning of a plant's development, the germination of the seed, the microbiome imbibed within the seed and the microbes inhabiting the soil impact the physiology and fitness of the plant. Even as the first root emerges, there are microbes associated and bacterial communities establish themselves along the root surface (Ofek *et al.*, 2011). These associated microbes are of such importance that they can be passed from one generation to the next. As is the case for the plant genus *Atriplex*, endophytic fungi are imbibed in the seed and establish a stable residence within the plant such that they are passed from parent to progeny through the seeds (Lucero *et al.*, 2011).

Plants, like animals, have different sets of microbes inhabiting different anatomical areas. The phyllosphere encompasses the area immediately surrounding the aerial parts of plants and includes any microbes located on the aerial surface of the

plant. This includes bacteria found on the epidermis as well as bacteria within the apoplast of the leaf mesophyll. Since the apoplast is space between cells, but not within, bacteria inhabiting these areas are still considered epiphytes. However, it is important to note that bacteria inhabiting the apoplast are more likely to be pathogenic than commensal. On the leaf surface, colonization is not uniform and the highest colonization is seen in crevices, along leaf veins, and at the base of trichomes (Hirano and Upper, 2000). Within the phyllosphere, there are frequent fluctuations in temperature, humidity, light, and moisture. Most plant surfaces are exposed to a high level of solar radiation, and the bacterial communities on the leaf surface often contain pigmented strains of species such as *Pantoea agglomerans* or *Pseudomonas fluorescens* (Sundin and Jacobs, 1999). There is evidence that more than half of the bacteria on leaf surfaces are pigmented and resistant to ultra violet (UV) radiation. If the pigmentation is lost, these bacteria are no longer able to survive under high levels of UV radiation (Sundin and Jacobs, 1999). Nutrients on the plant surface consist of simple sugars such as glucose and fructose (Leveau and Lindow, 2001) that seep from the plant (Tukey, 1970) as well as amino acids which along with sugars can escape through wounds caused by insects or frost damage (Hirano and Upper, 2000). Bacteria, fungi, nematodes, and even algae inhabit the phyllosphere (Andrews and Harris, 2000) although bacteria predominate. As with any microbiome, inhabitants can be neutral, beneficial, or harmful, and one unique way in which bacteria on the plant surface can harm the plant is through ice nucleation, in which bacteria serve to initiate the formation of ice crystals on the leaf surface at higher temperatures than ice would normally form, causing frost damage to the plant (Lindow *et al.*, 1982).

In contrast to the epiphytes that inhabit the surface, there are also endophytes which reside within the aerial parts of the plants. The environment within the leaf is much more constant than outside the leaf, particularly in terms of humidity. The waxy coating on leaves prevents desiccation leading to increase in water holding capacity in plants (Hirano and Upper, 2000). Often, endophytic bacteria are able to enter through openings in the leaf surface, such as stomata and hydathodes (Hugouvieux *et al.*, 1998), and bacteria communities are often found within the vascular bundle of leaves (Hirano and Upper, 2000). Endophytic fungi are also found within leaves, and often have a mutualistic relationship with plants such that they receive nutrients from the plant and offer the plant protection. One mode of protection is the production of mycotoxins that are active against some pathogenic microbes and animals that would otherwise feed off of the plant (Carroll, 1988). Although this type of protection can be systemic, it is often a localized event (Arnold *et al.*, 2003).

In contrast to aerial parts, belowground plant-microbe rhizospheric interactions are much more complex and poorly understood. Plant roots, along with the soil that immediately surrounds them comprise the rhizosphere. The rhizospheric environment is more amenable to bacterial growth than the phyllosphere because of physical conditions including reduced temperature, moisture, and light changes (Brencic and Winans, 2005). In addition, there are a multitude of nutritional sources found in the rhizosphere. Sugars as well as amino acids have been repeatedly found in root exudates which are deposited into the rhizosphere. As further evidence that the rhizosphere is a particularly amenable environment for bacterial growth, it has been noted numerous times that significantly more bacteria are found in rhizospheric soil

than in bulk soil, and this has been termed the “rhizosphere effect” (Smalla *et al.*, 2001).

In addition to root-associated bacteria, there are also endophytes which enter through tiny cracks in the root or areas of the root which are actively growing such as root primordia (Bloemberg *et al.*, 1997). Within the root, bacteria have a more constant environment than in the rhizosphere, but they must be able to cope with or evade the plant’s immune response. More often than not, endophytic microbes within plant roots are harmful to the plant. These types of interactions are far less common than colonization on the outside of the root, and evidence suggests that a complex coevolution has occurred to allow these bacteria to remain inside the plant (Carroll, 1995, Schardl *et al.*, 1997).

The composition of the microbiome is dynamic and controlled by multiple factors. In the case of the rhizosphere, temperature, pH, and the presence of chemical signals from bacteria, plants, and nematodes all shape the environment and influence which organisms will flourish. As the environment changes, so does the composition of organisms inhabiting it. This provides a basis for plants and their microbiomes to selectively associate with one another. In addition to abiotic influences, plants and microbes also modify the microbiome.

1.2.1 Role of plants in shaping their microbiome

Soil is typically devoid of energy sources for bacteria and there is competition for the scarce nutritional resources. Unlike bulk soil, the rhizosphere contains a unique and fluctuating composition of nutrient rich compounds exuded from the plant. This comes at a high energetic cost to the plant, and it is therefore likely that plant secretions are more than just a passive exudation of secondary metabolites and serve

to benefit the plant. The identity and quantity of secretions varies from plant to plant, as does the composition of microbes found in the surrounding rhizosphere (Kravchenko *et al.*, 2011). When plant root secretions are present, the diversity of bacteria in the soil increases, and the composition of the secretions is correlated with which taxa will dominate (Shi *et al.*, 2011). As further evidence, plants with genetically altered starch metabolism, and thus altered root secretions, attracted a different mixture of bacteria to their rhizosphere (Gschwendtner *et al.*, 2011).

Root secretions contain both low and high molecular weight compounds. The abundant low molecular weight compounds include amino acids, organic acids, phenolic compounds, simple sugars, and other small secondary metabolites. The high molecular weight compounds are proteins and polysaccharides which can make up the mucilage coating on roots (Walker *et al.*, 2003). One function of root exudates is to repel pathogens, pests, and competing plants (Shi *et al.*, 2011). Plants can rapidly produce phytoalexins, antimicrobial compounds that are toxic to organisms which cannot catabolize them. Momilactones are an example of a plant secondary metabolite that also serves as a phytoalexin (Kato-Noguchi, 2009). In addition to repulsion of pathogens and competitors, there is also evidence that plants attract beneficials using chemicals found in root secretions (Rudrappa *et al.*, 2008).

The bacterial proteome changes distinctly in response to signals from plants (Knief *et al.*, 2011). The ability to metabolize different carbon sources as well as the efficiency at which the bacteria can utilize these nutrients determines which microbes will be successful in a particular rhizosphere. *Pseudomonas fluorescens*, a common inhabitant of the rhizosphere and plant beneficial, has a variety of nutrient acquisition genes which are up-regulated in the rhizosphere, highlighting the importance and

specificity of plant nutrient sources in attracting specific microbes (Rainey, 1999). In multiple plants, it has been shown that bacteria are actively attracted, *via* chemotaxis, to the rhizosphere. For example, strigolactones, plant hormones that regulate shoot branching and are found in root secretions, attract beneficial mycorrhizal fungi (Xie and Yoneyama, 2010). Sugars from *Fragaria ananassa* root exudates attract *Azospirillum brasilense* (Pedraza *et al.*, 2010) and malic acid, found in root exudates of *Arabidopsis thaliana*, attracts the beneficial Gram positive *Bacillus subtilis* (Rudrappa *et al.*, 2008). Further, *Citrullus lanatus* secretions contain malic acid, citric acid, and oxalic acid, though malic acid was mainly responsible for the chemoattraction of biocontrol bacteria *Paenibacillus polymyxa* (Ling *et al.*, 2011).

Pseudomonads use swarming motility to efficiently colonize roots, and swarming can be initiated or augmented by specific amino acids in root secretions. Aspartate, glutamate, isoleucine, and lysine strongly induce swarming (Kohler *et al.*, 2000). The presence of these amino acids is also required for root colonization in some interactions (Simons *et al.*, 1997). Other amino acids have no effect on bacterial motility, emphasizing the specificity of underground chemical communications. The levels of amino acids in root exudates are not sufficient for sustenance of bacteria, highlighting the function of these molecules as chemical messengers. In fact, higher amino acid concentrations inhibit swarming while specific combinations of amino acid and carbon sources most strongly induce swarming (Kohler *et al.*, 2000). It is likely that each plant's secretions contain a unique cocktail of compounds to maximally entice specific microbes.

Within the rhizosphere, there are smaller microenvironments which are created along the root length due to differences in the composition and abundance of

secretions (Fukui *et al.*, 1994) resulting in distinct bacterial communities (Ofek *et al.*, 2011). For example, the growing root tip leaks much more simple sugars like sucrose than older areas of the root. Contrastingly, mature areas of the root near the base have higher secretions of amino acids such as tryptophan through cracks in the epidermis where lateral roots have emerged (Jaeger *et al.*, 1999). As more research is completed on the identity and role of root exudates, we gain a better understanding of how plants communicate in the rhizosphere. Another important aspect of that communication involves bacterial chemical messengers.

1.2.2 Role of microbes in shaping the microbiome

In addition to root exudates from plants, bacteria also secrete compounds into the rhizosphere which influence the composition of the microbiome. One important facet of bacterial communications is the formation of biofilms. Biofilms consist of multiple bacteria, attracted to the same location via chemotaxis, which associate with one another and surround themselves with a matrix composed of polysaccharides, protein, and DNA (Ramey *et al.*, 2004). Biofilm inhabitants can be from the same species or phylogenetically diverse and can include pathogens or beneficials. The three dimensional shape of the biofilm is due in part to the variety and availability of nutrients and chemical signals (Rudruppa *et al.*, 2008). Within a biofilm, communications between bacteria are frequent and necessary. Through these communications, bacteria modulate their activities to suit existence in such a multicellular environment, including changing their metabolism. The changes are specific to not only a multicellular lifestyle, but also particular to that bacterium's location and role within the biofilm (Danhorn and Fuqua, 2007). Roots are a prime site for biofilm formation due to their unique variety of nutrients and chemical signals,

although biofilms are also found on aerial parts of the plant as well as in intracellular spaces, though these biofilms are often associated with disease (Morris and Monier, 2003). The bacteria within a biofilm gain capabilities that bacteria in free standing colonies do not have, such as enhanced antibiotic resistance conferred by the protective extracellular biofilm matrix. The production of antibiotics such as pyrrolnitrin can also be dependent on quorum sensing and the formation of biofilms (Liu *et al.*, 2007). Biofilm forming bacteria are persistent and form longer lasting associations with the plant than free living colonies, making them an important presence within the microbiome. This is mainly due to the strong adherence of biofilms to the plant surfaces, and their resistance to being washed off (Ramey *et al.*, 2004). Seeds and seedlings also provide a surface for biofilm formation and often the bacteria found within seed biofilms will remain associated with the plant as it ages. Seed biofilms are also of economic importance, as farmers can coat seeds with biofilm forming beneficials to promote plant health and growth once the seeds are planted. Conversely, pathogens can form biofilms on seeds which persist once the seed germinates, that are not only detrimental to plant health and growth but also can render the plant unsafe for consumption (Ramey *et al.*, 2004). Biofilms are a fascinating multicellular organization within the microbiome and it is important to mention not only the communications within biofilms, but also the communications involved in initiating biofilms.

Quorum sensing is a fundamental way in which bacteria communicate with each other, and is necessary for establishment of biofilms. Quorum sensing involves the secretion of acetylated homoserine lactones, called autoinducers, which are sensed through bacterial LuxR receptors. Bacteria can produce multiple autoinducers and

sense the differences between them. Also, the way in which each species responds to a particular autoinducer may differ, leading to a very specific method of communication (Waters and Bassler, 2005). The rhizosphere is an environment in which chemical signals can accumulate, and a fitting site for quorum sensing to occur. Particularly, if plants are attracting a species of bacteria to the rhizosphere, the bacterial population is more likely to reach the critical level to produce enough autoinducers so that quorum sensing will be initiated (Miller and Bassler, 2001, Whitehead *et al.*, 2001). Additionally, some plants are capable of secreting chemicals analogous to certain bacterial autoinducers, which enhances quorum sensing. This serves to shape the microbiome such that those bacteria are more prevalent in microbiomes of plants that can mimic their autoinducers. Conversely, plants can also release compounds to inhibit the auto-inducers to interfere with quorum sensing of pathogens (Rasmussen and Givskov, 2006). It has been clearly shown several times that chemotaxis, rather than random motility is necessary for competitive colonization of roots (Lugtenberg *et al.*, 2001, Kohler *et al.*, 2000, Pratt and Kolter, 1998, de Weert *et al.*, 2002) and plant secretions as well as bacterial secretions provide the chemoattractants for this to occur. Thus, both plants and bacteria have crucial roles in assembling the microbiome.

1.2.3 Microbiome specificity and evidence for coevolution between microbiome and host

There is a complex and specific communication between microbiota and their host which reveals a co-evolutionary history between plants and their microbial neighbors. The specificity is maintained by complex discriminatory recognition systems in which all inhabitants of the rhizosphere must be able to recognize and

differentiate between other organisms present. Oftentimes, beneficial bacteria are recognized by patterns quite similar to those of pathogens, yet plants can differentiate and respond differently to pathogens versus beneficials. Lipopolysaccharide (LPS), a component of Gram-negative bacterial cell walls, is involved not only in pathogen recognition by the immune system, but also serves as an identifier of bacterial strains. Bacterial LPS has an O antigen region which can contain a varying number of repeats. Each bacterial strain has multiple variants of LPS, differing in the number of repeats in the O chain. When run on an SDS-PAGE gel, the different LPS molecules separate depending on the length of the O antigen, and the resulting pattern, referred to as a ladder, is fairly unique and can be used to identify different strains of bacteria. This difference is even seen between different plant-growth-promoting *Psuedomonas sp* (Deweger *et al.*, 1987) which may indicate a very specific recognition system exists in plants for identification of bacteria, to even the extent that plants can differentiate between strains of the same species. Not all bacteria have differing LPS variants, and such bacteria are deficient in root colonization, possibly because the plant does not recognize them. Hence, having a distinct identity marker on the cell surface is important in the communication with plants during the establishment of bacteria on the plant surface.

A particularly well studied example of coevolution between plant host and microbe is the recognition of specific symbiotic bacteria by leguminous plants. This process shares conserved similarities with the recognition of symbiotic mycorrhizal fungi, but has diverged into a distinct pathway. It is crucial that the plant share nutrient resources with the bacteria or fungi only if the plant is receiving nitrogen or phosphorous, respectively, in return. The initial step in recognizing these symbionts is

similar, but as the recognition process proceeds it becomes more specific. Leguminous plants entice their symbionts by releasing flavonoids and the rhizobia respond via secretion of uniquely decorated Nod factors, which are recognized by plant receptors. The recognition of the correct Nod factors is essential for the initiation of root hair curling and this starts the process of nodule development in which the symbiosis occurs (Radutoiu *et al.*, 2003). The stringent specificity of the Nod factors and plant receptors is the basis for such selective associations, and the most logical explanation of how this arose is that the receptors and ligands evolved together (Martinez-Romero, 2009).

Specificities between plant and pathogen have been well noted within microbiomes as well, and lend credence to the hypothesis that continuous associations have led to co-evolution between plants and their microbial neighbors. Only certain bacteria are capable of evading a plant's immune system and gaining entry. Others will be ward off by the plant's defense response, and the detection of pathogens by the plant becomes more specific as the pathogen develops additional virulence strategies. The immunity or resistance of a plant host is directly related to its phylogenetic divergence time from other plant hosts of the pathogen (Schulze-Lefert and Panstruga, 2011). Briefly, plant innate immunity is a process which is quickly initiated and involves recognition of pathogen associated molecular patterns (PAMPS) by plant pattern recognition receptors (PRRs). This leads to PAMP-triggered immunity (PTI). However, some pathogens have developed an ability to deliver effector proteins into the plant through a type III secretion system, and these proteins serve to inhibit PTI. This is an indication that the pathogens have been associated with the plant long enough to find ways to overcome the plant's initial defense

response. Further, plants have developed resistance proteins (R proteins) such as nucleotide-binding leucine-rich repeat (NB-LRR) proteins that recognize the pathogenic effectors and trigger a stronger immune response, called effector-triggered immunity (ETI) (Zhou and Chai, 2008, Chisholm *et al.*, 2006). Many specific plant-pathogen examples have been examined including tomato and *Pseudomonas syringae* (Cai *et al.*, 2011), soybean and *Phytophthora sojae* (Dong *et al.*, 2011a, Dong *et al.*, 2011b), wheat and *Mycosphaerella graminicola* (Marshall *et al.*, 2011), and rice and *Magnaporthe oryzae* (Li *et al.*, 2009). Additionally, pathogens have been shown to be more adapted to local hosts than the same host from a different geographical location (Gandon *et al.*, 1996). Speciation of a strain of pathogen can even occur if one strain is able to reproduce in isolation on a plant host for a long enough time. The ability of a pathogen to evade the hosts immune response and proliferate depends on which effector proteins it produces, and therefore the ability of one strain of a pathogen to make a particular effector protein can lead to its speciation (Schulze-Lefert and Panstruga, 2011).

1.3 Microbes Can Promote Plant Growth

Microbiota can positively influence plant growth in multiple ways. Some bacteria can secrete plant growth hormones such as auxins and cytokinins (Costacurta and Vanderleyden, 1995). For example, a natural auxin, indole acetic acid (IAA), is produced by several genera of rhizospheric bacteria and some bacteria have multiple pathways for synthesizing IAA. This growth regulator alters the root morphology by increasing lateral root growth while decreasing root length (Ahmed *et al.*, 2010, Patten and Glick, 1996), which can allow the plant access to additional soil nutrients. Other

bacteria release siderophores, small compounds that chelate iron, that allow plants to uptake nutrients in the soil that would otherwise be unavailable (Burd *et al.*, 2000).

Microbiota can also increase plant fitness by limiting the effect of abiotic stresses such as heat, frost, drought, and flooding. Some bacteria can release compounds into the soil that help plants to withstand abiotic environmental stresses and may also trigger plants to synthesize compounds involved in tolerance to abiotic stresses, such as osmoprotectants and heat shock proteins (Grover *et al.*, 2011). Also, siderophores can protect plants against heavy metal toxicity in soils that have high levels of lead, nickel, or zinc (Burd *et al.*, 2000) thus helping plants with both the uptake of insoluble nutrients and with the exclusion of toxins. Clearly, the microorganisms within a plant's microbiome have the ability to help or harm the plant, and play a vital role in plant fitness.

1.4 Microbes Can Confer Protection Against Phytopathogens

Aptly named 'disease-suppressive soils' contain predictable communities of microbes, namely a few genera of gamma proteobacteria, which consistently show the ability to reduce plant diseases caused by bacterial and fungal pathogens (Mendes *et al.*, 2011). General mechanisms of disease suppression include competition with pathogens for scarce nutrient resources as well as niche exclusion in which benign or beneficial microbes occupy spaces to limit colonization by pathogens. Another straightforward mechanism by which microbiota can protect against pathogens is through secretion of antibiotic compounds.

1.4.1 Microbial secretion of antibiotics can impede rhizospheric colonization by phytopathogens

Bacteria can produce a multitude of antibiotic compounds to lessen their own competition in the soil as well as to reduce the number of phytopathogens (Burgess *et al.*, 1999, Linares *et al.*, 2006). Pseudomonads often possess biocontrol capabilities (Daval *et al.*, 2011; Dowling and Ogara, 1994), and collectively they produce several well characterized antibiotic compounds, although the blend of these bioactive compounds varies from strain to strain. Antifungal metabolites commonly produced by pseudomonads include hydrogen cyanide (HCN), 2,4-diacetylphloroglucinol (2,4-DAPG), pyrrolnitrin, phenazines, and pyoluteorin (Bais *et al.*, 2006). Less commonly, pseudomonads have been reported to produce antifungal cyclic lipopeptides such as viscosinamide and tensin (Nielsen *et al.*, 1999). The biosynthesis of these compounds is regulated by a two component system, coded for by *GacA/GacS*, in which an environmental sensor will activate an internal cytoplasmic response factor once certain environmental conditions are met (Haas *et al.*, 2002). Thus, the synthesis and secretion of these antibiotics depends on what chemical messages are present in the rhizosphere.

Cyanide is perhaps the most well studied antimicrobial produced in pseudomonads. The *HCNABC* operon in bacteria produces three enzymes which convert glycine to CN and CO₂. Cyanogenesis in bacteria is tightly regulated and does not appear to play any significant role in general metabolism in cyanogenic bacteria. The pKa value for cyanide is 9.3, and therefore at a physiological pH of about 7, it is mainly in its volatile form, HCN. HCN interferes with cellular respiration by binding and inhibiting the activity of an enzyme in the electron transport chain, cytochrome C

oxidase, interfering with the transfer of electrons to oxygen, and therefore preventing the cell from aerobically producing ATP (Blumer and Haas, 2000).

Another well studied example of an antimicrobial produced by pseudomonads is 2,4-DAPG. It is produced by a wide range of *P. fluorescens* isolated from geographically diverse soils (Keel *et al.*, 1996). The synthesis of 2,4-DAPG is controlled by the *PHL* gene cluster, and it is closely related to other phloroglucinol compounds (Yang and Cao, 2012) although its direct method of action has not been fully elucidated. In 1964, the compound pyrrolnitrin was identified as a new antibiotic produced by pseudomonads (Arima *et al.*, 1964). Four genes encode the proteins necessary for biosynthesis of pyrrolnitrin and a phenyl pyrrole derivative of pyrrolnitrin has been used agriculturally as a fungicide (Hammer *et al.*, 1997). The biosynthesis of antifungal compound pyoluteorin requires ten genes and its structure includes a dichloropyrrol, derived from proline, and a resorcinol ring (Nowak-Thompson *et al.*, 1999). These compounds are the most commonly described antibiotics produced by biocontrol pseudomonads for direct antagonism of phytopathogens, but for most of these compounds the mechanism of action is not clearly defined. Although these are commonly found in biocontrol pseudomonads, they do not account for total biocontrol ability and there are many other antibiotic compounds produced by pseudomonads that have yet to be identified.

1.4.2 Microbes can induce systemic resistance in plants against phytopathogens

In addition to the direct secretion of antimicrobials, biocontrol bacteria can also act through triggering ISR, which works through a whole-plant signaling mechanism in which events triggered in plant roots can lead to protection even in aerial portions of the plant. Plant defense signaling pathways are complex and

intertwined. There is considerable variation in these pathways, depending on the plant, the organism triggering the plant's defense response, and the range of pathogens which will be prevented from causing disease. The initial recognition between a plant and a beneficial is the same as with any microbe and depends on microbe-associated molecular patterns (MAMPs) being recognized by PRRs on the plant cell surface. The subsequent ISR signaling prepares a plant for pathogen attack without the energetic costs of constitutively expressing defense genes. When ISR is triggered in a plant, it is often referred to as priming and results in production of plant phytoalexins to combat potential pathogens as well as reinforcement of the cell wall to hinder the ability of pathogens to penetrate plant cells (Heil and Bostock, 2002). There are parallels with systemic acquired resistance (SAR), a response triggered by pathogens, but ISR does not lead to negative consequences in the plant as seen in pathogen infections, such as necrosis. The hypersensitive response (HR) response, a fast localized event, also differs from ISR because it involves an oxidative burst and necrosis of the affected tissue (Bordiec *et al.*, 2011; Lamb and Dixon, 1997). Bacteria differ in their ability to trigger ISR in various plants (van Loon *et al.*, 1998) and this induction can occur through several pathways. The most well studied pathways for inducing ISR are mediated through either salicylic acid (SA) or ethylene (ETH) and jasmonic acid (JA). Typically, SA mediated signaling is associated with SAR but it can also be involved in ISR. The role of SA in SAR is to ultimately up-regulate pathogenesis-related (PR) genes in the plant. Typically, ISR is independent of SA signaling and induction of PR genes, although this is not always the case (Pieterse *et al.*, 1996). The SA induced signaling for SAR crosstalks with other pathways including ISR as well as ET and JA dependent pathways involved in response to herbivores and necrotrophic pathogens

(Durrant and Dong, 2004). Transgenic rice lines have been created which are deficient in SA, ETH, or JA signaling. These lines were used to show that ISR induction by *P. fluorescens* WCS374r is dependent on JA and ETH signaling but not SA (De Vleeschauwer *et al.*, 2008). Conflicting with this, a rice line impaired in JA signaling led to PR gene expression and increased resistance to *M. oryzae* infections (Mei *et al.*, 2006) indicating that it was SA signaling which was important for the resistance, and that the lack of JA positively influenced resistance. JA signaling has been shown to antagonize SA signaling (Adie *et al.*, 2007), partially explaining the increased expression of PR genes in JA deficient rice lines.

Overall, the signaling involved in ISR is becoming clearer, though it varies from system to system. However, common genes are involved, and overlap is seen with other defense pathways. Much of the research that has been done on ISR is based on the model system of *A. thaliana* and there is much to be explored in monocot crop plants.

1.5 Multifaceted Functions of Absciscic Acid in Plant Signaling

Absciscic acid (ABA) is a plant hormone that is most well-studied for its role in plant response to abiotic stress, particularly drought. ABA was first discovered for its role in abscission, the natural process by which plants drop leaves, fruit, flowers, or seeds, although it was later shown that this is not true in all plants (Craker and Abeles, 1969). ABA also acts antagonistically to gibberellic acid (GA) to maintain seed dormancy and prevent precocious germination. During seed development, it also promotes the biosynthesis of storage compounds (Koornneef *et al.*, 2002, Kim, 2007).

Beyond normal physiological processes in plants, ABA signaling is a crucial component of abiotic stress tolerance. Drought, low temperature, and high salinity all

induce ABA-mediated responses such as the accumulation of compatible solutes and biosynthesis of proteins such as dehydrins which prevent water loss in tissues and prevent plasmolysis (Sreenivasulu *et al.*, 2012). Elevated ABA levels results in reduced photosynthesis and during long-term stress can severely restrict plant growth. ABA is biosynthesized in shoots during cold stress to inhibit growth and protect plants against low temperatures (Jiang *et al.*, 2013, Roychoudhury *et al.*, 2013). ABA is also biosynthesized in roots, particularly during heat, drought, or high salinity, and it is then translocated to leaves where it closes stomata to slow transpiration and prevent water loss. The closure of stomata occurs when the plasma membranes of the two guard cells become depolarized, resulting in an outflow of potassium which changes the osmotic potential of the cells causing them to shrink, thus closing the stomata (Kim 2014).

ABA is detected by the PYR/PYL/RCAR family of receptors. Binding of ABA results in the separation of dimerized receptors, and the monomer which has ABA bound to it will latch around the ABA molecule, surrounding it. This creates a site which can bind PP2Cs. In the absence of ABA, PP2Cs bind and inhibit the activity of SnRK2s. In the presence of ABA, SnRK2s are released from PP2Cs and can be activated through phosphorylation. Active SnRK2s can enter the nucleus and phosphorylate transcription factors which will promote expression of genes involved in abiotic stress tolerance (Santiago *et al.*, 2012) (Figure 1.1).

In contrast to the canonical defense hormones SA, JA, and ETH, elevated levels of ABA play a mainly negative role in plant immunity. ABA acts antagonistically to SA, JA, and ETH and as a result suppresses ISR and SAR (Figure 1.2). In general, high levels of ABA have been associated with reduced resistance to

disease while low levels of ABA result in increased resistance (Koga *et al.*, 2004, Jiang *et al.*, 2010, Yazawa *et al.*, 2012, Xu *et al.*, 2013) particularly at very early stages of disease (Yazawa *et al.*, 2012) and during later stages of disease in the case of hemibiotrophic pathogen *Cercospora beticola* (Schmidt *et al.*, 2008).

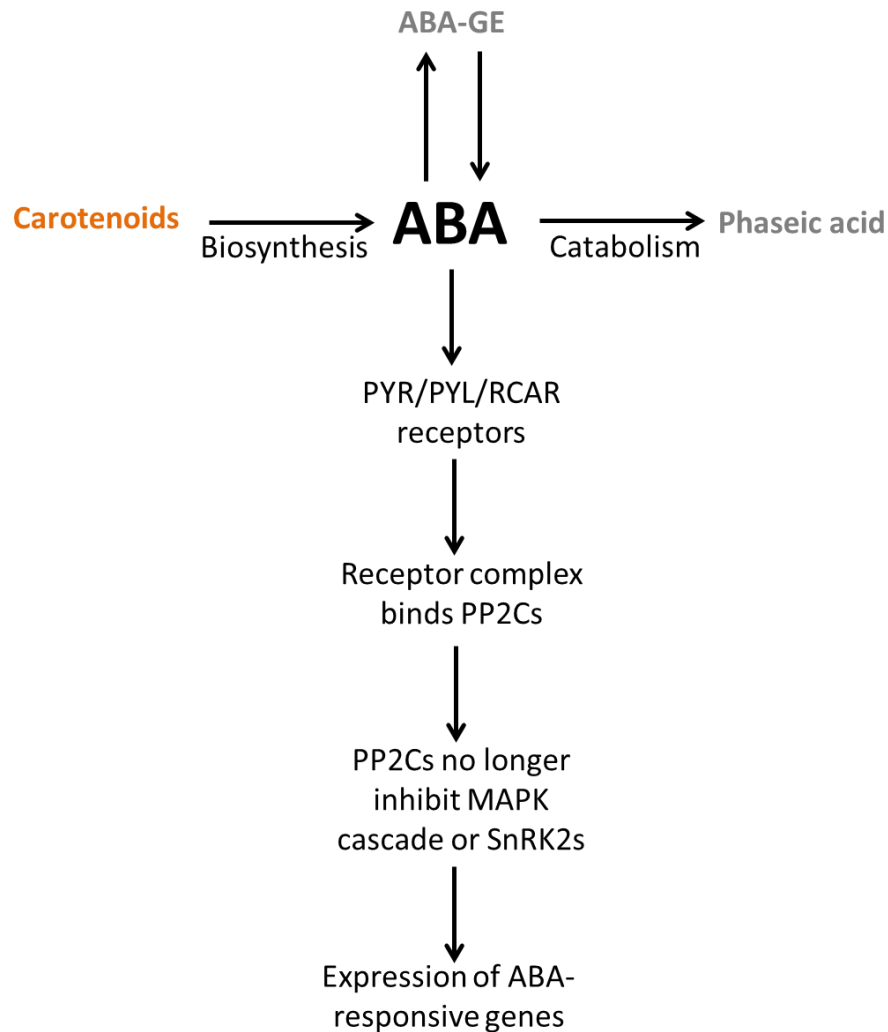


Figure 1.1. **Schematic of ABA signaling in plants.** Carotenoids are used as precursors for ABA biosynthesis in plants, and ABA can be later degraded into phaseic acid. The conjugation of glucosyl esters to ABA prevents ABA signaling from occurring, but this inactivation is reversible. The inactivation of ABA can be used as a mechanism to quickly regulate downstream ABA signaling events. ABA receptors exist in dimerized form, but dissociate from one another once bound to ABA. The ABA receptor complex can bind PP2Cs, which otherwise serve to inhibit a MAPK cascade and SnRK2s. Once the MAPK cascade and SnRK2s are activated, it results in phosphorylation of bZIP transcription factors, thereby activating them, which promotes transcription of ABA responsive genes.

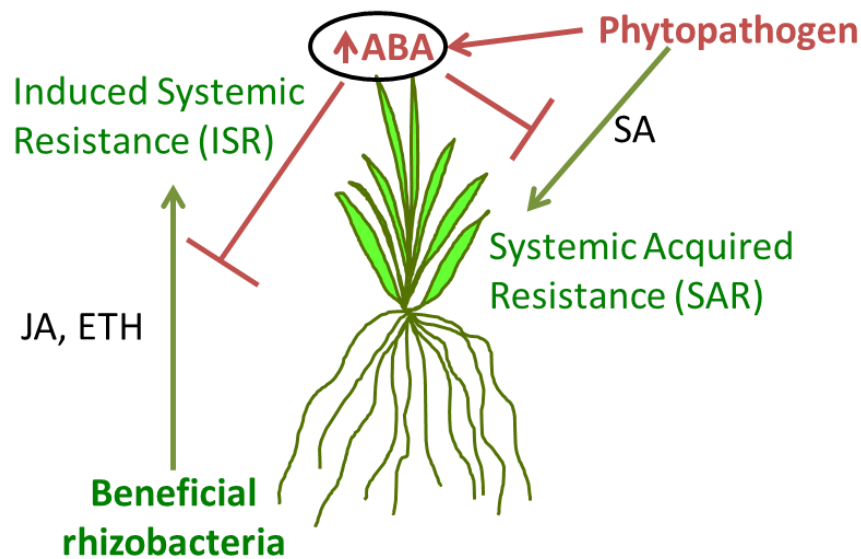


Figure 1.2. **Mechanisms by which ABA impedes plant defenses.** High levels of ABA, which can be induced in plants by phytopathogens, decreases resistance in plants by inhibiting SA, JA, and ETH signaling, thereby blocking Induced Systemic Resistance and Systemic Acquired Resistance.

There is also evidence that ABA can play a positive role in defense to certain herbivores and microbial pathogens during specific stages of infection. For example, some pathogens enter through open wounds or stomata. Since ABA closes stomata, it prevents the entry of those pathogens into the host (Ton *et al.*, 2009). This is not the case however with *M. oryzae*, which enters rice by physically forcing its way through the cuticle. There are conflicting reports regarding whether ABA inhibits or promotes the deposition of callose to fortify cell walls in response to pathogen entry (Ton *et al.*, 2009, Oide *et al.*, 2013). Usually, ABA signaling induces the formation of reactive oxygen species (ROS) which are a part of the plant HR, though there are times when ABA signaling can suppress ROS (Asselbergh *et al.*, 2007). However, *M. oryzae*,

which has the capacity to tolerate and detoxify large quantities of ROS (Samalova et al, 2014, Donofrio and Wilson, 2014), also utilizes ROS for cytoskeletal rearrangements during invasion and cannot cause disease in the absence of ROS (Egan et al, 2007, Ryder et al, 2013). Therefore, the production of ROS does not deter *M. oryzae* and can enhance virulence.

1.6 ABA in Fungi

ABA is likely to be an ancient signaling molecule which was present in an early unicellular eukaryotic ancestor (Hauser *et al.*, 2011). Several phytopathogenic fungi, including *M. oryzae*, retain the ability to synthesize ABA. In plants, ABA is synthesized through cleavage of carotenoids in the ‘indirect’ or ‘non-mevalonate’ pathway (Schwartz *et al.*, 1997, Loitenberg *et al.*, 1999, Schwartz *et al.*, 2003). In fungi, biosynthesis proceeds through the mevalonate pathway, involving 4 key enzymes (Siewers et al, 2006). There is evidence that the majority of fungal-produced ABA is secreted and the role for ABA in fungi is still largely a mystery. It has not been linked to stress response, contrary to what is seen in plants (Hartung *et al.*, 2010).

Recently, it was shown that an endophytic fungus, *Nigrospora sp.*, which lives in the medicinal plant *Fragaria virginiana*, produces ABA which serves to inhibit mycobacteria including *Mycobacterium tuberculosis*. The inhibitory activity was only moderate, but Mycobacteria have a characteristically strong cell wall which makes them resistant to most antibiotics, and it was shown that even relatively high levels of ABA were not toxic to mammalian cells (Clark *et al.*, 2013). However, from the fungal perspective, the role of ABA is still unclear.

As previously mentioned, ABA can promote ROS, which is required for virulence in *M. oryzae*. ROS is required for the rearrangement of the fungal

cytoskeleton during the progression of blast disease and is generated by NADPH oxidases, three of which are found in *M. oryzae* and named *Nox1-3* (Egan *et al.*, 2007, Ryder *et al.*, 2013). Although *Nox* mutants retain the ability to form appressoria, they are unable to penetrate the host (Egan *et al.*, 2007). However, ABA can also play a role in the suppression of ROS (Asselbergh *et al.*, 2007), and ROS can be harmful to many fungi. Therefore, it is unlikely that the main role of ABA in fungi is to generate ROS. ABA is likely to contribute to fungal fitness or virulence, but as of yet this area of research remains unexplored.

1.7 References

- Adie B, Chico JM, Rubio-Somoza I, Solano R** (2007) Modulation of plant defenses by ethylene. *Journal of Plant Growth Regulation* **26**: 160-177
- Ahmed M, Stal LJ, Hasnain S** (2010) Production of Indole-3-Acetic Acid by the *Cyanobacterium Arthrospira platensis* Strain MMG-9. *Journal of Microbiology and Biotechnology* **20**: 1259-1265
- Ahn IP, Kim S, Lee YH, Suh SC** (2007) Vitamin B-1-induced priming is dependent on hydrogen peroxide and the *NPR1* gene in Arabidopsis. *Plant Physiology* **143**: 838-848
- Aktar W, Sengupta D, Chowdhury A** (2009) Impact of pesticides use in agriculture: their benefits and hazards. *Interdisciplinary toxicology* **2**: 1-12
- Andrews JH, Harris RF** (2000) The ecology and biogeography of microorganisms of plant surfaces. *Annual Review of Phytopathology* **38**: 145-180
- Arima K, Fukuta A, Imanaka H, Kousaka M, Tamura G** (1964) Pyrrolnitrin new antibiotic substance produced by *Pseudomonas*. *Agricultural and Biological Chemistry* **28**: 575-&
- Arnold AE, Mejia LC, Kylo D, Rojas EI, Maynard Z, Robbins N, Herre EA** (2003) Fungal endophytes limit pathogen damage in a tropical tree. *Proceedings of the National Academy of Sciences of the United States of America* **100**: 15649-15654
- Asselbergh B, Curvers K, Franca SC, Audenaert K, Vuylsteke M, Van Breusegem F, Hofte M** (2007) Resistance to *Botrytis cinerea* in sitiens, an abscisic acid-deficient tomato mutant, involves timely production of hydrogen peroxide and cell wall modifications in the epidermis. *Plant Physiology* **144**: 1863-1877
- Audenaert K, Monbaliu S, Deschuyffeleer N, Maene P, Vekeman F, Haesaert G, De Saeger S, Eeckhout M** (2012) Neutralized electrolyzed water efficiently reduces *Fusarium* spp. in vitro and on wheat kernels but can trigger deoxynivalenol (DON) biosynthesis. *Food Control* **23**: 515-521
- Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM** (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. *In Annual Review of Plant Biology*, Vol 57, pp 233-266

- Bloemberg GV, Otoole GA, Lugtenberg BJJ, Kolter R** (1997) Green fluorescent protein as a marker for *Pseudomonas* spp. Applied and Environmental Microbiology **63**: 4543-4551
- Blumer C, Haas D** (2000) Mechanism, regulation, and ecological role of bacterial cyanide biosynthesis. Archives of Microbiology **173**: 170-177
- Bordiec S, Paquis S, Lacroix H, Dhondt S, Barka EA, Kauffmann S, Jeandet P, Mazeyrat-Gourbeyre F, Clement C, Baillieul F, Dorey S** (2011) Comparative analysis of defence responses induced by the endophytic plant growth-promoting rhizobacterium *Burkholderia phytofirmans* strain PsJN and the non-host bacterium *Pseudomonas syringae* pv. pisi in grapevine cell suspensions. Journal of Experimental Botany **62**: 595-603
- Brencic A, Winans SC** (2005) Detection of and response to signals involved in host-microbe interactions by plant-associated bacteria. Microbiology and Molecular Biology Reviews **69**: 155-+
- Burd GI, Dixon DG, Glick BR** (2000) Plant growth-promoting bacteria that decrease heavy metal toxicity in plants. Canadian Journal of Microbiology **46**: 237-245
- Burgess JG, Jordan EM, Bregu M, Mearns-Spragg A, Boyd KG** (1999) Microbial antagonism: a neglected avenue of natural products research. Journal of Biotechnology **70**: 27-32
- Cai RM, Lewis J, Yan SC, Liu HJ, Clarke CR, Campanile F, Almeida NF, Studholme DJ, Lindeberg M, Schneider D, Zaccardelli M, Setubal JC, Morales-Lizcano NP, Bernal A, Coaker G, Baker C, Bender CL, Leman S, Vinatzer BA** (2011) The plant pathogen *Pseudomonas syringae* pv. tomato is genetically monomorphic and under strong selection to evade tomato immunity. Plos Pathogens **7**
- Cani PD, Delzenne NM** (2011) The gut microbiome as therapeutic target. Pharmacology & Therapeutics **130**: 202-212
- Carroll G** (1988) Fungal endophytes in stems and leaves: From latent pathogen to mutualistic symbiont. Ecology **69**: 2-9
- Carroll G** (1995) Forest endophytes - pattern and process. Canadian Journal of Botany-Revue Canadienne De Botanique **73**: S1316-S1324
- Chisholm ST, Coaker G, Day B, Staskawicz BJ** (2006) Host-microbe interactions: Shaping the evolution of the plant immune response. Cell **124**: 803-814

- Clark TN, Ellsworth K, Li H, Johnson JA, Gray CA** (2013) Isolation of the plant hormone (+) - abscisic acid as an antimycobacterial constituent of the medicinal plant endophyte, *Nigrospora sp.* *Natural Product Communications* **8**: 1673-1674
- Coca M, Bortolotti C, Rufat M, Penas G, Eritja R, Tharreau D, del Pozo AM, Messeguer J, San Segundo B** (2004) Transgenic rice plants expressing the antifungal AFP protein from *Aspergillus giganteus* show enhanced resistance to the rice blast fungus *Magnaporthe grisea*. *Plant Molecular Biology* **54**: 245-259
- Colborn T, Saal FSV, Soto AM** (1993) Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environmental Health Perspectives* **101**: 378-384
- Costacurta A, Vanderleyden J** (1995) Synthesis of phytohormones by plant-associated bacteria. *Critical Reviews in Microbiology* **21**: 1-18
- Craker LE, Abeles FB** (1969) Abscission - Role of abscisic acid. *Plant Physiology* **44**: 1144
- Danhorn T, Fuqua C** (2007) Biofilm formation by plant-associated bacteria. *Annual Review of Microbiology* **61**: 401-422
- Daval S, Lebreton L, Gazengel K, Boutin M, Guillermin-Erckelboudt AY, Sarniguet A** (2011) The biocontrol bacterium *Pseudomonas fluorescens* Pf29Arp strain affects the pathogenesis-related gene expression of the take-all fungus *Gaeumannomyces graminis* var. *tritici* on wheat roots. *Molecular Plant Pathology* **12**: 839-854
- De Vleeschauwer D, Djavaheiri M, Bakker P, Hofte M** (2008) *Pseudomonas fluorescens* WCS374r-induced systemic resistance in rice against *Magnaporthe oryzae* is based on pseudobactin-mediated priming for a salicylic acid-repressible multifaceted defense response. *Plant Physiology* **148**: 1996-2012
- de Weert S, Vermeiren H, Mulders IHM, Kuiper I, Hendrickx N, Bloemberg GV, Vanderleyden J, De Mot R, Lugtenberg BJJ** (2002) Flagella-driven chemotaxis towards exudate components is an important trait for tomato root colonization by *Pseudomonas fluorescens*. *Molecular Plant-Microbe Interactions* **15**: 1173-1180

- Dean R, Van Kan JAL, Pretorius ZA, Hammond-Kosack KE, Di Pietro A, Spanu PD, Rudd JJ, Dickman M, Kahmann R, Ellis J, Foster GD** (2012) The Top 10 fungal pathogens in molecular plant pathology. *Molecular Plant Pathology* **13**: 414-430
- deJong JC, McCormack BJ, Smirnoff N, Talbot NJ** (1997) Glycerol generates turgor in rice blast. *Nature* **389**: 244-245
- Del Sorbo G, Schoonbeek HJ, De Waard MA** (2000) Fungal transporters involved in efflux of natural toxic compounds and fungicides. *Fungal Genetics and Biology* **30**: 1-15
- Deweger LA, Jann B, Jann K, Lugtenberg B** (1987) Lipopolysaccharides of *Pseudomonas* spp that stimulate plant-growth - composition and use for strain identification. *Journal of Bacteriology* **169**: 1441-1446
- Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM, Zoeller RT, Gore AC** (2009) Endocrine-disrupting chemicals: An endocrine society scientific statement. *Endocrine Reviews* **30**: 293-342
- Dong SM, Yin WX, Kong GH, Yang XY, Qutob D, Chen QH, Kale SD, Sui YY, Zhang ZG, Dou DL, Zheng XB, Gijzen M, Tyler BM, Wang YC** (2011) *Phytophthora sojae* avirulence effector Avr3b is a secreted NADH and ADP-ribose pyrophosphorylase that modulates plant immunity. *Plos Pathogens* **7**
- Dong SM, Yu D, Cui LK, Qutob D, Tedman-Jones J, Kale SD, Tyler BM, Wang YC, Gijzen M** (2011) Sequence variants of the *Phytophthora sojae* RXLR Effector Avr3a/5 are differentially recognized by Rps3a and Rps5 in soybean. *Plos One* **6**
- Donofrio NM, Wilson RA** (2014) Redox and rice blast: newtools for dissecting molecular fungal-plant interactions. *New Phytologist* **201**: 367-369
- Dowling DN, Ogara F** (1994) Metabolites of *Pseudomonas* involved in the biocontrol of plant-disease. *Trends in Biotechnology* **12**: 133-141
- Dridi B, Raoult D, Drancourt M** (2011) Archaea as emerging organisms in complex human microbiomes. *Anaerobe* **17**: 56-63
- Durrant WE, Dong X** (2004) Systemic acquired resistance. *Annual Review of Phytopathology* **42**: 185-209

- Egan MJ, Wang ZY, Jones MA, Smirnov N, Talbot NJ** (2007) Generation of reactive oxygen species by fungal NADPH oxidases is required for rice blast disease. *Proceedings of the National Academy of Sciences of the United States of America* **104**: 11772-11777
- Farooq M, Kobayashi N, Wahid A, Ito O, Basra SMA** (2009) Strategies for producing more rice with less water. *In* DL Sparks, ed, *Advances in Agronomy*, Vol 101, Vol 101. Elsevier Academic Press Inc, San Diego, pp 351-388
- Fukui R, Poinar EI, Bauer PH, Schroth MN, Henderson M, Wang XL, Hancock JG** (1994) Spatial colonization patterns and interaction of bacteria on inoculated sugar-beet seed. *Phytopathology* **84**: 1338-1345
- Fuller R** (1989) Probiotics in man and animals. *Journal of Applied Bacteriology* **66**: 365-378
- Gandon S, Capowiez Y, Dubois Y, Michalakis Y, Olivieri I** (1996) Local adaptation and gene-for-gene coevolution in a metapopulation model. *Proceedings of the Royal Society of London Series B-Biological Sciences* **263**: 1003-1009
- Gibson GR, Roberfroid MB** (1995) Dietary modulation of the human colonic microbiota - introducing the concept of prebiotics. *Journal of Nutrition* **125**: 1401-1412
- Giraldo MC, Dagdas YF, Gupta YK, Mentlak TA, Yi M, Martinez-Rocha AL, Saitoh H, Terauchi R, Talbot NJ, Valent B** (2013) Two distinct secretion systems facilitate tissue invasion by the rice blast fungus *Magnaporthe oryzae*. *Nature Communications* **4**
- Grover M, Ali SZ, Sandhya V, Rasul A, Venkateswarlu B** (2011) Role of microorganisms in adaptation of agriculture crops to abiotic stresses. *World Journal of Microbiology & Biotechnology* **27**: 1231-1240
- Gschwendtner S, Esperschuetz J, Buegger F, Reichmann M, Muller M, Munch JC, Schlöter M** (2011) Effects of genetically modified starch metabolism in potato plants on photosynthate fluxes into the rhizosphere and on microbial degraders of root exudates. *Fems Microbiology Ecology* **76**: 564-575
- Haas D, Keel C, Reimann C** (2002) Signal transduction in plant-beneficial rhizobacteria with biocontrol properties. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **81**: 385-395

- Hamada M, Hashimoto T, Takahashi S, Yoneyama M, Miyake T, Takeuchi Y, Okami Y, Umezawa H** (1967) Antifungal activity of kasugamycin. *J. Antibiot. B.* **20**: 424-426
- Hammer PE, Hill DS, Lam ST, VanPee KH, Ligon JM** (1997) Four genes from *Pseudomonas fluorescens* that encode the biosynthesis of pyrrolnitrin. *Applied and Environmental Microbiology* **63**: 2147-2154
- Hartung W** (2010) The evolution of abscisic acid (ABA) and ABA function in lower plants, fungi and lichen. *Functional Plant Biology* **37**: 806-812
- Hauser F, Waadt R, Schroeder JI** (2011) Evolution of Absciscic Acid Synthesis and Signaling Mechanisms. *Current Biology* **21**: R346-R355
- Heil M, Bostock RM** (2002) Induced systemic resistance (ISR) against pathogens in the context of induced plant defences. *Annals of Botany* **89**: 503-512
- Hess RA, Nakai M** (2000) Histopathology of the male reproductive system induced by the fungicide benomyl. *Histology and Histopathology* **15**: 207-224
- Hirano SS, Upper CD** (2000) Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae* - a pathogen, ice nucleus, and epiphyte. *Microbiology and Molecular Biology Reviews* **64**: 624-+
- Howard RJ, Valent B** (1996) Breaking and entering: Host penetration by the fungal rice blast pathogen *Magnaporthe grisea*. *Annual Review of Microbiology* **50**: 491-512
- Hugouvieux V, Barber CE, Daniels MJ** (1998) Entry of *Xanthomonas campestris* pv. *campestris* into hydathodes of *Arabidopsis thaliana* leaves: A system for studying early infection events in bacterial pathogenesis. *Molecular Plant-Microbe Interactions* **11**: 537-543
- Imamura T, Yasuda M, Kusano H, Nakashita H, Ohno Y, Kamakura T, Taguchi S, Shimada H** (2010) Acquired resistance to the rice blast in transgenic rice accumulating the antimicrobial peptide thanatin. *Transgenic Research* **19**: 415-424
- Iwata M** (2001) Probenazole- a plant defense activator. *Pesticide outlook*: 28-31
- Jaeger CH, Lindow SE, Miller S, Clark E, Firestone MK** (1999) Mapping of sugar and amino acid availability in soil around roots with bacterial sensors of sucrose and tryptophan. *Applied and Environmental Microbiology* **65**: 2685-2690

- Jansen C, von Wettstein D, Schafer W, Kogel KH, Felk A, Maier FJ** (2005) Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted *Fusarium graminearum*. Proceedings of the National Academy of Sciences of the United States of America **102**: 16892-16897
- Jha S, Chattoo BB** (2010) Expression of a plant defensin in rice confers resistance to fungal phytopathogens. Transgenic Research **19**: 373-384
- Jiang CJ, Shimono M, Sugano S, Kojima M, Yazawa K, Yoshida R, Inoue H, Hayashi N, Sakakibara H, Takatsuji H** (2010) Abscissic acid interacts antagonistically with salicylic acid signaling pathway in rice-*Magnaporthe grisea* interaction. Molecular Plant-Microbe Interactions **23**: 791-798
- Jiang JH, Yun YZ, Fu J, Shim WB, Ma ZH** (2011) Involvement of a putative response regulator FgRrg-1 in osmotic stress response, fungicide resistance and virulence in *Fusarium graminearum*. Molecular Plant Pathology **12**: 425-436
- Jiang Y, Peng D, Bai LP, Ma H, Chen LJ, Zhao MH, Xu ZJ, Guo ZF** (2013) Molecular switch for cold acclimation - anatomy of the cold-inducible promoter in plants. Biochemistry-Moscow **78**: 342-354
- Jung HI, Kuk YI, Kim HY, Back K, Lee DJ, Lee S, Burgos NR** (2010) Resistance levels and fitness of protoporphyrinogen oxidase (PROTOX) inhibitor-resistant transgenic rice in paddy fields. Field Crops Research **115**: 125-131
- Kaplan JL, Shi HN, Walker WA** (2011) The role of microbes in developmental immunologic programming. Pediatric Research **69**: 465-472
- Kato H** (2001) Rice blast disease. Pesticide Outlook **12**: 23-25
- Kato-Noguchi H** (2009) Stress-induced allelopathic activity and momilactone B in rice. Plant Growth Regulation **59**: 153-158
- Keel C, Weller DM, Natsch A, Defago G, Cook RJ, Thomashow LS** (1996) Conservation of the 2,4-diacetylphloroglucinol biosynthesis locus among fluorescent *Pseudomonas* strains from diverse geographic locations. Applied and Environmental Microbiology **62**: 552-563
- Kim SY** (2007) Recent advances in ABA signaling. Journal of Plant Biology **50**: 117-121

- Kim TH** (2014) Mechanism of ABA signal transduction: Agricultural highlights for improving drought tolerance. *Journal of Plant Biology* **57**: 1-8
- Knief C, Delmotte N, Vorholt JA** (2011) Bacterial adaptation to life in association with plants - A proteomic perspective from culture to in situ conditions. *Proteomics* **11**: 3086-3105
- Koga H, Dohi K, Mori M** (2004) Absciscic acid and low temperatures suppress the whole plant-specific resistance reaction of rice plants to the infection of *Magnaporthe grisea*. *Physiological and Molecular Plant Pathology* **65**: 3-9
- Kohler T, Curty LK, Barja F, van Delden C, Pechere JC** (2000) Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. *Journal of Bacteriology* **182**: 5990-5996
- Kong HDH** (2011) Skin microbiome: genomics-based insights into the diversity and role of skin microbes. *Trends in Molecular Medicine* **17**: 320-328
- Koornneef M, Bentsink L, Hilhorst H** (2002) Seed dormancy and germination. *Current Opinion in Plant Biology* **5**: 33-36
- Kravchenko LV, Shapozhnikov AI, Makarova NM, Azarova TS, L'Vova KA, Kostyuk, II, Lyapunova OA, Tikhonovich IA** (2011) Exometabolites of bread wheat and tomato affecting the plant-microbe interactions in the rhizosphere. *Russian Journal of Plant Physiology* **58**: 936-940
- Kulik T, Lojko M, Jestoi M, Perkowski J** (2012) Sublethal concentrations of azoles induce tri transcript levels and trichothecene production in *Fusarium graminearum*. *Fems Microbiology Letters* **335**: 58-67
- Lamb C, Dixon RA** (1997) The oxidative burst in plant disease resistance. *Annual Review of Plant Physiology and Plant Molecular Biology* **48**: 251-275
- Lee YJ, Yamamoto K, Hamamoto H, Nakaune R, Hibi T** (2005) A novel ABC transporter gene ABC2 involved in multidrug susceptibility but not pathogenicity in rice blast fungus, *Magnaporthe grisea*. *Pesticide Biochemistry and Physiology* **81**: 13-23
- Leveau JHJ, Lindow SE** (2001) Appetite of an epiphyte: Quantitative monitoring of bacterial sugar consumption in the phyllosphere. *Proceedings of the National Academy of Sciences of the United States of America* **98**: 3446-3453

- Li H, Zhou SY, Zhao WS, Su SC, Peng YL** (2009) A novel wall-associated receptor-like protein kinase gene, OsWAK1, plays important roles in rice blast disease resistance. *Plant Molecular Biology* **69**: 337-346
- Li P, Pei Y, Sang XC, Ling YH, Yang ZL, He GH** (2009) Transgenic indica rice expressing a bitter melon (*Momordica charantia*) class I chitinase gene (McCHIT1) confers enhanced resistance to *Magnaporthe grisea* and *Rhizoctonia solani*. *European Journal of Plant Pathology* **125**: 533-543
- Li SH, Oakley CE, Chen GF, Han XY, Oakley BR, Xiang X** (2005) Cytoplasmic dynein's mitotic spindle pole localization requires a functional anaphase-promoting complex, gamma-tubulin, and NUDF/LIS1 in *Aspergillus nidulans*. *Molecular Biology of the Cell* **16**: 3591-3605
- Li W, Wang BH, Wu J, Lu GD, Hu YJ, Zhang X, Zhang ZG, Zhao Q, Feng QY, Zhang HY, Wang ZY, Wang GL, Han B, Wang ZH, Zhou B** (2009) The *Magnaporthe oryzae* avirulence gene AvrPiz-t encodes a predicted secreted protein that triggers the immunity in rice mediated by the blast resistance gene Piz-t. *Molecular Plant-Microbe Interactions* **22**: 411-420
- Linares JF, Gustafsson I, Baquero F, Martinez JL** (2006) Antibiotics as intermicrobial signaling agents instead of weapons. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 19484-19489
- Lindow SE, Arny DC, Upper CD** (1982) Bacterial ice nucleation - A factor in frost injury to plants. *Plant Physiology* **70**: 1084-1089
- Ling N, Raza W, Ma JH, Huang QW, Shen QR** (2011) Identification and role of organic acids in watermelon root exudates for recruiting *Paenibacillus polymyxa* SQR-21 in the rhizosphere. *European Journal of Soil Biology* **47**: 374-379
- Liotenberg S, North H, Marion-Poll A** (1999) Molecular biology and regulation of abscisic acid biosynthesis in plants. *Plant Physiology and Biochemistry* **37**: 341-350
- Liu HJ, Colavitti R, Rovira, II, Finkel T** (2005) Redox-dependent transcriptional regulation. *Circulation Research* **97**: 967-974
- Liu XG, Bimerew M, Ma YX, Mueller H, Ovadis M, Eberl L, Berg G, Chernin L** (2007) Quorum-sensing signaling is required for production of the antibiotic pyrrolnitrin in a rhizospheric biocontrol strain of *Serratia plymuthica*. *Fems Microbiology Letters* **270**: 299-305

- Lucero ME, Unc A, Cooke P, Dowd S, Sun SL** (2011) Endophyte microbiome diversity in micropropagated *Atriplex canescens* and *Atriplex torreyi* var *griffithsii*. *Plos One* **6**: 12
- Lugtenberg BJJ, Dekkers L, Bloemberg GV** (2001) Molecular determinants of rhizosphere colonization by *Pseudomonas*. *Annual Review of Phytopathology* **39**: 461-+
- Mares D, Romagnoli C, Andreotti E, Forlani G, Guccione S, Vicentini CB** (2006) Emerging antifungal azoles and effects on *Magnaporthe grisea*. *Mycological Research* **110**: 686-696
- Marshall R, Kombrink A, Motteram J, Loza-Reyes E, Lucas J, Hammond-Kosack KE, Thomma B, Rudd JJ** (2011) Analysis of two in planta expressed LysM effector homologs from the fungus *Mycosphaerella graminicola* reveals novel functional properties and varying contributions to virulence on wheat. *Plant Physiology* **156**: 756-769
- Martinez-Romero E** (2009) Coevolution in rhizobium-legume symbiosis? *DNA and Cell Biology* **28**: 361-370
- Mei CS, Qi M, Sheng GY, Yang YN** (2006) Inducible overexpression of a rice allene oxide synthase gene increases the endogenous jasmonic acid level, PR gene expression, and host resistance to fungal infection. *Molecular Plant-Microbe Interactions* **19**: 1127-1137
- Mendes R, Kruijt M, de Bruijn I, Dekkers E, van der Voort M, Schneider JHM, Piceno YM, DeSantis TZ, Andersen GL, Bakker P, Raaijmakers JM** (2011) Deciphering the rhizosphere microbiome for disease-suppressive bacteria. *Science* **332**: 1097-1100
- Miller MB, Bassler BL** (2001) Quorum sensing in bacteria. *Annual Review of Microbiology* **55**: 165-199
- Morris CE, Monier JM** (2003) The ecological significance of biofilm formation by plant-associated bacteria. *Annual Review of Phytopathology* **41**: 429-453
- Nandi S, Suzuki YA, Huang JM, Yalda D, Pham P, Wu LY, Bartley G, Huang N, Lonnerdal B** (2002) Expression of human lactoferrin in transgenic rice grains for the application in infant formula. *Plant Science* **163**: 713-722
- Nielsen TH, Christophersen C, Anthoni U, Sorensen J** (1999) Viscosinamide, a new cyclic depsipeptide with surfactant and antifungal properties produced by *Pseudomonas fluorescens* DR54. *Journal of Applied Microbiology* **87**: 80-90

- Nikolaev ON, Averyanov AA** (1991) Involvement of superoxide radical in the mechanism of fungicidal action of phthalide and probenazol. *Soviet Plant Physiology* **38**: 375-381
- Nomoto S, Mori K** (1997) Synthetic microbial chemistry .30. Synthesis of acetophthalidin, a fungal metabolite which inhibits the progression of the mammalian cell cycle. *Liebigs Annalen-Recueil*: 721-723
- Nowak-Thompson B, Chaney N, Wing JS, Gould SJ, Loper JE** (1999) Characterization of the pyoluteorin biosynthetic gene cluster of *Pseudomonas fluorescens* Pf-5. *Journal of Bacteriology* **181**: 2166-2174
- Ofek M, Hadar Y, Minz D** (2011) Colonization of cucumber seeds by bacteria during germination. *Environmental Microbiology* **13**: 2794-2807
- Oide S, Bejai S, Staal J, Guan N, Kaliff M, Dixelius C** (2013) A novel role of PR2 in abscisic acid (ABA) mediated, pathogen-induced callose deposition in *Arabidopsis thaliana*. *New Phytologist* **200**: 1187-1199
- Pareja L, Colazzo M, Perez-Parada A, Besil N, Heinzen H, Bocking B, Cesio V, Fernandez-Alba AR** (2012) Occurrence and distribution study of residues from pesticides applied under controlled conditions in the field during rice processing. *Journal of Agricultural and Food Chemistry* **60**: 4440-4448
- Patten CL, Glick BR** (1996) Bacterial biosynthesis on indole-3-acetic acid. *Canadian Journal of Microbiology* **42**: 207-220
- Pedraza RO, Motok J, Salazar SM, Ragout AL, Mentel MI, Tortora ML, Guerrero-Molina MF, Winik BC, Diaz-Ricci JC** (2010) Growth-promotion of strawberry plants inoculated with *Azospirillum brasilense*. *World Journal of Microbiology & Biotechnology* **26**: 265-272
- Pieterse CMJ, vanWees SCM, Hoffland E, vanPelt JA, vanLoon LC** (1996) Systemic resistance in *Arabidopsis* induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. *Plant Cell* **8**: 1225-1237
- Pimbucha R, Kaiser HM** (2009) Measuring the effectiveness of US rice export promotion programs. *Agribusiness* **25**: 215-230
- Podila GK, Sreedasyam A, Muratet MA** (2009) Populus rhizosphere and the ectomycorrhizal interactome. *Critical Reviews in Plant Sciences* **28**: 359-367

- Porras-Alfaro A, Bayman P** (2011) Hidden fungi, emergent properties: Endophytes and microbiomes. *In* NK VanAlfen, G Bruening, JE Leach, eds, Annual Review of Phytopathology, Vol 49, Vol 49. Annual Reviews, Palo Alto, pp 291-315
- Pratt LA, Kolter R** (1998) Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. *Molecular Microbiology* **30**: 285-293
- Radutoiu S, Madsen LH, Madsen EB, Felle HH, Umehara Y, Gronlund M, Sato S, Nakamura Y, Tabata S, Sandal N, Stougaard J** (2003) Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. *Nature* **425**: 585-592
- Rainey PB** (1999) Adaptation of *Pseudomonas fluorescens* to the plant rhizosphere. *Environmental Microbiology* **1**: 243-257
- Ramey BE, Koutsoudis M, von Bodman SB, Fuqua C** (2004) Biofilm formation in plant-microbe associations. *Current Opinion in Microbiology* **7**: 602-609
- Rasmussen TB, Givskov M** (2006) Quorum sensing inhibitors: a bargain of effects. *Microbiology-Sgm* **152**: 895-904
- Roinestad KS, Montville TJ, Rosen JD** (1994) Mechanism for sodium-bicarbonate inhibition of trochothecene biosynthesis in *Fusarium tricinctum*. *Journal of Agricultural and Food Chemistry* **42**: 2025-2028
- Rosenberg E, Sharon G, Atad I, Zilber-Rosenberg I** (2010) The evolution of animals and plants via symbiosis with microorganisms. *Environmental Microbiology Reports* **2**: 500-506
- Roychoudhury A, Paul S, Basu S** (2013) Cross-talk between abscisic acid-dependent and abscisic acid-independent pathways during abiotic stress. *Plant Cell Reports* **32**: 985-1006
- Rudrappa T, Biedrzycki ML, Bais HP** (2008) Causes and consequences of plant-associated biofilms. *Fems Microbiology Ecology* **64**: 153-166
- Rudrappa T, Czymmek KJ, Pare PW, Bais HP** (2008) Root-Secreted malic acid recruits beneficial soil bacteria. *Plant Physiology* **148**: 1547-1556

- Ryder LS, Dagdas YF, Mentlak TA, Kershaw MJ, Thornton CR, Schuster M, Chen JS, Wang ZH, Talbot NJ** (2013) NADPH oxidases regulate septin-mediated cytoskeletal remodeling during plant infection by the rice blast fungus. *Proceedings of the National Academy of Sciences of the United States of America* **110**: 3179-3184
- Sakurai H, Naito H, Fujita S** (1976) Sensitivity distribution of phytopathogenic bacteria and fungi to antibiotics. *Journal of Antibiotics* **29**: 1230-1236
- Samalova M, Meyer AJ, Gurr SJ, Fricker MD** (2014) Robust anti-oxidant defences in the rice blast fungus *Magnaporthe oryzae* confer tolerance to the host oxidative burst. *New Phytologist* **201**: 556-573
- Sanogo I, Amadou MM** (2010) Rice market integration and food security in Nepal: The role of cross-border trade with India. *Food Policy* **35**: 312-322
- Santiago J, Dupeux F, Betz K, Antoni R, Gonzalez-Guzman M, Rodriguez L, Marquez JA, Rodriguez PL** (2012) Structural insights into PYR/PYL/RCAR ABA receptors and PP2Cs. *Plant Science* **182**: 3-11
- Schardl CL, Leuchtmann A, Chung KR, Penny D, Siegel MR** (1997) Coevolution by common descent of fungal symbionts (*Epichloe* spp) and grass hosts. *Molecular Biology and Evolution* **14**: 133-143
- Schmidt K, Pflugmacher M, Klages S, Maser A, Mock A, Stahl DJ** (2008) Accumulation of the hormone abscisic acid (ABA) at the infection site of the fungus *Cercospora beticola* supports the role of ABA as a repressor of plant defence in sugar beet. *Molecular Plant Pathology* **9**: 661-673
- Schroth MN, Hancock JG** (1982) Disease-suppressive soil and root-colonizing bacteria. *Science* **216**: 1376-1381
- Schulze-Lefert P, Panstruga R** (2011) A molecular evolutionary concept connecting nonhost resistance, pathogen host range, and pathogen speciation. *Trends in Plant Science* **16**: 117-125
- Schwartz SH, Qin XQ, Zeevaart JAD** (2003) Elucidation of the indirect pathway of abscisic acid biosynthesis by mutants, genes, and enzymes. *Plant Physiology* **131**: 1591-1601
- Schwartz SH, Tan BC, Gage DA, Zeevaart JAD, McCarty DR** (1997) Specific oxidative cleavage of carotenoids by VP14 of maize. *Science* **276**: 1872-1874

- Shi SJ, Richardson AE, O'Callaghan M, DeAngelis KM, Jones EE, Stewart A, Firestone MK, Condon LM** (2011) Effects of selected root exudate components on soil bacterial communities. *Fems Microbiology Ecology* **77**: 600-610
- Shinjo A, Araki Y, Hirano K, Arie T, Ugaki M, Teraoka T** (2011) Transgenic rice plants that over-express the mannose-binding rice lectin have enhanced resistance to rice blast. *Journal of General Plant Pathology* **77**: 85-92
- Siewers V, Kokkelink L, Smedsgaard J, Tudzynski P** (2006) Identification of an abscisic acid gene cluster in the grey mold *Botrytis cinerea*. *Applied and Environmental Microbiology* **72**: 4619-4626
- Simons M, Permentier HP, deWeger LA, Wijffelman CA, Lugtenberg BJJ** (1997) Amino acid synthesis is necessary for tomato root colonization by *Pseudomonas fluorescens* strain WCS365. *Molecular Plant-Microbe Interactions* **10**: 102-106
- Singh P, Rathinasamy K, Mohan R, Panda D** (2008) Microtubule assembly dynamics: An attractive target for anticancer drugs. *Iubmb Life* **60**: 368-375
- Smalla K, Wieland G, Buchner A, Zock A, Parzy J, Kaiser S, Roskot N, Heuer H, Berg G** (2001) Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: Plant-dependent enrichment and seasonal shifts revealed. *Applied and Environmental Microbiology* **67**: 4742-4751
- Sundin GW, Jacobs JL** (1999) Ultraviolet radiation (UVR) sensitivity analysis and UVR survival strategies of a bacterial community from the phyllosphere of field-grown peanut (*Arachis hypogaea* L.). *Microbial Ecology* **38**: 27-38
- Suzuki F, Arai M, Yamaguchi J** (2007) Genetic analysis of *Pyricularia grisea* population by rep-PCR during development of resistance to scytalone dehydratase inhibitors of melanin biosynthesis. *Plant Disease* **91**: 176-184
- Takagaki M, Kaku K, Watanabe S, Kawai K, Shimizu T, Sawada H, Kumakura K, Nagayama K** (2004) Mechanism of resistance to carpropamid in *Magnaporthe grisea*. *Pest Management Science* **60**: 921-926
- Talbot NJ** (2003) On the trail of a cereal killer: Exploring the biology of *Magnaporthe grisea*. *Annual Review of Microbiology* **57**: 177-202
- Tilg H, Kaser A** (2011) Gut microbiome, obesity, and metabolic dysfunction. *Journal of Clinical Investigation* **121**: 2126-2132

- Tisdale S, Nelson W** (1975) Soil fertility and fertilizers. Macmillan Publishing, New York, U.S.A.
- Ton J, Flors V, Mauch-Mani B** (2009) The multifaceted role of ABA in disease resistance. *Trends in Plant Science* **14**: 310-317
- Toppari J, Larsen JC, Christiansen P, Giwercman A, Grandjean P, Guillette LJ, Jegou B, Jensen TK, Jouannet P, Keiding N, Leffers H, McLachlan JA, Meyer O, Muller J, Rajpert-DeMeyts E, Scheike T, Sharpe R, Sumpter J, Skakkebaek NE** (1996) Male reproductive health and environmental xenoestrogens. *Environmental Health Perspectives* **104**: 741-803
- Tukey HB** (1970) Leaching of substances from plants. *Annual Review of Plant Physiology* **21**: 305-&
- Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI** (2007) The human microbiome project. *Nature* **449**: 804-810
- van Loon LC, Bakker P, Pieterse CMJ** (1998) Systemic resistance induced by rhizosphere bacteria. *Annual Review of Phytopathology* **36**: 453-483
- Vitousek PM, Aber JD, Howarth RW, Likens GE, Matson PA, Schindler DW, Schlesinger WH, Tilman D** (1997) Human alteration of the global nitrogen cycle: Sources and consequences. *Ecological Applications* **7**: 737-750
- Walker TS, Bais HP, Grotewold E, Vivanco JM** (2003) Root exudation and rhizosphere biology. *Plant Physiology* **132**: 44-51
- Waters CM, Bassler BL** (2005) Quorum sensing: Cell-to-cell communication in bacteria. *Annual Review of Cell and Developmental Biology* **21**: 319-346
- Whitehead NA, Barnard AML, Slater H, Simpson NJL, Salmond GPC** (2001) Quorum-sensing in gram-negative bacteria. *Fems Microbiology Reviews* **25**: 365-404
- Wilson RA, Talbot NJ** (2009) Under pressure: investigating the biology of plant infection by *Magnaporthe oryzae*. *Nature Reviews Microbiology* **7**: 185-195
- Xie XN, Yoneyama K** (2010) The Strigolactone Story. *Annual Review of Phytopathology*, Vol 48 **48**: 93-117
- Xu J, Audenaert K, Hofte M, De Vleeschauwer D** (2013) Absciscic acid promotes susceptibility to the rice leaf blight pathogen *Xanthomonas oryzae* pv *oryzae* by suppressing salicylic acid-mediated defenses. *Plos One* **8**: 10

- Yamada N, Motoyama T, Nakasako M, Kagabu S, Kudo T, Yamaguchi I** (2004) Enzymatic characterization of scytalone dehydratase Val75Met variant found in melanin biosynthesis dehydratase inhibitor (MBI-D) resistant strains of the rice blast fungus. *Bioscience Biotechnology and Biochemistry* **68**: 615-621
- Yang F, Cao YJ** (2012) Biosynthesis of phloroglucinol compounds in microorganisms-review. *Applied Microbiology and Biotechnology* **93**: 487-495
- Yang XD, Anderson NJ, Dong XH, Shen J** (2008) Surface sediment diatom assemblages and epilimnetic total phosphorus in large, shallow lakes of the Yangtze floodplain: their relationships and implications for assessing long-term eutrophication. *Freshwater Biology* **53**: 1273-1290
- Yazawa K, Jiang CJ, Kojima M, Sakakibara H, Takatsuji H** (2012) Reduction of abscisic acid levels or inhibition of abscisic acid signaling in rice during the early phase of *Magnaporthe oryzae* infection decreases its susceptibility to the fungus. *Physiological and Molecular Plant Pathology* **78**: 1-7
- Ye XD, Al-Babili S, Kloti A, Zhang J, Lucca P, Beyer P, Potrykus I** (2000) Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. *Science* **287**: 303-305
- Yoon MY, Kim YS, Ryu SY, Choi GJ, Choi YH, Jang KS, Cha B, Han SS, Kim JC** (2011) In vitro and in vivo antifungal activities of decursin and decursinol angelate isolated from *Angelica gigas* against *Magnaporthe oryzae*, the causal agent of rice blast. *Pesticide Biochemistry and Physiology* **101**: 118-124
- Yukioka H, Inagaki S, Tanaka R, Katoh K, Miki N, Mizutani A, Masuko M** (1998) Transcriptional activation of the alternative oxidase gene of the fungus *Magnaporthe grisea* by a respiratory-inhibiting fungicide and hydrogen peroxide. *Biochimica Et Biophysica Acta-Gene Structure and Expression* **1442**: 161-169
- Yukioka H, Tanaka R, Inagaki S, Katoh K, Miki N, Mizutani A, Masuko M, Kunoh H** (1997) Mutants of the phytopathogenic fungus *Magnaporthe grisea* deficient in alternative, cyanide-resistant, respiration. *Fungal Genetics and Biology* **22**: 221-228
- Zemanick ET, Sagel SD, Harris JK** (2011) The airway microbiome in cystic fibrosis and implications for treatment. *Current Opinion in Pediatrics* **23**: 319-324
- Zhou JM, Chai J** (2008) Plant pathogenic bacterial type III effectors subdue host responses. *Current Opinion in Microbiology* **11**: 179-185

ENDNOTES

¹ Short excerpts adapted from **Spence C, and Bais H.** (2013) Probiotics for plants: Rhizospheric microbiome and plant fitness. In Frans J. de Bruijn, Molecular Microbial Ecology of the Rhizosphere. 713-721.

Chapter 2

NATURAL RICE RHIZOSPHERIC MICROBES SUPPRESS RICE BLAST INFECTIONS²

2.1 Abstract

The natural interactions between plant roots and their rhizospheric microbiome are vital to plant fitness, modulating both growth promotion and disease suppression. In rice (*Oryza sativa*), a globally important food crop, as much as 30% of yields are lost due to blast disease caused by fungal pathogen *Magnaporthe oryzae*. Capitalizing on the abilities of naturally occurring rice soil bacteria to reduce *M. oryzae* infections could provide a sustainable solution to reduce the amount of crops lost to blast disease.

Naturally occurring root-associated rhizospheric bacteria were isolated from California field grown rice plants (M-104), eleven of which were taxonomically identified by 16S rRNA gene sequencing and fatty acid methyl ester (FAME) analysis. Bacterial isolates were tested for biocontrol activity against the devastating foliar rice fungal pathogen, *M. oryzae* pathovar 70-15. *In vitro*, a *Pseudomonas* isolate, EA105, displayed antibiosis through reducing appressoria formation by nearly 90% as well as directly inhibiting fungal growth by 76%. Although hydrogen cyanide (HCN) is a volatile commonly produced by biocontrol pseudomonads, the activity of EA105 seems to be independent of its HCN production. During *in planta* experiments, EA105 reduced the number of blast lesions formed by 33% and *Pantoea agglomerans* isolate, EA106 by 46%. The data also show both EA105 and EA106 trigger jasmonic

acid (JA) and ethylene (ETH) dependent induced systemic resistance (ISR) response in rice.

Out of 11 bacteria isolated from rice soil, pseudomonad EA105 most effectively inhibited the growth and appressoria formation of *M. oryzae* through a mechanism that is independent of cyanide production. In addition to direct antagonism, EA105 also appears to trigger ISR in rice plants through a mechanism that is dependent on JA and ETH signaling, ultimately resulting in fewer blast lesions. The application of native bacteria as biocontrol agents in combination with current disease protection strategies could aid in global food security.

2.2 Introduction

With a burgeoning world population, food security and crop protection are of utmost importance. One of the most important staple food crops is rice, which over 3.5 billion people are dependent on for daily energy consumption. Rice blast disease, caused by the wide-spread foliar fungal pathogen *Magnaporthe oryzae*, occurs in more than 85 countries and causes devastating crop loss. Each year this disease destroys enough rice to feed an estimated 60 million people (Zeigler, 1998) and, unfortunately, there are currently no effective means to provide lasting, adequate control of the pathogen.

Current low cost protection strategies include planting of uninfected seeds, limiting nitrogen fertilizers, perpetual field flooding, and post-harvest burning of plant remains (Skamnioti and Gurr, 2009); however, these strategies can neither eliminate infections nor resolve situations when a field does become infected. Rice varieties with genetic resistance to rice blast, for example, a cultivar carrying the *Pi-ta* R-gene are effective in initiating a gene-for-gene interaction with the corresponding *M. oryzae*

avirulence (AVR) gene and conferring resistance; yet the pathogen rapidly overcomes plant-encoded resistance (Bonman *et al.*, 1992; Chuma *et al.*, 2011). Chemical pesticides offer marginal protection from the disease, yet pose environmental risks and may put non-pathogenic organisms, including humans, at risk (Aktar *et al.*, 2009). Thus, the control strategies currently employed are limited in effectiveness and may lead to further problems. An alternative means of crop protection would be through the use of biological control agents (BCA).

An effort is underway to describe the microbiome that associates with plants and their impact on plant health and productivity. As with the gut microflora in humans, rhizospheric microbial communities aid in nutrient acquisition and control soil pathogens through competition for nutrients and production of antimicrobials (Lugtenberg and Kamilova, 2009). Some gram-negative *Pseudomonas* species are well-studied biocontrol bacteria that have been shown to produce a number of antimicrobial secondary metabolites (Silby *et al.*, 2011). These include but are not limited to phenazines (Thomashow and Weller, 1988), hydrogen cyanide (Voisard *et al.*, 1989; Rudrappa *et al.*, 2008), 2,4-diacetylphloroglucinol (Raaijmakers *et al.*, 1997), pyrrolnitrin (Howell and Stipanovic, 1979), and pyoluteorin (Howell and Stipanovic, 1980), as well as the cyclic lipopeptides tensin (Nielsen *et al.*, 2000) and viscosinamide (Nielsen *et al.*, 1999). The most well studied Gram-positive biocontrol bacteria are within the genus *Bacillus*, and have been shown to produce low molecular weight surfactins with antifungal activity (Vitullo *et al.*, 2012) as well as antifungal lipopeptides called kurstakins (Bechet *et al.*, 2012).

BCA also help protect plants against foliar pathogens by altering of host immunity for quicker defense responses. This induced systemic resistance (ISR)

response occurs through root to shoot long distance intra-plant signaling, priming the plants to better resist pathogen attack (van Loon, 2007). In most cases ISR depends on jasmonic acid (JA) and ethylene (ETH) plant signaling and not salicylic acid (SA) signaling as seen with systemic acquired resistance (Van der Ent *et al.*, 2009). Priming occurs when the plant recognizes microbial cell components, secretions, or volatiles (Ryu *et al.*, 2004). Upon attack by a pathogen, primed plants have more rapid cellular defense responses (Van Wees *et al.*, 2008). This is due to increased accumulation of inactive transcription factors as a response to microbial colonization, that are then activated during pathogen attack, creating enhanced expression of defense genes (Pozo *et al.*, 2008). *Pseudomonas fluorescens* strain WCS417r was the first bacterium documented to induce a systemic response in carnation (*Dianthus caryophyllus* L.) allowing it to be more resistant to *Fusarium* wilt (Vanpeper *et al.*, 1991).

Schroth *et al.* (Schroth and Hancock, 1982) described how plants grown in certain soils are less prone to disease. These disease-suppressive soils can occur naturally due to their physiochemical properties promoting colonization of biological control (hereafter biocontrol) microbes, or can be established through plant recruitment of beneficial microbes to the roots, regardless of soil type, when under biotic stress. For example, *Arabidopsis thaliana* infection by the foliar bacterial pathogen *Pseudomonas syringae* pv *tomato* DC3000 (hereafter DC3000) induces root secretion of L-malic acid, which attracts the beneficial rhizobacterium *Bacillus subtilis* FB17 to the roots (Rudrappa *et al.*, 2008; Lakshmanan *et al.*, 2012). FB17 then triggers the expression of defense-related genes in *A. thaliana* leaves, including

pathogenesis-related protein *PR1* and plant defensin *PDF1.2*, reducing DC3000 growth and disease incidence (Rudrappa *et al.*, 2008; Lakshmanan *et al.*, 2012).

Understanding and manipulating natural associations between rice plants and their rhizospheric communities, in combination with current disease control strategies, would be a comprehensive and effective way to reduce infection and increase food production. The objective of this study is to isolate and characterize naturally occurring and closely associated rhizospheric rice bacteria in order to identify possible biocontrol bacteria for *M. oryzae*. The bacteria and bacteria-derived components could then be used as fungal suppressors. We have identified a *Pseudomonas* isolate, EA105, which appears to inhibit *M. oryzae* through direct antagonism as well as through the induction of systemic resistance in rice.

2.3 Materials and Methods

2.3.1 DNA extraction from rhizospheric soil and processing for metagenomic analysis

Field grown rice plants were harvested for root associated microbial DNA for cloning and sequencing of 16S rRNA sequences. The majority of the aerial part of the rice plants was removed and a clump of soil encompassing the root ball was retained for processing. Individual roots from single plants were processed one at a time until sufficient root material was obtained for this plant. A single complete root, considered untouched during harvest, was excised from the middle of the root ball. Excess soil was removed from the root using gloved hands until only tightly bound soil remained. The root was then added to 30 ml of PBS buffer (pH 7.0). Further roots from the same plant were added until volume of roots collected approximated 12 ml. Roots in PBS buffer were vortexed, and about 16 ml of the root wash soil

suspension (rice rhizosphere soil) was spun down and the pellets stored at -80°C until DNA extraction. Microbial DNA was extracted from 0.25 to 1 gram of rhizospheric soil using the MoBio UltraClean Soil DNA Isolation Kit with use of the maximum yield 'Alternative Protocol'. Amplification of 16S rDNA was performed using the primers 27F(AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT). The sequences were screened for possible chimeras using Mallard (Ashelford *et al.*, 2006) and then passing sequences classified against the taxonomic reference set available from the Ribosomal Database Project (RDP) resource (<http://rdp.cme.msu.edu/>). Specifically, the sequences were classified using the java based RDP Naïve Bayesian rRNA Classifier Version 2.1 (Wang *et al.*, 2007) with the taxonomic reference set RDP 10.18 (Wickham, 2009). The R package ggplot2 (Cole *et al.*, 2009) was used to generate the barplots depicting taxonomic composition. The amplified product was gel purified, and cloned using the Topo TA vector. Colonies with inserts were purified, and the insert DNA sequences were obtained by Sanger sequencing.

2.3.2 Isolation and identification of cultured rhizobacteria

Natural rhizobacteria were isolated from root-associated soil and roots of M-104 rice plants, a temperate japonica cultivar widely grown in California. M-104 roots were harvested and the soil adhering to the root was removed using a sterile spatula and collected as the root-associated soil sample. The root was then rinsed, crushed and processed as the root sample, which included endophytic bacteria as well as tightly bound root bacteria. The samples were suspended in sterile water (0.1 g/ml) and serial dilutions were dispensed on LB (Bertani, 1951), TY (Beringer, 1974), or CP + benzoate (Wahlund and Madigan, 1995) agar plates. They were incubated for 48

hours at 30 °C and single colonies were selected based on morphology and re-streaked on fresh agar plates. Isolate identification was initiated by sequencing the 16S rDNA using colony PCR and the universal primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT). Taxonomic assignments were determined using the RDP website classifier. Further identification was done by MIDI, Inc (midi-inc.com) through a fatty acid methyl ester (FAME) analysis. A similarity (SIM) index of 1.000 means an exact species match determined by fatty acid make-up. The lower the SIM index, the more varied the fatty acid content. SIM Index cutoff of 0.600 was used to determine confident species match, unless otherwise noted.

2.3.3 Plant materials and growth conditions

Oryza sativa ‘M-104’ seeds were a gift from Dr. Thomas Tai (University of California-Davis). The seeds were dry planted in a Davis field where rice had been previously grown for several years. The field was flooded soon after emergence, and the roots were harvested for sampling at about 1 month after planting. *O. sativa* ‘Maratelli’, a susceptible variety to blast fungus *M. oryzae* strain 70-15 was used for the studies. All plants were grown in a growth chamber with a daily cycle of 16 hr light (28°C, 80% RH), and 8 hr dark (26°C, 60% RH).

2.3.4 *In-vitro* antibiosis assay

Two experimental designs were created using petri dishes to determine the antagonistic activity of bacterial isolates. First is the diffusible assay, whereby sterile petri dishes were filled with autoclaved complete media (CM) agar, consisting of 10 g sucrose, 6 g yeast extract, 6 g casaminoacids, 15 g agar, and 1 ml *Aspergillus nidulans*

trace elements in 1 L water. Five mm plugs of *M. oryzae* 70-15 or guy11 mycelia were placed 4 cm from 5 μ l of 5×10^5 bacterial cells. The plates were sealed with parafilm and put in the dark in a 25 °C incubator. Photographs were taken after 5 days and the diameter of the mycelium growing out from the plug was measured using ImageJ software. Percentage (%) inhibition was calculated by the formula: % inhibition = $[(C - T) \times 100] / C$, where C = fungal diameter (cm) in the control plate, and T = fungal diameter (cm) in the bacterial treated plates. Three biological replicates were performed and an average was taken. Second, the volatile (compartment) assay used compartmentalized petri dishes where the bacteria were grown on LB agar or LB liquid and *M. oryzae* was grown on CM agar in separate compartments. Three biological replicates were performed and an average was taken. The activated charcoal assay used the same experimental design as the volatile assay, except the remaining two compartments were each filled with 1g of activated charcoal (Darco[®], 20–40 mesh particle size, granular, Aldrich, Milwaukee, WI) wrapped in KimWipes. Two biological replicates were performed and an average was taken. For the heat killed and spent media assay, bacterial isolate EA105 was grown overnight in 10 mL of LB liquid in a 50 mL falcon tubes and optical density at 600 nm (OD₆₀₀) was measured. The culture was either placed in a 65 °C water bath for 24 hours, or spun down (centrifuged for 8 minutes at 4000 rpm) and the supernatant passed through a 0.45 μ m filter (Millipore, Billerica, MA). Sterile filter discs were placed on CM agar plates 4 cm away from a 5 mm plug of *M. oryzae* 70-15. The filter discs were inoculated with 50 μ l of LB liquid, 50 μ l of EA105 heat-killed cells, or 50 μ l of EA105 supernatant (cell-free spent media). Two biological replicates were performed and an

average was taken. All fungal diameters were measured using ImageJ, and % inhibition was calculated as described above.

2.3.5 Bacterial motility

To evaluate the bacterial motility, swimming and swarming assays were performed with rice isolates as per the published protocol (Rashid and Kornberg, 2000). Briefly, bacterial stabs were placed on swimming plates (5 g/L NaCl, 10 g/L tryptone, and 0.03% (w/v) agarose), and swarming plates (8 g/L nutrient broth, 5 g/L glucose, with 0.5% (wt/vol) agar and after incubation at 30°C the diameter of bacterial growth was measured.

2.3.6 Cyanide measurement

Cyanide production in bacterial culture supernatant was measured using the Lazar Model LIS-146CNCM micro cyanide ion electrode from Lazar Research Laboratories, Inc. Bacterial cultures were grown in LB for 24 hours shaking at 200 rpm at 30°C. Optical density at 600 nm (OD₆₀₀) was recorded. The cells were centrifuged (8 minutes at 4000 rpm) and supernatant was taken for measurement. The electrode was conditioned prior to use, and rinsed with 70% ethanol then water between each sample reading. Two biological replicates were performed.

2.3.7 Construction of cyanide mutant D5

The D5 mutant was constructed using the Targetron gene knockout system (Sigma-Aldrich) to disrupt a region of the hydrogen cyanide biosynthetic operon that encompassed both the *hcnB* and *hcnC* genes. Primers for the insertion sites of the group II intron were chosen by a Sigma-Aldrich computer algorithm based on an input sequence from the *hcnBC* genes. These primers (IBS, EBS1d, and EBS2) as well as

the EBS universal primer were used to amplify the intron template. The resulting amplicon was purified using the QiaQuick PCR purification kit (Qiagen), double digested with HindIII and BsrGI, and then ligated into the linear pACD4K-C vector using T4 DNA ligase and 2X Rapid ligation buffer (Promega) with a 1:2 molar ratio of vector to insert DNA. Transformation was performed according to Targetron's suggestions, with exception of the heat shock being extended to 60 seconds, the recovery period being extended to 3 hours, and the incubation temperature being at 30°C. Induction of the group II intron insertion using IPTG was performed as per the Targetron protocol. Potential transformants were selected using colony PCR and absence of cyanide production was confirmed using the LIS-146 Micro Cyanide probe (Lazar Research Laboratories).

2.3.8 Solid-phase microextraction-gas chromatography mass-spectrometry (SPME-GC-MS)

Volatile metabolites produced by EA105 were extracted using an SPME fused silica fiber coated with 65 µm of polydimethylsiloxane/divinylbenzene (Sigma-Adrich). EA105 was grown on LB agar for 2 days and then the fiber was exposed for 24 hours to the headspace above EA105. The fiber was then manually injected into an Agilent 6890 GC with a 5973N MS detector (Agilent Technologies), installed with a HP-5MS capillary column (30 m x 0.25 mm, 0.5µm) and a flame ionization detector. Inlet temperature was 250°C. Oven conditions started at 40°C for 2 min, ramped at 10°C/min to 250°C, and held for 2 min. VOCs were identified using the mass spectral library (NIST). Standard curves of the identified compounds were created using commercially available compounds. They were diluted in methanol and 2 µl was injected into the GC. The concentration of the volatiles produced was determined by

comparing peak heights of the EA105 profile to the standard curve. Four biological replicates were performed.

2.3.9 Spore germination and appressoria formation

Plastic coverslips were sterilized with ethanol and used as hydrophobic surfaces for the conidiospores. *M. oryzae* 70-15 spores grown on oatmeal agar for 10 days were suspended in water and filtered through Miracloth. For S-methyl thioester treatments, a 100 mM stock of the compounds in 100% methanol was used, and compared to a control treatment with the same final amount of methanol. For cyanide treatments, potassium cyanide was dissolved in 35 mM KOH to make a 100 mM stock, which was further diluted in water. A 1:1 (v:v) solution of spores plus compound were made with a final concentration of 10^5 spores/ml in compound concentrations ranging from 1-500 μ M. For bacterial treatments, a final concentration of $OD_{600}=0.02$ ($\sim 1 \times 10^7$ cells/mL) was used. Five plastic coverslips were placed into a petri dish containing a wet filter disc in the center to maintain humidity. A 50 μ L drop of treated spores was placed on each coverslip. For indirect bacterial treatment, a drop of bacterial cells was placed next to each coverslip and a 50 μ L drop of untreated spores was placed on the coverslip. Petri dishes were parafilmed and placed in the dark at room temperature. Percent germination was determined at 3 hours post treatment and percent appressorium formation was determined 24 hours post treatment using the Zeiss Axioscope2 upright light microscope. Five images were taken at different locations on each coverslip for a total of 25 images per treatment. Percentage germination was calculated by counting the number of germinated spores and the total number of spores in the images. Percentage appressorium formation was determined

by counting the number of germinated conidia which had produced an appressorium. Three biological replicates were examined following the protocol described above.

2.3.10 Evaluation of rhizobacterial-mediated ISR

Rhizobacterial isolates were grown overnight in LB at 30°C shaking at 200 rpm. Cells were spun down by centrifugation (8 minutes at 4000 rpm) and the supernatant discarded. Cells were washed in sterile water twice, then resuspended to an OD₆₀₀ of 0.5 (~2.5x10⁸ cells/mL). Three-week old soil-grown Maratelli rice plants were root primed with 2 mL of the rhizobacterial suspension per plant. Eight replicates were used per treatment. Mock plants were treated with 2 mL of sterile water. After 24 hours, the shoots (stems and leaves) of each plant were sprayed with 1 mL of *M. oryzae* strain 70-15 at a concentration of 10⁵ spores per mL. Ten-day old spores were suspended in sterile water, filtered through Miracloth, and counted using a hemocytometer. Spores were adjusted to a concentration of 1x10⁵ spores/mL water and a 1:10 (v:v) of 0.2% gelatin was added to the suspension. Plants were sprayed inside of plastic bags containing wet paper towels using an artist's air brush, sealed to maintain humidity, and covered with plastic bins for 24 hours of darkness. As a precautionary measure, pathogen-inoculated plants were transferred to separate growth chambers and grown in identical growth conditions as the other treatment groups. Photographs of leaves were taken after 1 week and the number of lesions on the second youngest leaf was counted using the image analysis program ImageJ to facilitate accurate scoring. Four biological replicates were performed.

To test gene expression changes in rice, M-104 seeds were sterilized and germinated in petri dishes. At 7 days post germination, seedlings were transferred to clear, sterile boxes containing 50 mL of Hoagland's liquid medium. The pH of the

medium was maintained at 5.7. At 14 days post germination, the liquid medium was inoculated with bacteria which had been washed in water, to a final concentration of 10^6 cells/mL. At 24 hours post treatment, leaf tissue was frozen in liquid nitrogen and RNA was extracted using the Bio Basic EZ-10 Spin Column Plant RNA Mini-Prep Kit. RNA was treated with Turbo DNase (Ambion) and the High Capacity cDNA Reverse Transcription Kit (Ambion) was used to synthesize cDNA, using 500 ng of RNA. PCR was carried out using standard Taq Polymerase (New England Biolabs). Primers to test for SA responsive genes *PR1* and *WRKY77*, JA responsive genes *JAR1* and *WRKY30*, and ETH responsive genes *EIL1* and *ERF1* were designed using Primer Blast (NCBI) of Nipponbare gene sequences (Table 2.1). PCR products were run on a 1.4% agarose gel, stained with ethidium bromide, and imaged using an Alpha Imager system. Band intensities were quantified using ImageJ. A ubiquitin control was used to normalize all samples. Each biological replicate was pooled from 9 plants, and there were 3 biological replicates per treatment.

Table 2.1. Primer sequences.

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
<i>PR1</i>	TCGTATGCTATGCTACGTGTTT	CACTAAGCAAATACGGCTGACA
<i>WRKY77</i>	CTGTGTCCAGCTACCTCTCC	TGAAGAGAGCGATCACCTC
<i>JAR1</i>	TCTCCCCAGCCTTAACCGTA	CTAAACGCGACGACAAACCC
<i>WRKY30</i>	ACTTCTTGAGTCGCCGGTTT	GCTTCTGGGATGCTCACTGT
<i>EIL1</i>	ATCACCAGCGCCATATCGTT	CACGGTTGTTTCAGCATCAGC
<i>ERF1</i>	CATATCACCTTGACGCCCCA	ACCCTCACAACTCACTCGG

2.3.11 Statistical analysis

The statistical software JMP 10 was used to analyze data. To compare across treatments, the Tukey's HSD test was used and results were considered to be statistically different when $p < 0.05$.

2.4 Results

2.4.1 Isolation and Identification of rhizobacteria

Rhizospheric soil samples from California field-grown M-104 rice plants were sequenced for bacterial 16S rDNA and distributions of the phyla (Figure 2.1) and genera (Figure 2.2) of bacteria present in the soil samples were determined. There were 8 to 10 phyla (among Acidobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Gemmatimonadetes, Nitrospira, Planctomycetes, Proteobacteria, Verrucomicrobia) that were considered abundant for the 2008 and 2009 data respectively (Figure 2.1). For these, the 16S rRNA sequences each individually make up greater than 1% of the total. Apart from the Proteobacteria that make up 44% and 50% of the 16S sequences, the second-most abundant phylum was Acidobacteria making up 24% and 30% of the sequences in the 2008 and 2009 samples respectively. Other phyla making up greater than 4% of the sequences were Actinobacteria, Bacteroidetes and Firmicutes. At the rank of genera, the top 1% of sequences (99th percentile) were comprised of Acidobacteria subdivisions Gp1, Gp3, Gp4, and Gp6, and also Nitrosospora, a member of the Betaproteobacteria (Figure 2.2). From the same soil samples, naturally occurring root-associated and root-bound rhizospheric bacteria were isolated (Table 2.2). Strains labeled EA101-EA108 were isolated on TY agar, and strains labeled EA201-EA202 were isolated on LB agar. One bacterium, labeled EA303, was isolated using *Chlorobium* plating (CP) agar

plates with benzoate as the sole carbon source. A total of eleven isolates were taxonomically identified by fatty acid methyl ester (FAME) analysis and their identities were further confirmed using 16S rRNA gene sequencing (Table 2.2). Six out of the 11 isolates belonged to the class *Gamma-proteobacteria*, and of these, 5 were of the genus *Pseudomonas*. This may be due to their ability to be cultured and their natural abundance in the soil environment, including the rhizosphere.

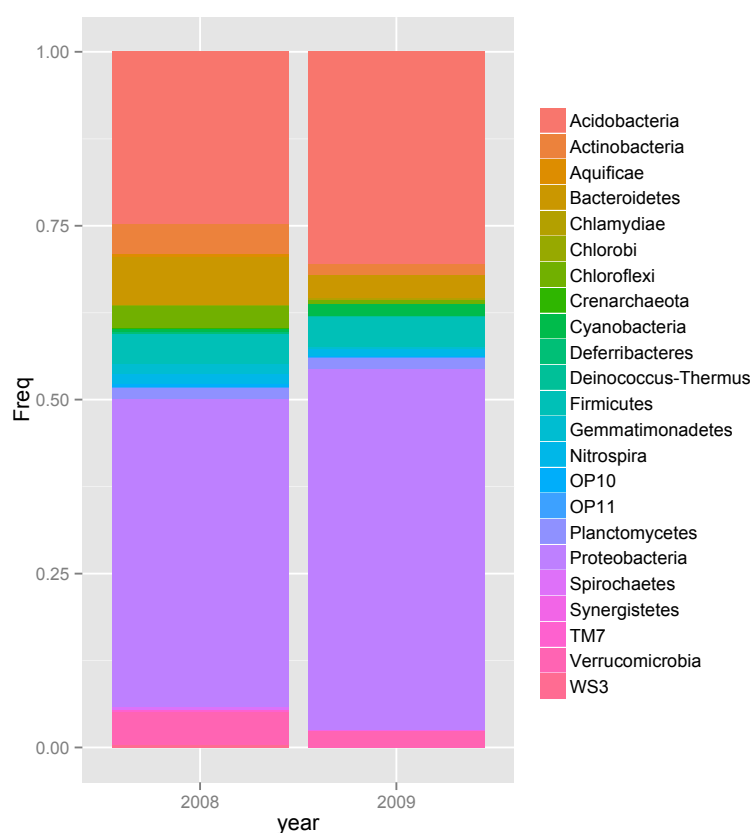


Figure 2.1. **Representation of bacteria phyla.** Relative abundance (frequency) of the major bacterial phyla present in the rice rhizosphere microbial community recorder over two-years. The frequencies shown were obtained via classification of 16S rDNA sequences corresponding to a total of 654 and 630 clones, for 2008 and 2009 respectively.

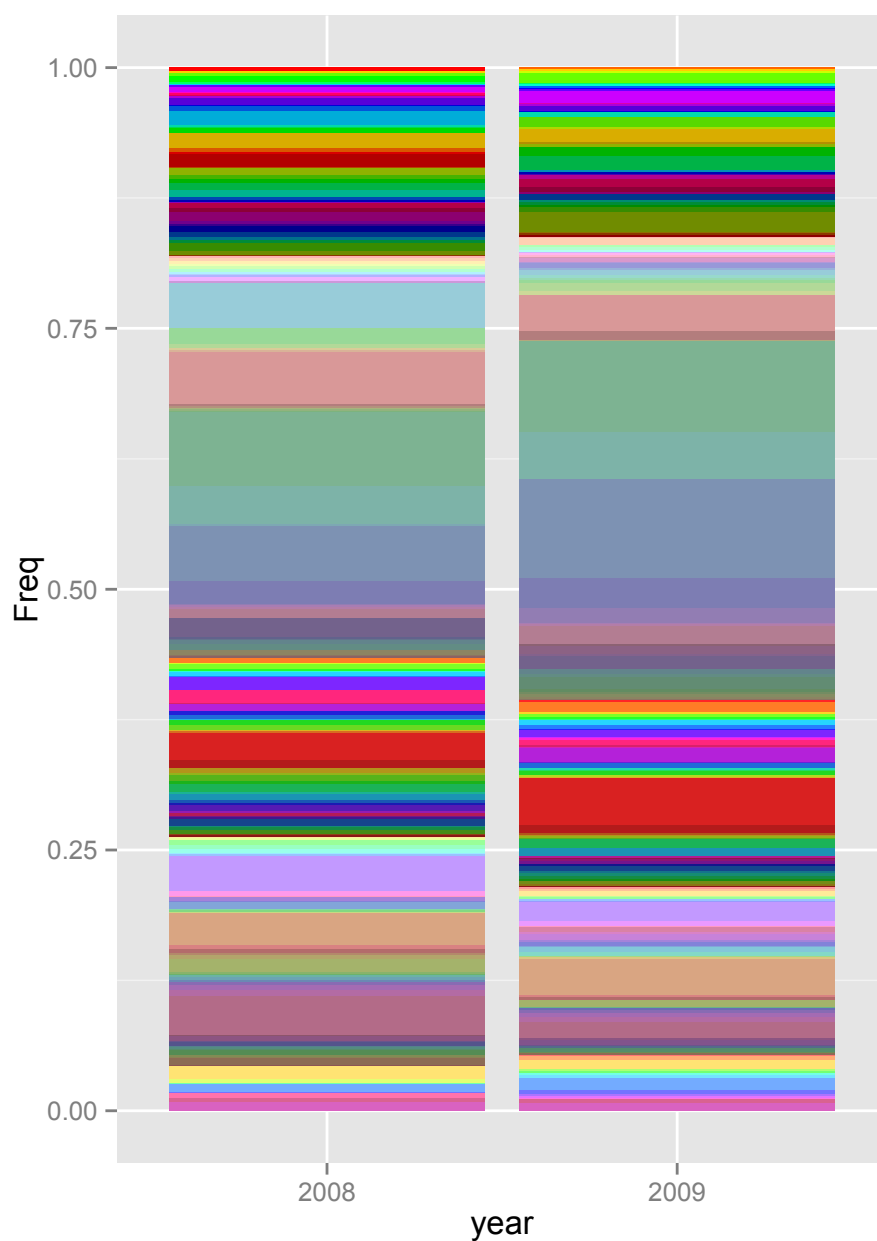


Figure 2.2. **Representation of bacterial genera.** Relative abundance (frequency) of the major bacterial genera in the rice rhizosphere microbial community recorded over a two-year period. The frequencies shown were obtained via classification of 16S rDNA sequences corresponding to a total of 654 and 630 clones, for 2008 and 2009 respectively.

Acanthopleuribacter	Dasania	Haliangium	Ottowia	Skermanella
Acetivibrio	Dechloromonas	Haliaea	Owenweeksia	Smithella
Achromobacter	Dehalobacter	Halicomenobacter	Oxalicibacterium	Sorangium
Acidaminobacter	Denitratisoma	Halothermothrix	Paenibacillus	Spartobacteria_genera_incertae_sedis
Acidovorax	Derxia	Herbaspirillum	Paenisporosarcina	Sphaerobacter
Acinetobacter	Desulfarculus	Herpetosiphon	Paludibacter	Sphingomonas
Adhaeribacter	Desulfatiferula	Hipaea	Paludibacterium	Sphingopyxis
Afipia	Desulfobacca	Holophaga	Parachlamydia	Spirochaeta
Aggregatibacter	Desulfocapsa	Hyalangium	Paraeggerthella	Spongiibacter
Agromonas	Desulfoglaeba	Hydrogenophaga	Parasegetibacter	Sporacetigenium
Alkanibacter	Desulforegula	Hylemonella	Paucibacter	Sporocytophaga
Anaerobacter	Desulfosporosinus	Hymenobacter	Paucimonas	Sporotalea
Anaeromyxobacter	Desulfovermiculus	Iamia	Paucisalibacillus	Staphylococcus
Anaerophaga	Desulfovibrio	Ideonella	Pedobacter	Stella
Andersenella	Desulfovirga	Ilumatobacter	Pelomonas	Steroidobacter
Aquicella	Diaphorobacter	Isosphaera	Persicobacter	Sterolibacterium
Aquiflexum	Dokdonella	Jonquetella	Phaselicystis	Subdivision3_genera_incertae_sedis
Aquimonas	Ecotiorhodospinus	Kofleria	Phenyllobacterium	Succiniclasticum
Aquicola	Eliaera	Lacibacter	Pimelobacter	Sulfurospirillum
Archangium	Enhygromyxa	Leptolinea	Pirellula	Syntrophobacter
Arenimonas	Enterobacter	Leptothrix	Planctomyces	Syntrophorhabdus
Arthrobacter	Erythrobacter	Levilina	Plesiocystis	Syntrophothermus
Aspromonas	Erythromicrobium	Lewinella	Procabacter	Syntrophus
Azomonas	Exiguobacterium	Lishizhenia	Prolixibacter	Tepidiphilus
Azonexus	Ferribacterium	Longilinea	Propionibacterium	Terrabacter
Azospira	Ferruginibacter	Luteimonas	Propionispora	Terrimonas
Azovibrio	Fervidicola	Luteolibacter	Propionivibrio	Tetrasphaera
Bacillus	Filibacter	Lysobacter	Prosthecochloris	Thalassobacillus
Balnearium	Filomicrobium	Macromonas	Prosthecomicrobium	Thermithiobacillus
Bdellovibrio	Flavisolibacter	Martellella	Pseudolabrys	Thermobrachium
Bellilinea	Formivibrio	Massilia	Pseudomonas	Thermodesulfovibrio
Bhargavaea	Gemmata	Melittangium	Pseudospirillum	Thermofilum
Blastomonas	Gemmatimonas	Mesorhizobium	Puniceicoccus	Thioalkalibacter
Bradyrhizobium	Geobacter	Methylibium	Pusillimonas	Thiobacillus
Burkholderia	Geothermobacter	Methylobacillus	Quatronicoccus	Thiobacter
Byssovorax	Goodfellowiella	Methylophilus	Ramlibacter	TM7_genera_incertae_sedis
Caenimonas	Gordonibacter	Methyloversatilis	Rhizobacter	Tolomonas
Caldiilinea	Gp1	Methylovorus	Rhizobium	Truepera
Caldimonas	Gp10	Microscilla	Rhodocyclus	Turneriella
Caldithrix	Gp16	Modestobacter	Rhodoferax	Uliginosibacterium
Chelatococcus	Gp17	Nannocystis	Roseateles	Undibacterium
Chitinilyticum	Gp18	Niastella	Roseomonas	Variovorax
Chitinophaga	Gp21	Nitratireductor	Rubrivorax	Verrucomicrobium
Chondromyces	Gp25	Nitrilireductor	Rubrobacter	Wautersia
Clostridium	Gp3	Nitrobacter	Rudaea	Weeksella
Collimonas	Gp4	Nitrosospira	Schlegelella	Wolinella
Conexibacter	Gp5	Nitrospira	Schlesneria	WS3_genera_incertae_sedis
Craurococcus	Gp6	Oceanibaculum	Shuttleworthia	Xylophilus
Croceicoccus	Gp7	OP10_genera_incertae_sedis	Silanimonas	Zavarzinella
Curvibacter	GpI	OP11_genera_incertae_sedis	Singularimonas	
Cystobacter	GpIV	Opiritut	Singulisphaera	
Dactylosporangium	GpIX	Oscillibacter	Sinobaca	

Figure 2.2 Continued

Table 2.2. Identification of rice soil isolates.

Genus	Species ^a	Similarity Index	Confidence Level	Strain Label	
<i>Pseudomonas</i>	<i>corrugata</i>	0.761	Species inconclusive	EA104	Root Associated
	<i>chlororaphis</i>	0.598	Genus	EA105	Root
	<i>chlororaphis</i>	0.77	Species	EA107	Root
	<i>putida</i>	0.785	Species	EA108	Root
	-	0.232	No match*	EA303	Root Associated
<i>Pantoea</i>	<i>agglomerans</i>	0.896	Species	EA106	Root
<i>Dyadobacter</i>	-		Genus*	EA202	Root Associated
<i>Pedobacter</i>	<i>heparinus</i>	0.682	Species	EA101	Root Associated
<i>Chryseobacterium</i>	<i>balustinum</i>	0.776	Species	EA102	Root Associated
<i>Rhodococcus</i>	<i>rubripertincta</i>	0.807	Species	EA103	Root Associated
<i>Arthrobacter</i>	<i>oxydans</i>	0.758	Species	EA201	Root Associated
^a Closest match in MIDI library as determined by FAME analysis					
- Inconclusive match					
* Genus solely determined by 16S rRNA gene sequencing					

2.4.2 *In-vitro* antifungal properties of rice rhizospheric bacterial isolates

The effect of naturally associated rice rhizobacteria on growth and development of *M. oryzae* strain 70-15 was assessed using petri dish assays. A diffusible assay evaluated the effect, if any, of bacterial-derived diffusible compounds on *M. oryzae* 70-15 (hereafter 70-15) without direct contact. The two microbes could communicate and interact through both volatile compounds and diffusible compounds. All isolates were tested and five *Pseudomonas* isolates (EA104, EA105, EA107, EA108, and EA303) showed significant inhibition of 70-15 growth (Figure 2.3). The most dramatic effect was seen by the *Pseudomonas* isolate EA105, inhibiting fungal growth by 65% after 5 days, relative to the control (Figure 2.3).

Bacterial volatiles have been receiving increasing attention for their roles not only as odors, but as phyto stimulants, antimicrobials, and compounds involved in inducing a systemic resistance response as well (Haas and Keel, 2003; Rudrappa *et al.*, 2010). To examine whether volatile antifungal metabolites were playing a role in the observed hindering of 70-15 growth, a volatile (compartment) plate assay was performed using petri dishes that were divided into four quadrants. *M. oryzae* and rice bacterial isolates were placed in opposite compartments where they shared the same headspace, yet there was no exchange of diffusible compounds. Any inhibition observed was therefore due to volatile compounds. All of the *Pseudomonas* isolates significantly reduced growth to about the same degree as seen in direct plates, except for EA105, whose inhibition effect was reduced in compartment plates (Figure 2.3). Bacterial motility allows for a number of beneficial activities, including acquiring more nutrients, maneuvering away from toxic substances, and colonizing in optimal environments (Ryu *et al.*, 2003). EA105 is able to spread across plates quickly through swimming and swarming (Figure 2.4) and restriction to one quadrant of a

plate could have contributed to the reduction in inhibition. A similar reduction in EA105's inhibitory activity was seen when EA105 was grown on CM agar instead of LB agar, and in liquid culture as opposed to agar (Table 2.3).

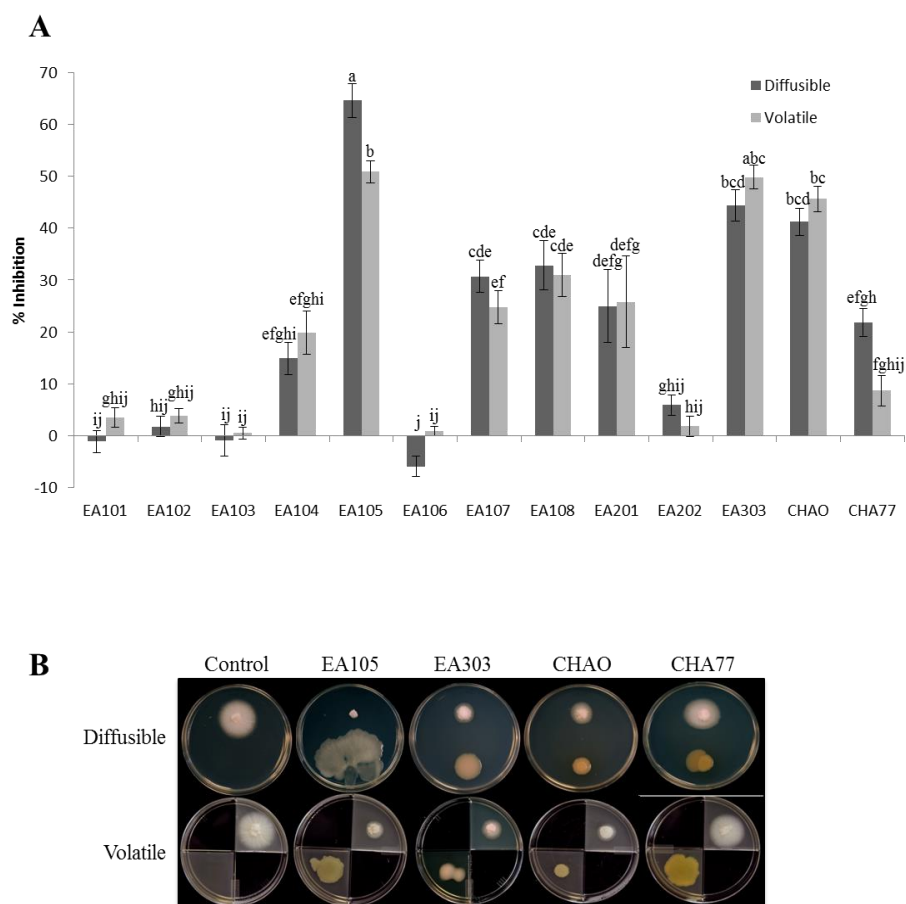


Figure 2.3. **Inhibition of *M. oryzae* vegetative growth by rice soil isolates.** **A)** Antimicrobial assay showing the degree of inhibition of *M. oryzae* 70-15 by naturally isolated rice rhizobacteria as well as *P. fluorescens* CHAO and cyanide mutant CHA77. Error bars indicate standard error. Different letters indicate statistically significant differences between treatments (Tukey's HSD). **B)** Representative images of the fungal inhibitory effect seen when 70-15 was exposed to bacterial diffusible and volatile compounds (diffusible plates), or solely through volatile compounds (volatile plates).

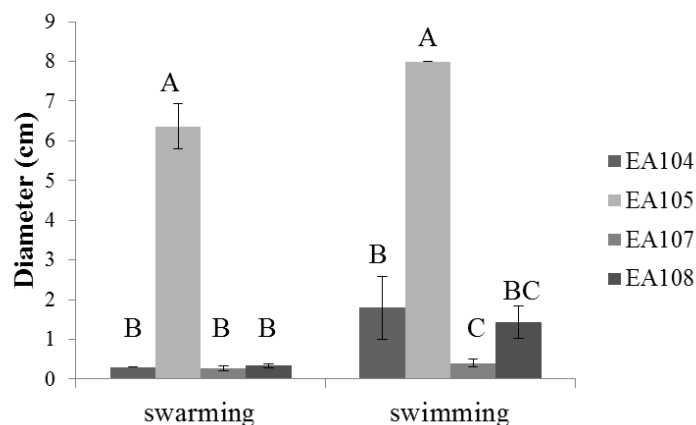


Figure 2.4 **Swimming and swarming motility of *Pseudomonas* isolates.** Cells were grown on motility plates for 24 hours as described (Rashid and Kornberg, 2000). Means comparisons for all pairs were done using Tukey-Kramer HSD statistical test, where means with the same letter do not differ significantly (n=3). Treatments were compared within swarming plates, and within swimming plates.

Table 2.3. Comparison of fungal inhibition elicited by EA105.

	% Inhibition at 3 dpi
EA105 diffusible on CM agar	62% * (n=30)
EA105 volatile on LB agar	50% * (n=42)
EA105 volatile on CM agar	66% * (n=4)
EA105 compartment in liquid	61% * (n=5)
* Indicates significant inhibition (Student's t-test, $p < 0.0001$)	

To see if metabolically active cells are needed for the direct antagonism exhibited by EA105, a control experiment was performed using the same diffusible assay set-up, except heat killed EA105 cells or the spent media (cell-free supernatant) were used in place of live cells. Neither the heat killed cells nor the spent media showed any significant effect on fungal growth (Figure 2.5), indicating that active

cells are needed for fungal inhibition. To further examine the nature of EA105-derived inhibition, *M. oryzae* 70-15 plugs were taken from plates where 70-15 had been exposed to EA105 (inhibited) and were subcultured onto fresh CM agar. When no longer exposed to the bacteria, 70-15 grew normally (Figure 2.5), indicating the fungistatic nature of EA105.

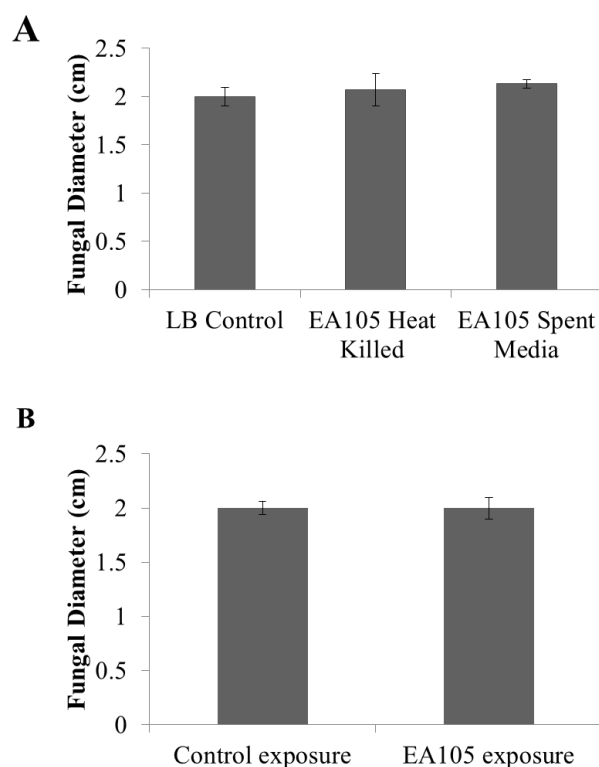


Figure 2.5. **Growth of *M. oryzae* treated with heat killed cells and growth after inhibition by EA105.** A) Effect of heat killed cells and cell-free spent media on fungal inhibition at three days post treatment. Error bars indicate standard deviation. There was no significant difference between the control and treatments using Student's t-test and a p -value of <0.05 . B) Recovery of *M. oryzae* 70-15 growth after exposure to EA105 volatiles. Fungal plugs were re-plated onto fresh CM agar after previously being exposed to EA105. Diameter was measured after three days. There was no significant difference between the control and previously exposed 70-15. Error bars indicate standard error.

One frequently reported toxin produced by some pseudomonad species is hydrogen cyanide (HCN), which binds to cytochrome C oxidase and blocks cellular respiration (Blumer and Haas, 2000). HCN can exist in either a gaseous or aqueous state, suggesting that it can be released by the bacteria as a volatile, as well as secreted into the media. Therefore, the tolerance of 70-15 to a known cyanide (CN) producer, *Pseudomonas fluorescens* CHAO (Pessi and Haas, 2000), and its HCN production negative mutant, *P. fluorescens* CHA77 (Laville *et al.*, 1998) was tested. In diffusible plates, CHAO significantly reduced fungal growth by 46% (Figure 2.3); however, this was not as drastic of an inhibition effect as seen by EA105. CHA77 also significantly reduced fungal growth, but only by 22% directly and 10% through volatiles (Figure 2.3).

Since many of the known pseudomonads, including *P. fluorescens* strain CHAO (Pessi and Haas, 2000), produce CN as a major antimicrobial component, bacterial CN production in stationary phase culture supernatants of all rice isolates was quantified using the Lazar Model LIS-146CN-CM micro cyanide ion electrode (Zlosnik and Williams, 2004). As controls, CN generated by *P. fluorescens* CHAO and CHA77 was also measured. EA105 produced around 500 μM cyanide after 24 hours of incubation, while EA303 and CHAO produced around 700 μM (Figure 2.6). As expected, CN production was severely diminished in CHA77, which has a disrupted CN biosynthesis operon (Figure 2.6). Even though EA105 produced less cyanide, it inhibited *M. oryzae* vegetative growth more than CHAO, indicating the involvement of other antifungal metabolites.

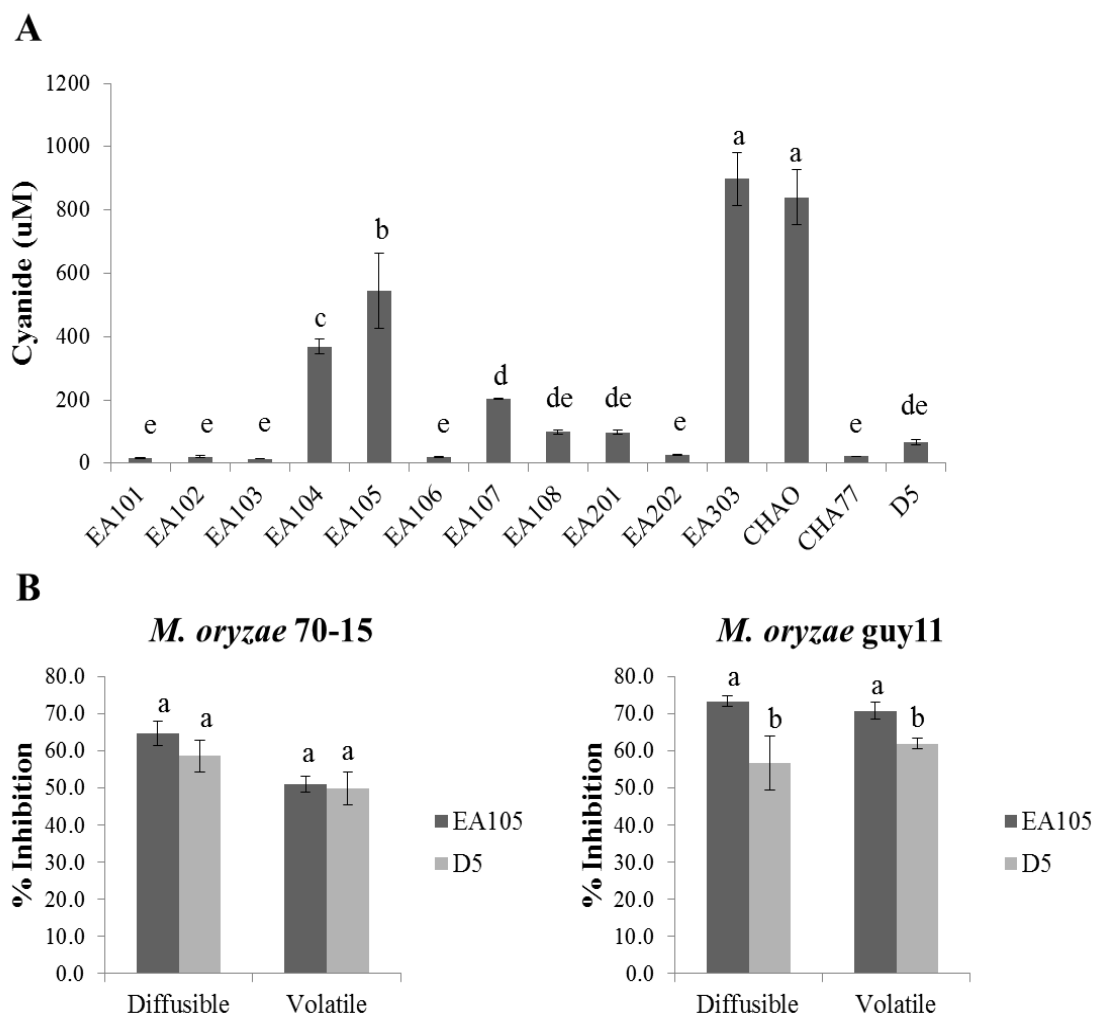


Figure 2.6. **Cyanide production by rice isolates and activity of cyanide mutant D5 against *M. oryzae*.** **A)** Bacterial cyanide production of all rice isolates, D5, CHAO, and CHA77 was measured after 24 hour incubation using the Lazar Model LIS-146CNCM micro cyanide ion electrode. Different letters indicate statistical significance (Tukey's HSD). **B)** Antimicrobial assay against *M. oryzae* strains 70-15 and guy11 with EA105 and its cyanide deficient mutant, D5. Different letters indicate statistical significance (Tukey's HSD).

Additionally, a HCN biosynthetic mutant, D5, was created in EA105 in which the *hcnABC* operon involved in CN synthesis was disrupted and CN generation was diminished (Figure 2.6). The two plate-based bioassays were utilized to evaluate the importance of CN in EA105 antibiosis against 70-15. EA105 and the D5 mutant attenuated the growth of 70-15 and guy11 to a similar degree under both diffusible and volatile assays (Figure 2.6). CHAO's cyanide deficient mutant, CHA77, showed a drastic reduction in ability to inhibit *M. oryzae* (Figure 2.3), while EA105's cyanide deficient mutant, D5, only showed minimal reduction in antifungal activity, suggesting that EA105 and CHAO have different mechanisms of antibiosis. This also indicates that the restriction of *M. oryzae* growth by EA105 is mainly independent of CN, and requires an unidentified bacteria-derived compound.

Both organic and inorganic volatile compounds produced by bacteria have been shown to provide biocontrol activity against plant pathogens (Howell *et al.*, 1988; Fernando *et al.*, 2005). To determine whether the antifungal activity seen by EA105 volatiles is due to organic or inorganic compounds, or both, the volatile (compartment) plate design was used. As previously described, *M. oryzae* 70-15 and the bacteria were placed in opposite compartments; however, the two remaining compartments were filled with activated charcoal/carbon, which will adsorb organic bacterial volatiles. The plates amended with activated charcoal showed normal fungal growth and no inhibition through bacterial volatile compounds (Figure 2.7). This implies that the active antifungal volatiles are organic compound(s), and henceforth referred to as volatile organic compounds (VOCs).

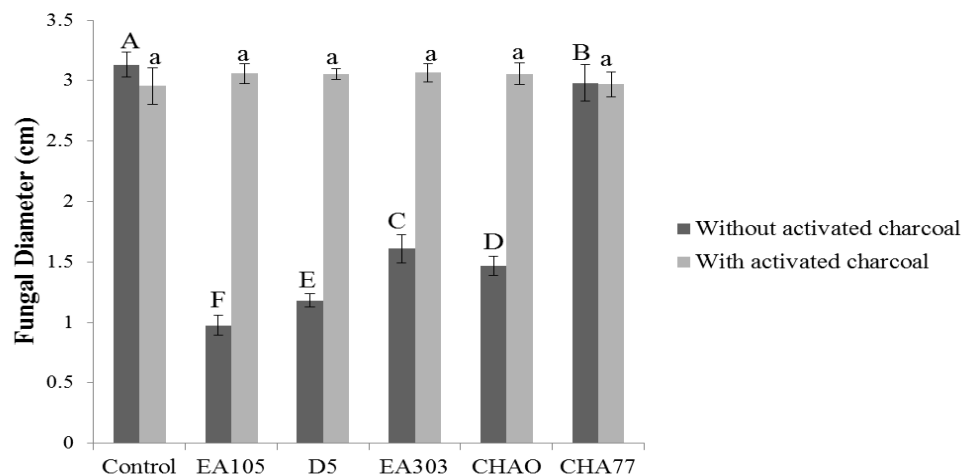


Figure 2.7. **Activity of volatile compounds produced by bacteria in the presence of activated charcoal.** Inhibitory effect through bacterial volatiles was abolished in the presence of activated charcoal. Error bars indicate standard deviation. Means with the same letter do not differ significantly as per Student's t-test, $p < 0.05$. Capital letters were used for plates without activated charcoal, and lower case letters were used for plates amended with activated charcoal.

In addition to the effect rhizobacterial isolates have on vegetative growth, these bacteria also affect development of conidia into a specialized infection structure called the appressorium. During pathogenesis, a penetration peg develops at the tip of the appressoria, which enables physical puncturing of the plant cuticle and infection of the host (Wilson and Talbot, 2009). EA105 inhibited 70-15 appressorial formation by nearly 90% compared to the control; while a known biocontrol strain of *P. fluorescens*, CHAO, inhibited about 60% through direct treatment (Figure 2.8). An unexpected observation was that both cyanide mutants, D5 and CHA77, inhibited appressorial formation slightly more than their cyanide-producing counterparts, EA105 and CHAO, respectively. Although it has not been shown in fungi, there is evidence that sub-lethal concentrations of cyanide can trigger defense mechanisms in

nematodes (Neidig *et al.*, 2011). Through indirect treatment, CHAO completely failed to inhibit appressorial formation while EA105 was still able to reduce appressorial formation by about 20% (Figure 2.8). This indicates that volatile compounds may be involved in the inhibition of vegetative growth as well as in the reduction of appressorial formation in the case of EA105.

To gain a better understanding of the effectiveness of EA105's antimicrobial potential against diverse phytopathogens, EA105 was tested against a variety of naturally isolated pathogens. Both EA105 and CHAO inhibited other phytopathogens to a similar and lesser degree than *M. oryzae*; however EA105 was able to restrict *M. oryzae* growth to a significantly greater degree than CHAO (Figure 2.9). This suggests the antimicrobial activity seen by EA105 is more specific and effective against a rice pathogen compared to other non-specific pathogens.

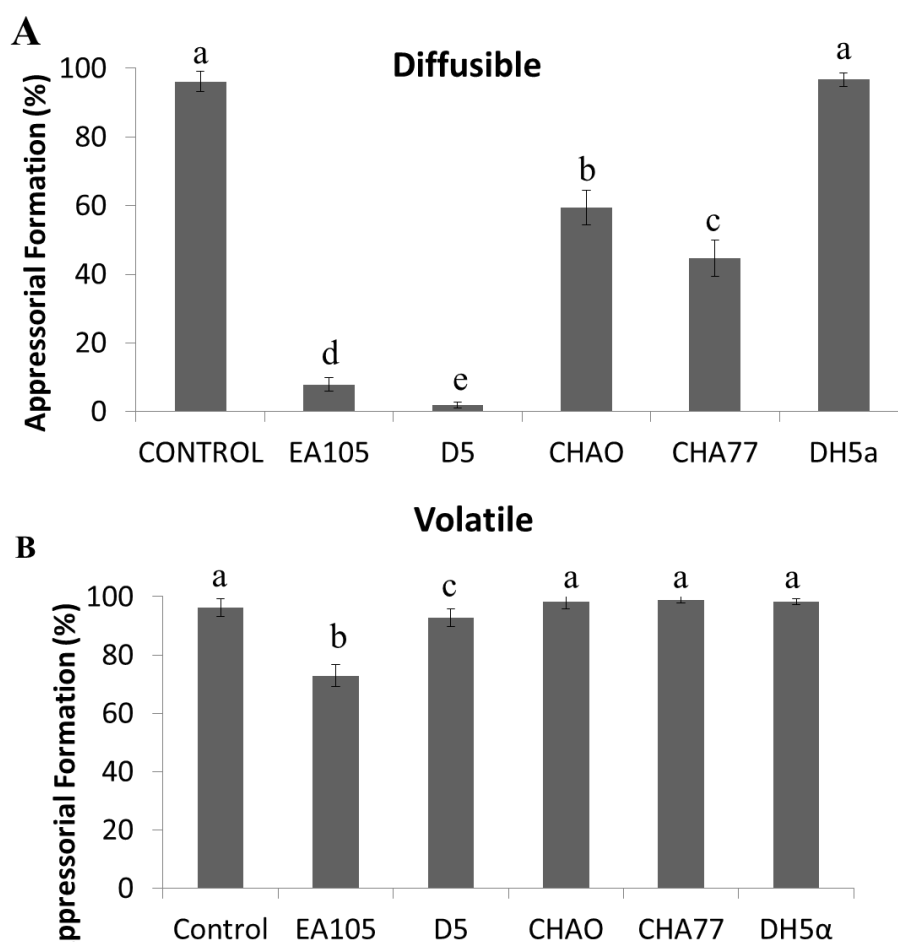


Figure 2.8. **Inhibition of *M. oryzae* appressoria after bacterial treatment.** Effect of bacteria on *M. oryzae* 70-15 appressorial formation through **A)** direct bacterial treatment, or through **B)** indirect (or volatile) bacterial treatment. Germinated conidia were incubated in a 50uL drop with bacterial treatment (EA105, cyanide mutant D5, CHAO, cyanide mutant CHA77, or *E. coli* DH5α) or placed in a drop next to the bacterial treatment for the indirect assay. Error bars represent standard deviation. Different letters indicate a significant difference (Tukey's HSD).

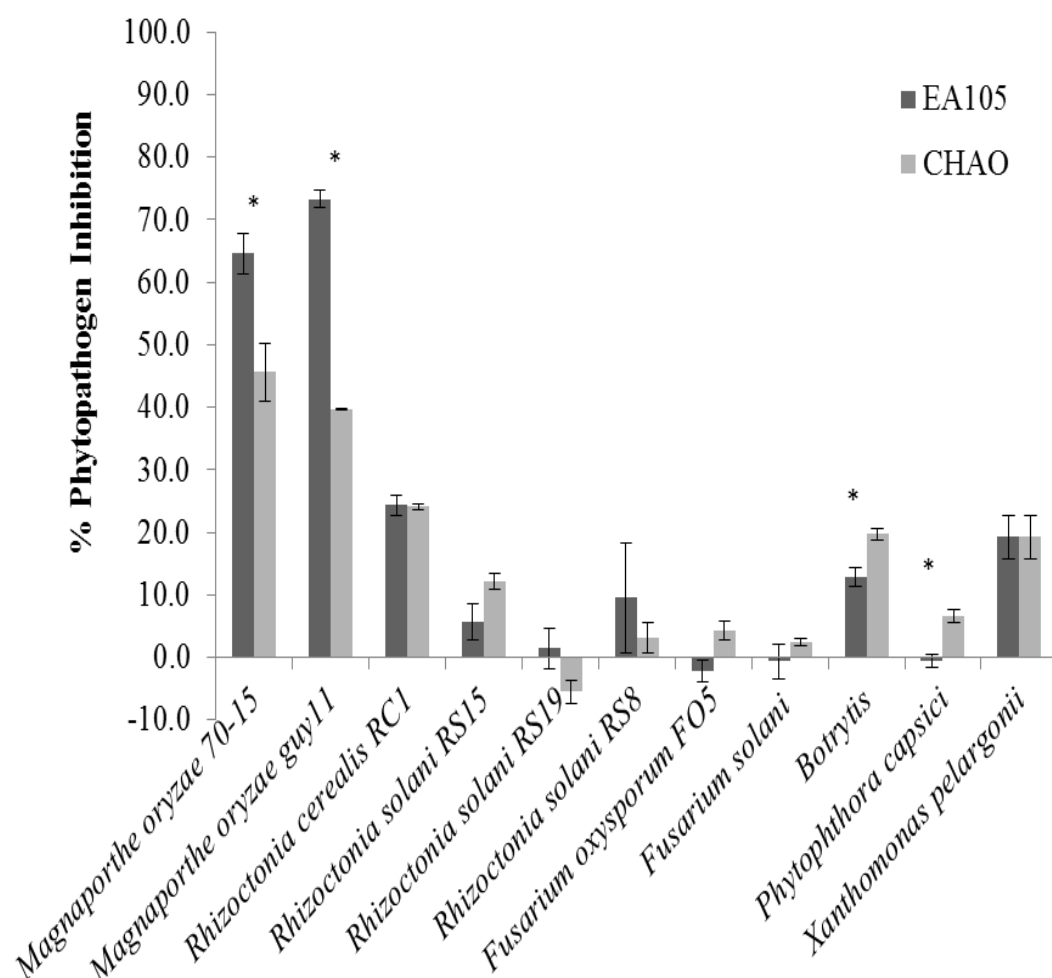


Figure 2.9. **Activity of EA105 against naturally isolated phytopathogens.** Inhibition of naturally isolated phytopathogens by EA105 and CHAO in comparison to *M. oryzae*. With the exception of lab strains *M. oryzae* 70-15 and *F. oxysporum* FO5, all pathogens were isolated from infected plants or soil, and acquired from Nancy Gregory at the University of Delaware. Error bars represent standard error. Asterisks indicate significant differences between EA105 and CHAO treatment (Student's t-test, $p < 0.05$).

2.4.3 Characterization of antifungal metabolites from EA105

Volatile organic compounds (VOCs) produced by EA105 were identified using solid-phase microextraction-gas chromatography mass-spectrometry (SPME-GC-MS) (Table 2.4). The most abundant peak in the headspace profile of EA105 was identified as 1-undecene, being produced at a concentration of 270 μM over 24 hours based on commercial standards (Table 2.5; Figure 2.10). Past antimicrobial studies with 1-undecene shows it has no effect on *Sclerotinia sclerotiorum* (Fernando *et al.*, 2005) and a small effect on *Fusarium culmorum* (Kai *et al.*, 2009). S-methyl thioesters were also identified in the volatile profile of EA105, producing around 30 μM in 24 hours (Table 2.5; Figure 2.10). Antifungal activity against 70-15 by these compounds was examined and no significant growth reduction was seen at biologically relevant concentrations (Figure 2.10), suggesting these compounds are not responsible for EA105's antifungal activity.

Although not directly correlated to vegetative growth reduction, we were interested to see if EA105-derived thiol-esters could reduce virulence; therefore the effect on 70-15 conidial germination and ability to form appressorium was examined post EA105 treatment. Even though a large effect was not seen, there was significant reduction in appressorial formation by all compounds at 100 μM concentration (Table 2.6).

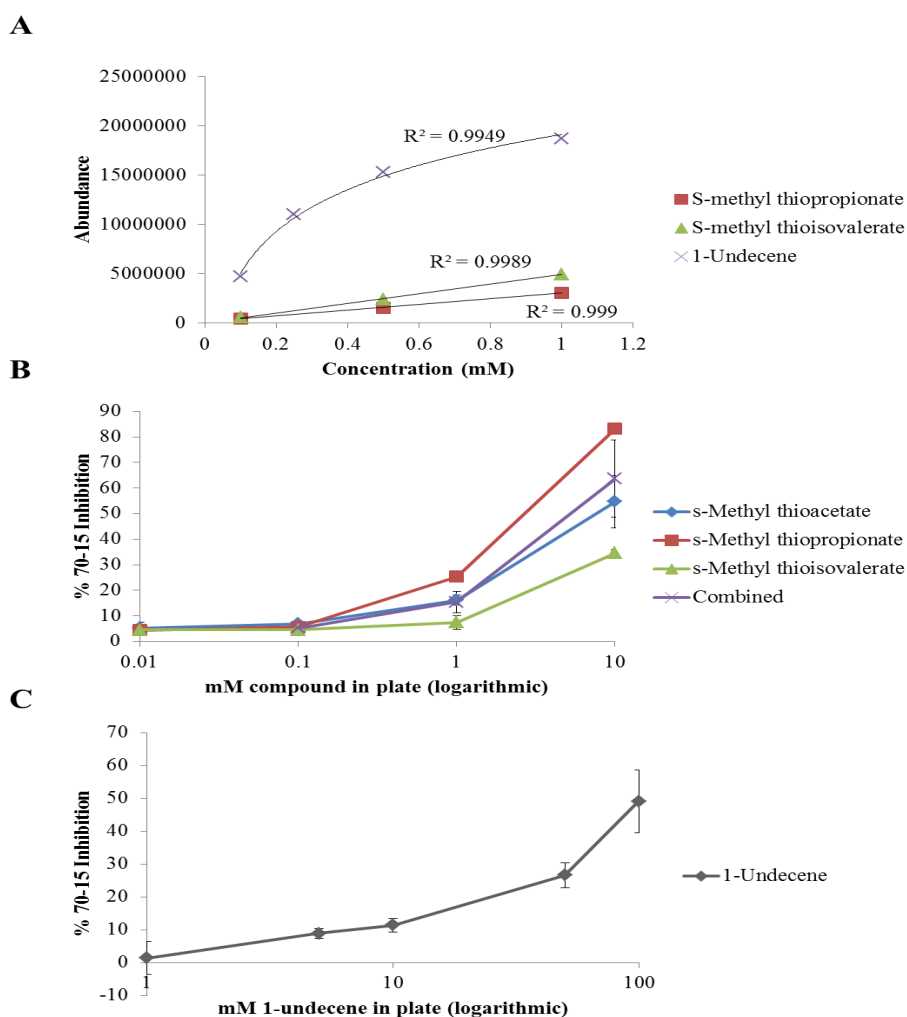


Figure 2.10. **Inhibition of *M. oryzae* by S methyl thioesters and 1-undecene.** **A)** Standard curves used to calculate biological concentrations of volatiles produced by EA105. Commercially available compounds were diluted in methanol (S-methyl thiopropionate, S-methyl thioisovalerate), or chloroform (1-undecene) and injected into a GC-MS for analysis. **B)** Growth of *M. oryzae* 70-15 after 5 days on plates containing different concentrations of S-methyl thioesters in the media. Significant inhibition occurred by 1 mM for all except S-methyl thioisovalerate (Student's *t*-test, $p < 0.05$) Error bars indicate standard error. **C)** Growth of *M. oryzae* 70-15 after 5 days on plates containing different concentrations of 1-undecene in the media. Significant inhibition occurred by 5 mM 1-undecene (Student's *t*-test, $p < 0.05$). Error bars indicate standard error.

Table 2.4. List of EA105 VOCs

RT (min)	Compound
<i>Alcohols</i>	
14.07	2-Undecanol
<i>Hydrocarbons</i>	
7.28	Cyclopropane, 1-methyl-2-pentyl-
10.77	1,4-Octadiene
10.89	1-Undecene
12.42	1-Dodecene
13.71	Cyclodecene
13.91	1-Tridecene
<i>Ketones</i>	
13.94	2-Undecanone
16.67	2-Tridecanone
<i>S-containing compounds</i>	
3.72	Methyl thiolacetate
4.5	Dimethyl disulfide
5.54	S-methyl propanethioate
8.27	S-methyl 3-methylbutanethioate

Table 2.5. Volatile metabolite concentrations.

Compound	Peak Height	Biosynthetic concentration (μM)
S-methyl thioacetate	149,550	^A
S-methyl thiopropionate	195,230	30
S-methyl thioisovalerate	170,940	33
1-Undecene	12,169,570	270
^A Not determined; compound and solvents eluted at same retention time.		

Table 2.6. Spore germination and appressoria formation.

Treatment	% Germination Inhibition	% Appressorium Inhibition
<i>10 μM</i>		
S-methyl thioacetate	4.5	0.9
S-methyl thiopropionate	-1.6	9.7*
S-methyl thioisovalerate	0.1	10.2*
Combined	-1.2	2.1
<i>100 μM</i>		
S-methyl thioacetate	3.0	8.4*
S-methyl thiopropionate	-0.4	6.2*
S-methyl thioisovalerate	-0.2	11.5*
S-methyl combined	2.8	6.9*

* indicates significant inhibition (Student's t-test, $p < 0.05$)

2.4.4 EA105 treatment on rice roots primes for against *M. oryzae*

Induced systemic resistance (ISR) is elicited by plant growth promoting rhizobacteria (PGPR) and results in increased disease resistance in plants. Our data previously showed that EA105 directly inhibits fungal growth by the production of an antifungal compound. Next, we tested if EA105 could also suppress *M. oryzae* indirectly by inducing changes in the host plant. Three-week old roots of soil-grown rice cv. Maratelli (highly susceptible to *M. oryzae*) were root inoculated with rhizobacteria and after 24 hours, the plants were challenged with *M. oryzae* 70-15 spores. In addition to EA105, rice isolates EA106, a *Pantoea agglomerans*, and EA201, an *Arthrobacter oxydans*, were also tested. Strikingly, the plants whose roots had been pretreated, or 'primed', with EA105 and EA106 showed a significantly

reduced number of blast lesions ($P \leq 0.0087$ and 0.0003 , respectively), as compared to the plants receiving no pretreatment (Figure 2.11). Interestingly, pretreatment with a previously characterized direct antagonist of *M. oryzae*, *P. fluorescence* CHAO (de Werra *et al.*, 2009), conferred no protection against disease formation on the leaves (Figure 2.11). Although it has previously been reported that CHAO induces ISR in *Arabidopsis thaliana* (Iavicoli *et al.*, 2003), rice is a non-native host of CHAO, being originally isolated from Swiss soils suppressive to black root rot (Stutz *et al.*, 1986). These results clearly support the hypothesis that root colonization by EA105 and EA106 induces plant-encoded mechanisms which prime rice for foliar attack by *M. oryzae*, enhancing a defense response which leads to reduction of *M. oryzae* infection on the aerial portion of the plant.

To further explore the mechanism by which isolates EA105 and EA106 reduce lesions through a plant-mediated mechanism, the expression of several key ISR genes were examined in rice at 24 hours post bacterial treatment. As a control, the effect of CHAO, which does not reduce the number of *M. oryzae* lesions on rice plants, was also examined. With EA105 or EA106 treatment, there was significant up-regulation of the JA responsive genes, *JAR1* and *WRKY30*, while CHAO treatment down regulated these genes. Similarly, ETH responsive genes, *EIL1* and *ERF1*, were also up-regulated with EA105 and EA106 treatment, but to a significantly lesser extent with CHAO treatment (Figure 2.12). A positive control with JA ($50 \mu\text{M}$) treatment also induced *JAR1* and *WRKY30* (data not shown). There was only slight induction of SA responsive genes *PR1* and *WRKY77* with the bacterial treatments (Figure 2.12). The SA responsive genes were also induced by SA treatment (1 mM). Of the 6 genes examined, expression patterns were similar between EA105 and EA106 treatments for

all genes except *PR1*. In rice treated with EA106, there was a significantly stronger induction of *PR1* than in rice plants treated with EA105. The data suggest that EA105 induces a JA and ETH dependent ISR that may protect plants against *M. oryzae*.

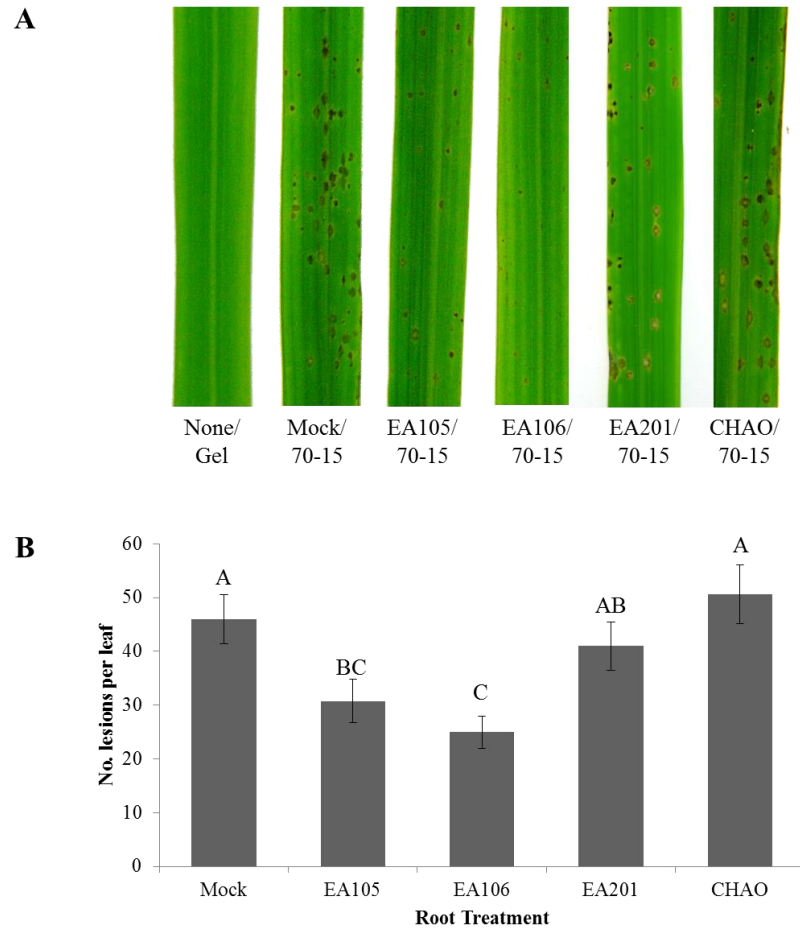


Figure 2.11. **The effect of rhizobacterial priming on rice blast lesion formation.** Spores were sprayed on 3-week old whole plants 24 hour after being root primed with mock, EA105, EA106, EA201 or CHAO suspension. **A)** Representative leaf segments of mock or rhizobacterial primed plants. **B)** The average number of lesions formed on the second youngest leaf of *O. sativa* cv. Maratelli. Error bars indicate standard error. Means with the same letter do not differ significantly (Tukey's HSD).

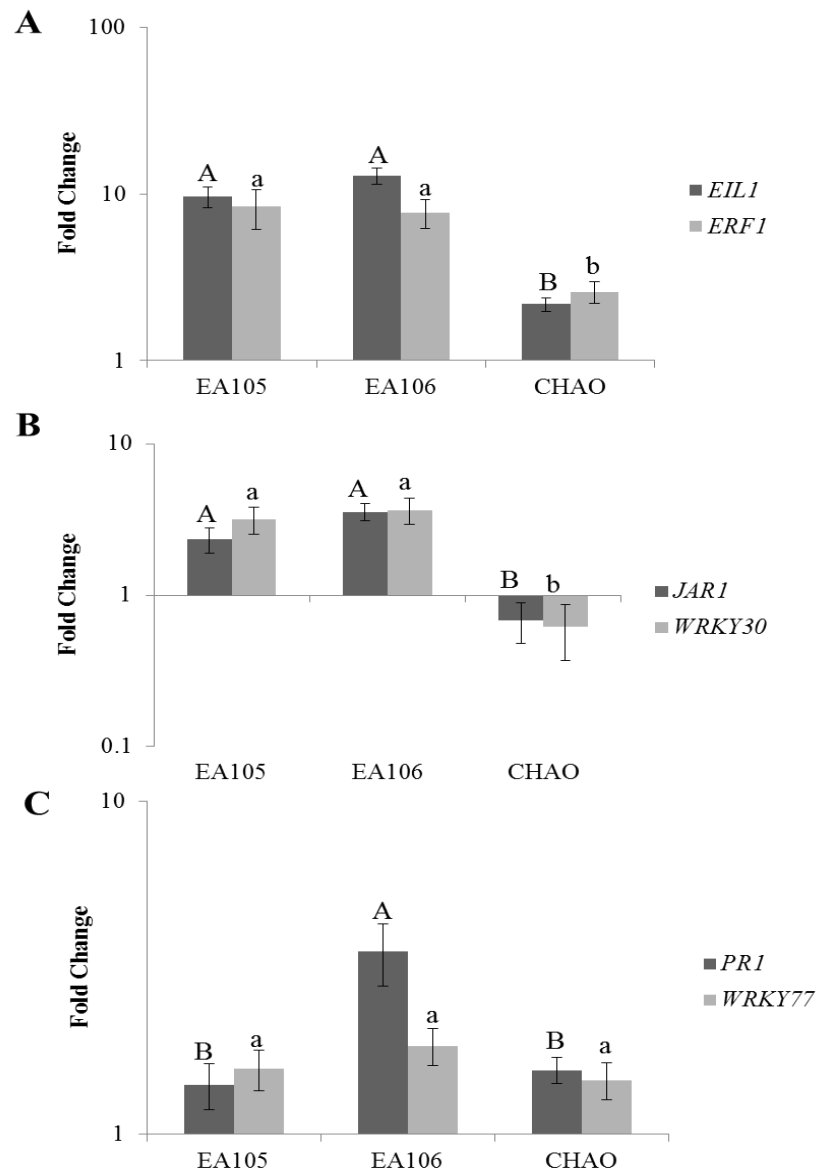


Figure 2.12. **Expression of defense related genes in rice plants treated with rhizobacteria EA105 and EA106.** Roots of aseptically grown rice plants were treated with EA105 or EA106. Leaf samples were collected at 24 hours post treatment and the expression of genes involved in **A)** ethylene, **B)** jasmonic acid (JA), or **C)** salicylic acid (SA) signaling was examined. Error bars indicate standard error. Means with the same letter do not differ significantly (Tukey's HSD).

2.5 Discussion

In order to make a significant impact on global food security, a biocontrol solution to rice blast disease must be developed that is both effective and sustainable while reducing or eliminating the need for synthetic chemical fungicides. We have found microbes from the rice rhizosphere that attenuate *M. oryzae* *in vitro* and *in planta*. Most notable is *P. chlororaphis* strain EA105, which has demonstrated the ability to severely restrict the growth of rice pathogen *M. oryzae*, and is therefore a strong candidate for a novel biocontrol agent against rice blast disease. Previously, *P. chlororaphis* isolates have been shown to be agriculturally important in the biocontrol of several plant pathogens including *Sclerotinia sclerotiorum* (Berry *et al.*, 2014), *Rhizoctonia cerealis* (Jiao *et al.*, 2013), *Seiridium cardinale* (Raio *et al.*, 2011), and *Leptosphaeria maculans* (Ramarathnam *et al.*, 2011). To our knowledge, this is the first report of *P. chlororaphis* reducing rice blast symptoms. In contrast to chemical fungicides, biocontrol bacteria produce a mixture of antifungal compounds which can fluctuate based on environmental cues (Hoitink and Boehm, 1999). The fungistatic activity of EA105 could lead to a longer-term, more effective strategy for reducing rice blast disease than current chemical fungicides, which exert stronger selective pressure for *M. oryzae* to develop resistance. Furthermore, as living organisms, these biocontrol microbes are continuing to evolve with their rhizospheric neighbors ensuring a more sustainable solution.

To gain a better understanding of the composition and diversity of the rice rhizospheric soil, we used a metagenomic approach to examine the phyla and genera that naturally inhabit this niche. Distribution of phyla was consistent across growing seasons, with the two predominant phyla being Acidobacteria and Proteobacteria. Acidobacteria have only recently been discovered and the vast majority are currently

unculturable. However, their abundance in soil has been documented, and they may be playing a crucial role in the rhizosphere that has yet to be determined (da Rocha *et al.*, 2009). Proteobacteria is a very broad phylum, encompassing a variety of bacteria, including pseudomonads which are gamma-proteobacteria (Rudramurthy *et al.*, 2011).

Evidence shows that stress to the aerial portions of plants can stimulate rhizodeposition of chemo-attractants to enhance colonization by rhizobacteria (Haas and Keel, 2003; Rudrappa *et al.*, 2008). Effective plant defense may be due to an ability of the host plant to modulate the composition of root exudates, attracting microbes which can trigger plant resistance. The recruitment of beneficial microbes can also alter physiological functions in plants to resist aerial pathogens (Kumar *et al.*, 2012). Although *M. oryzae* is most commonly a foliar pathogen, it also has the ability to infect roots (Marcel *et al.*, 2010) and is closely related to other root pathogens such as *M. poae*, *M. rhizophila*, and *Gaeumannomyces graminis* (Sesma and Osbourn, 2004). Root infection by *M. oryzae* is often followed by dispersal to the shoots and traditional blast lesion formation (Sesma and Osbourn, 2004). Therefore, the direct antifungal activity of EA105 against *M. oryzae* could have ecologically relevant implications in preventing blast infections.

Our data reveal that treatment of soil-grown rice plants with EA105 activates basal resistance mechanisms against 70-15 *in planta*. The precise mechanism by which rice rhizospheric microbes induce physiological effects on the host (rice) is not known, although some of these changes are modulated through the signaling of small molecules such as salicylic acid (SA), jasmonic acid (JA), or ethylene (ETH) (Chisholm *et al.*, 2006). The pathogenesis related, or PR, genes such as *PR1* and *WRKY77* are SA responsive (Quilis *et al.*, 2008) and are up-regulated during pathogen

infection, ultimately triggering a defense response and reducing disease symptoms (Han *et al.*, 2004). However, beneficial rhizobacteria such as *P. fluorescens* WCS374r have been shown to stimulate a defense response which induces resistance in rice to *M. oryzae*, but is completely independent of SA signaling (De Vleeschauwer *et al.*, 2008). Similar to this finding, our gene expression data suggest that EA105 triggers ISR in rice through a mechanism that involves both JA and ETH and to a lesser extent SA signaling. The JA responsive genes *JAR1* and *WRKY30* are crucial to JA signaling and are required for the stimulation of ISR in *A. thaliana* as well as rice (Pieterse *et al.*, 2001; Peng *et al.*, 2012) and both of these genes were highly expressed 24 hours after EA105 and EA106 treatment but not with CHAO treatment. We saw similar up-regulation of the ethylene responsive genes *EIL1* and *ERF1*, which have also been implicated in ISR signaling and reduction in disease susceptibility (Nakano *et al.*, 2006). Moreover, we demonstrate the ability of EA105 to severely restrict mycelial growth of 70-15 and almost completely halt appressorium formation on abiotic hydrophobic surfaces. This suggests that the beneficial microbiome of rice could attenuate the virulence of rice blast through multiple mechanisms; therefore, manipulation of the rhizosphere is a valuable and comprehensive manner in which to target biotic stresses.

Biocontrol agents are currently employed to control rice pathogens that cause fungal sheath blight (Kanjamaneesathian *et al.*, 1998; Vidhyasekaran and Muthamilan, 1999; Nandakumar *et al.*, 2001; Wiwattanapatapee *et al.*, 2004; Someya *et al.*, 2005) and a subset of fungal pathogens that cause rice blast (Gnanamanickam and Mew, 1992; Krishnamurthy and Gnanamanickam, 1998). With a few exceptions (Han *et al.*, 2005; Naureen *et al.*, 2009) the biocontrol agents tested were not isolated

from rice, as compared to the bacterial strain EA105, which was isolated from the rice rhizosphere. We speculate that a microbe which is confirmed to associate with field grown rice roots, such as EA105, may have better implications for rice protection compared to unrelated biocontrol isolates due to its ability to compete and survive in the rice rhizosphere. Previous studies have shown a relative of *Pseudomonas*, *Delftia tsuruhatensis*, to directly inhibit *M. oryzae* and also reduce lesions in rice by about 50%, however the mechanism of lesion reduction has not been examined (Han *et al.*, 2005). Isolates from the rice and millet rhizospheres, including 13 bacilli and 6 pseudomonads, did show direct inhibition and lesion reduction of Setaria blast, on the host plant Foxtail millet (*Setaria italica* L) though these isolates were not tested in rice (Karthikeyan and Gnanamanickam, 2008). There have also been reports of naturally isolated rice rhizobacteria reducing blast in aerobically grown rice in Brazil, though the isolates have not been identified and the mechanism by which they induce resistance has not yet been examined (Filippi *et al.*, 2011). Similarly, Naureen *et al.* investigated multiple isolates from bulk soil and the rice rhizosphere for their direct antagonism against *M. oryzae* and their ability to reduce lesions *in planta*, but the mechanisms underlying these activities have not yet been explored. Five of the isolates examined were *Pseudomonas* sp. but these 5 isolates were from bulk soil rather than the rice rhizosphere (Naureen *et al.*, 2009). Two isolates from the rhizosphere of *Lupinus hispanicus*, *Pseudomonas fluorescens* Aur 6 and *Chryseobacterium balustinum* Aur 9, showed the ability to reduce blast severity and increase rice production when co-inoculated (Lucas *et al.*, 2009) however, these isolates were not originally isolated from the rice rhizosphere and the way in which they reduce lesions has not yet been described. De Vleeschauwer *et al.*, 2008,

thoroughly examined the way in which *P. fluroescens* WCS374r induces resistance in rice, independent of SA signaling, and mediated through the ETH and octadecanoid pathways. Strain WCS374r is a spontaneous rifampicin mutant of lab strain WCS374 (De Vleeschauwer *et al.*, 2008). De Vleeschauwer *et al.* provide valuable insight into the mechanisms underlying ISR against *M. oryzae*, and we have shown that a natural rice isolate, EA105, shows parallels in its ability to trigger ETH signaling while minimally impacting SA signaling. We have, in a way, combined these stories to investigate how a natural rice isolate works in reducing blast both through direct and plant-mediated mechanisms.

Shimoi *et al.* examined a novel mechanism of blast reduction by selectively isolating phyllospheric microbes from rice, including one *P. geniculata* strain, which catabolizes collagen and gelatin. Some of these microbes were able to reduce blast symptoms when co-inoculated onto rice leaves, presumably by disrupting the adhesion of the spore tip mucilage and extracellular matrix from the leaf surface, preventing proper attachment by *M. oryzae* (Shimoi *et al.*, 2010). It would be interesting to test such a method in combination with a root-associated microbe such as EA105, which can induce resistance through plant based signaling.

Thorough groundwork has been laid in testing methods for introducing biocontrol bacteria to plants. Talc-based powder applications of *P. fluorescens* to rice seeds followed by foliar sprays on rice shoots have resulted in the most effective reduction of blast symptoms (Vidhyasekaran *et al.*, 1997). The survival of two strains of *P. fluorescens* was examined in 3 cultivars of rice, and bacterial treatment of seeds resulted in persistence of the bacteria throughout the 110 day experiment (Krishnamurthy and Gnanamanickam, 1998). However, the mode by which these two

strains were reducing blast symptoms has not been elucidated and appears to differ from the mechanism used by EA105. While we noted elevated JA and ETH signaling with minimal effect on SA, these two *Pseudomonas* isolates resulted in elevated SA levels in rice (Krishnamurthy and Gnanamanickam, 1998).

To our knowledge, this is the first report of a *Pseudomonas chlororaphis* isolate which can protect against rice blast, and this isolate shows two distinct mechanisms of action- direct antifungal activity and induction of resistance in the host. Beyond showing the ability of EA105 to inhibit vegetative growth of *M. oryzae*, we also show an ability to reduce *M. oryzae* pathogenesis by inhibiting appressoria formation. Interestingly, the activity of EA105 is largely independent of cyanide production, despite cyanide commonly being associated with biocontrol activity in pseudomonads.

Decades of research support the importance of microbes to animal health and immunity, across the various environments of the body including the skin and gut. There are compelling reasons to believe that root-associated microbes are equally important to plants. Plant roots encounter diverse microbial populations in soil and generate a unique ecological niche for microbes by the secretion of resources into the rhizosphere. These rhizospheric resources are limited in abundance, and some microbes have evolved antimicrobial traits to reduce competition from other microbes and to bolster the health of their plant host. However, we lack a clear understanding of the contribution conferred by individual microbial strains within a microbiome to plant growth and protection. Since biocontrol has proven to be a successful approach to crop protection, more efforts are needed to identify potential biocontrol agents from

the diverse pool of rhizospheric bacteria and to understand the mechanisms by which they positively influence plant productivity.

2.6 Conclusion

Eleven bacteria were isolated from rhizospheric rice soil and identified. Isolate EA105, *Psuedomonas chlororaphis*, showed the strongest biocontrol potential against blast pathogen *M. oryzae*. EA105 reduced mycelial growth, and almost completely halted appressoria formation in *M. oryzae*. A HCN mutant in EA105, D5, showed similar antagonistic abilities against *M. oryzae*, indicating a mechanism of action which is independent of HCN. Isolate EA105 as well as *Pantoea agglomerans* EA106 were able to reduce the number of blast lesions in rice, when roots were pre-treated with the bacteria prior to infection with *M. oryzae*. The response elicited in rice by EA105 and EA106 is mediated through JA and ETH signaling. Isolate EA105 was the only isolate which was effective both as a direct antagonist to *M. oryzae* as well as an elicitor of the ISR response in rice. Isolate EA105 shows promise as a potentially valuable biocontrol agent to reduce crop losses from blast disease. The resulting increase in rice yields could have a tremendous impact on global food security.

2.7 Acknowledgments

Support from NSF Award PGPR-0923806; Dr. Rovshan Mahmudov for his assistance with the GC-MS; Nancy Gregory for donating the naturally isolated phytopathogens; Adam Draper for his assistance with the inhibition experiments involving these strains; Dr. Thomas Hanson for his advice and guidance.

2.8 References

- Aktar W, Sengupta D, Chowdhury A** (2009) Impact of pesticides use in agriculture: their benefits and hazards. *Interdisciplinary toxicology* **2**: 1-12
- Ashelford KE, Chuzhanova NA, Fry JC, Jones AJ, Weightman AJ** (2006) New screening software shows that most recent large 16S rRNA gene clone libraries contain chimeras. *Applied and Environmental Microbiology* **72**: 5734-5741
- Bechet M, Caradec T, Hussein W, Abderrahmani A, Chollet M, Leclere V, Dubois T, Lereclus D, Pupin M, Jacques P** (2012) Structure, biosynthesis, and properties of kurstakins, nonribosomal lipopeptides from *Bacillus* spp. *Applied Microbiology and Biotechnology* **95**: 593-600
- Beringer JE** (1974) R factor transfer in *Rhizobia leguminosarum*. *Journal of General Microbiology* **84**: 188-198
- Berry CL, Nandi M, Manuel J, Brassinga AKC, Fernando WGD, Loewen PC, de Kievit TR** (2014) Characterization of the *Pseudomonas* sp DF41 quorum sensing locus and its role in fungal antagonism. *Biological Control* **69**: 82-89
- Bertani G** (1951) Studies on lysogenesis.1. The mode of phage liberation by lysogenic *Escherichia coli*. *Journal of Bacteriology* **62**: 293-300
- Blumer C, Haas D** (2000) Mechanism, regulation, and ecological role of bacterial cyanide biosynthesis. *Archives of Microbiology* **173**: 170-177
- Bonman JM, Khush GS, Nelson RJ** (1992) Breeding rice for resistance to pests. *Annual Review of Phytopathology* **30**: 507-528
- Chisholm ST, Coaker G, Day B, Staskawicz BJ** (2006) Host-microbe interactions: Shaping the evolution of the plant immune response. *Cell* **124**: 803-814
- Chuma I, Isobe C, Hotta Y, Ibaragi K, Futamata N, Kusaba M, Yoshida K, Terauchi R, Fujita Y, Nakayashiki H, Valent B, Tosa Y** (2011) Multiple translocation of the AVR-Pita effector gene among chromosomes of the rice blast fungus *Magnaporthe oryzae* and related species. *Plos Pathogens* **7**
- Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, Kulam-Syed-Mohideen AS, McGarrell DM, Marsh T, Garrity GM, Tiedje JM** (2009) The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Research* **37**: D141-D145

- da Rocha UN, van Overbeek L, van Elsas JD** (2009) Exploration of hitherto-uncultured bacteria from the rhizosphere. *Fems Microbiology Ecology* **69**: 313-328
- De Vleeschauwer D, Djavaheeri M, Bakker P, Hofte M** (2008) *Pseudomonas fluorescens* WCS374r-induced systemic resistance in rice against *Magnaporthe oryzae* is based on pseudobactin-mediated priming for a salicylic acid-repressible multifaceted defense response. *Plant Physiology* **148**: 1996-2012
- de Werra P, Pechy-Tarr M, Keel C, Maurhofer M** (2009) Role of gluconic acid production in the regulation of biocontrol traits of *Pseudomonas fluorescens* CHA0. *Applied and Environmental Microbiology* **75**: 4162-4174
- Fernando WGD, Ramarathnam R, Krishnamoorthy AS, Savchuk SC** (2005) Identification and use of potential bacterial organic antifungal volatiles in biocontrol. *Soil Biology & Biochemistry* **37**: 955-964
- Filippi MCC, da Silva GB, Silva-Lobo VL, Cortes M, Moraes AJG, Prabhu AS** (2011) Leaf blast (*Magnaporthe oryzae*) suppression and growth promotion by rhizobacteria on aerobic rice in Brazil. *Biological Control* **58**: 160-166
- Gnanamanickam S, Mew T** (1992) Biological control of blast disease of rice (*Oryza sativa* L.) with antagonistic bacteria and its mediation by a *Pseudomonas* antibiotic. *Japanese Journal of Phytopathology* **58**: 380-385
- Haas D, Keel C** (2003) Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance for biological control of plant disease. *Annual Review of Phytopathology* **41**: 117-153
- Han CU, Lee CH, Jang KS, Choi GJ, Lim HK, Kim JC, Ahn SN, Choi JE, Cha JS, Kim HT, Cho KY, Lee SW** (2004) Identification of rice genes induced in a rice blast-resistant mutant. *Molecules and Cells* **17**: 462-468
- Han JG, Sun L, Dong XZ, Cai ZQ, Sun XL, Yang HL, Wang YS, Song W** (2005) Characterization of a novel plant growth-promoting bacteria strain *Delftia tsuruhatensis* HR4 both as a diazotroph and a potential biocontrol agent against various plant pathogens. *Systematic and Applied Microbiology* **28**: 66-76
- Hoitink HAJ, Boehm MJ** (1999) Biocontrol within the context of soil microbial communities: A substrate-dependent phenomenon. *Annual Review of Phytopathology* **37**: 427-446

- Howell CR, Beier RC, Stipanovic RD** (1988) Production of ammonia by *Enterobacter-cloacae* and its possible role in the biological control of *Pythium* preemergence damping-off by the bacterium. *Phytopathology* **78**: 1075-1078
- Howell CR, Stipanovic RD** (1979) Control of *Rhizoctonia solani* on cotton seedlings with *Pseudomonas fluorescens* and with an antibiotic produced by the bacterium. *Phytopathology* **69**: 480-482
- Howell CR, Stipanovic RD** (1980) Suppression of *Pythium ultimum*-induced damping-off of cotton seedlings by *Pseudomonas fluorescens* and its antibiotic, pyoluteorin. *Phytopathology* **70**: 712-715
- Iavicoli A, Boutet E, Buchala A, Mettraux JP** (2003) Induced systemic resistance in *Arabidopsis thaliana* in response to root inoculation with *Pseudomonas fluorescens* CHA0. *Molecular Plant-Microbe Interactions* **16**: 851-858
- Jiao Z, Wu N, Hale L, Wu W, Wu D, Guo Y** (2013) Characterisation of *Pseudomonas chlororaphis* subsp *aurantiaca* strain Pa40 with the ability to control wheat sharp eyespot disease. *Annals of Applied Biology* **163**: 444-453
- Kai M, Haustein M, Molina F, Petri A, Scholz B, Piechulla B** (2009) Bacterial volatiles and their action potential. *Applied Microbiology and Biotechnology* **81**: 1001-1012
- Kanjanamaneesathian M, Kusonwiriawong C, Pengnoo A, Nilratana L** (1998) Screening of potential bacterial antagonists for control of sheath blight in rice and development of suitable bacterial formulations for effective application. *Australasian Plant Pathology* **27**: 198-206
- Karthikeyan V, Gnanamanickam SS** (2008) Biological control of *Setaria* blast (*Magnaporthe grisea*) with bacterial strains. *Crop Protection* **27**: 263-267
- Krishnamurthy K, Gnanamanickam SS** (1998) Biological control of rice blast by *Pseudomonas fluorescens* strain Pf7-14: Evaluation of a marker gene and formulations. *Biological Control* **13**: 158-165
- Krishnamurthy K, Gnanamanickam SS** (1998) Induction of systemic resistance and salicylic acid accumulation in *Oryza sativa*, L. in the biological suppression of rice blast caused by treatments with *Pseudomonas* spp. *World Journal of Microbiology & Biotechnology* **14**: 935-937
- Kumar AS, Lakshmanan V, Caplan JL, Powell D, Czymmek KJ, Levia DF, Bais HP** (2012) Rhizobacteria *Bacillus subtilis* restricts foliar pathogen entry through stomata. *Plant Journal* **72**: 694-706

- Lakshmanan V, Kitto SL, Caplan JL, Hsueh YH, Kearns DB, Wu YS, Bais HP** (2012) Microbe-associated molecular patterns-triggered root responses mediate beneficial rhizobacterial recruitment in *Arabidopsis*. *Plant Physiology* **160**: 1642-1661
- Laville J, Blumer C, Von Schroetter C, Gaia V, Defago G, Keel C, Haas D** (1998) Characterization of the hcnABC gene cluster encoding hydrogen cyanide synthase and anaerobic regulation by ANR in the strictly aerobic biocontrol agent *Pseudomonas fluorescens* CHAO. *Journal of Bacteriology* **180**: 3187-3196
- Lucas JA, Solano BR, Montes F, Ojeda J, Megias M, Manero FJG** (2009) Use of two PGPR strains in the integrated management of blast disease in rice (*Oryza sativa*) in Southern Spain. *Field Crops Research* **114**: 404-410
- Lugtenberg B, Kamilova F** (2009) Plant-Growth-Promoting Rhizobacteria. *Annual Review of Microbiology* **63**: 541-556
- Marcel S, Sawers R, Oakeley E, Angliker H, Paszkowski U** (2010) Tissue-adapted invasion strategies of the rice blast fungus *Magnaporthe oryzae*. *Plant Cell* **22**: 3177-3187
- Nakano T, Suzuki K, Fujimura T, Shinshi H** (2006) Genome-wide analysis of the ERF gene family in *Arabidopsis* and rice. *Plant Physiology* **140**: 411-432
- Nandakumar R, Babu S, Viswanathan R, Raguchander T, Samiyappan R** (2001) Induction of systemic resistance in rice against sheath blight disease by *Pseudomonas fluorescens*. *Soil Biology & Biochemistry* **33**: 603-612
- Naureen Z, Price AH, Hafeez FY, Roberts MR** (2009) Identification of rice blast disease-suppressing bacterial strains from the rhizosphere of rice grown in Pakistan. *Crop Protection* **28**: 1052-1060
- Neidig N, Paul RJ, Scheu S, Jousset A** (2011) Secondary metabolites of *Pseudomonas fluorescens* CHA0 drive complex non-trophic interactions with bacterivorous nematodes. *Microbial Ecology* **61**: 853-859
- Nielsen TH, Christophersen C, Anthoni U, Sorensen J** (1999) Viscosinamide, a new cyclic depsipeptide with surfactant and antifungal properties produced by *Pseudomonas fluorescens* DR54. *Journal of Applied Microbiology* **87**: 80-90

- Nielsen TH, Thrane C, Christophersen C, Anthoni U, Sorensen J** (2000) Structure, production characteristics and fungal antagonism of tensin - a new antifungal cyclic lipopeptide from *Pseudomonas fluorescens* strain 96.578. *Journal of Applied Microbiology* **89**: 992-1001
- Peng XX, Hu YJ, Tang XK, Zhou PL, Deng XB, Wang HH, Guo ZJ** (2012) Constitutive expression of rice *WRKY30* gene increases the endogenous jasmonic acid accumulation, PR gene expression and resistance to fungal pathogens in rice. *Planta* **236**: 1485-1498
- Pessi G, Haas D** (2000) Transcriptional control of the hydrogen cyanide biosynthetic genes hcnABC by the anaerobic regulator ANR and the quorum-sensing regulators LasR and RhlR in *Pseudomonas aeruginosa*. *Journal of Bacteriology* **182**: 6940-6949
- Pieterse CMJ, Van Pelt JA, Van Wees SCM, Ton J, Leon-Kloosterziel KM, Keurentjes JJB, Verhagen BWM, Knoester M, Van der Sluis I, Bakker P, Van Loon LC** (2001) Rhizobacteria-mediated induced systemic resistance: Triggering, signalling and expression. *European Journal of Plant Pathology* **107**: 51-61
- Pozo MJ, Van Der Ent S, Van Loon LC, Pieterse CMJ** (2008) Transcription factor MYC2 is involved in priming for enhanced defense during rhizobacteria-induced systemic resistance in *Arabidopsis thaliana*. *New Phytologist* **180**: 511-523
- Quilis J, Penas G, Messeguer J, Brugidou C, Segundo BS** (2008) The *Arabidopsis* AtNPR1 inversely modulates defense responses against fungal, bacterial, or viral pathogens while conferring hypersensitivity to abiotic stresses in transgenic rice. *Molecular Plant-Microbe Interactions* **21**: 1215-1231
- Raaijmakers JM, Weller DM, Thomashow LS** (1997) Frequency of antibiotic-producing *Pseudomonas* spp. in natural environments. *Applied and Environmental Microbiology* **63**: 881-887
- Raio A, Puopolo G, Cimmino A, Danti R, Della Rocca G, Evidente A** (2011) Biocontrol of cypress canker by the phenazine producer *Pseudomonas chlororaphis* subsp aureofaciens strain M71. *Biological Control* **58**: 133-138
- Ramarathnam R, Fernando WGD, de Kievit T** (2011) The role of antibiosis and induced systemic resistance, mediated by strains of *Pseudomonas chlororaphis*, *Bacillus cereus* and *B. amyloliquefaciens*, in controlling blackleg disease of canola. *Biocontrol* **56**: 225-235

- Rashid MH, Kornberg A** (2000) Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of *Pseudomonas aeruginosa*. Proceedings of the National Academy of Sciences of the United States of America **97**: 4885-4890
- Rudramurthy SM, Chakrabarti A, Geertsens E, Mouton JW, Meis JF** (2011) In vitro activity of isavuconazole against 208 *Aspergillus flavus* isolates in comparison with 7 other antifungal agents: assessment according to the methodology of the European Committee on Antimicrobial Susceptibility Testing. Diagnostic Microbiology and Infectious Disease **71**: 370-377
- Rudrappa T, Biedrzycki M, Kunjeti S, Donofrio N, Czymmek K, Pare P, Bais H** (2010) The rhizobacterial elicitor acetoin induced systemic resistance in *Arabidopsis thaliana*. Communicative & Integrative Biology **3**: 1-9
- Rudrappa T, Czymmek KJ, Pare PW, Bais HP** (2008) Root-secreted malic acid recruits beneficial soil bacteria. Plant Physiology **148**: 1547-1556
- Rudrappa T, Splaine RE, Biedrzycki ML, Bais HP** (2008) Cyanogenic pseudomonads influence multitrophic interactions in the rhizosphere. Plos One **3**
- Ryu CM, Farag MA, Hu CH, Reddy MS, Kloepper JW, Pare PW** (2004) Bacterial volatiles induce systemic resistance in *Arabidopsis*. Plant Physiology **134**: 1017-1026
- Ryu CM, Farag MA, Hu CH, Reddy MS, Wei HX, Pare PW, Kloepper JW** (2003) Bacterial volatiles promote growth in *Arabidopsis*. Proceedings of the National Academy of Sciences of the United States of America **100**: 4927-4932
- Schroth MN, Hancock JG** (1982) Disease-suppressive soil and root-colonizing bacteria. Science **216**: 1376-1381
- Sesma A, Osbourn AE** (2004) The rice leaf blast pathogen undergoes developmental processes typical of root-infecting fungi. Nature **431**: 582-586
- Shimoi S, Inoue K, Kitagawa H, Yamasaki M, Tsushima S, Park P, Ikeda K** (2010) Biological control for rice blast disease by employing detachment action with gelatinolytic bacteria. Biological Control **55**: 85-91
- Silby MW, Winstanley C, Godfrey SAC, Levy SB, Jackson RW** (2011) *Pseudomonas* genomes: diverse and adaptable. Fems Microbiology Reviews **35**: 652-680

- Skamnioti P, Gurr SJ** (2009) Against the grain: safeguarding rice from rice blast disease. *Trends in Biotechnology* **27**: 141-150
- Someya N, Nakajima M, Watanabe K, Hibi T, Akutsu K** (2005) Potential of *Serratia marcescens* strain B2 for biological control of rice sheath blight. *Biocontrol Science and Technology* **15**: 105-109
- Stutz EW, Defago G, Kern H** (1986) Naturally-occurring fluorescent pseudomonads involved in suppression of black root rot of tobacco. *Phytopathology* **76**: 181-185
- Thomashow LS, Weller DM** (1988) Role of a phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis* var tritici. *Journal of Bacteriology* **170**: 3499-3508
- Van der Ent S, Van Wees SCM, Pieterse CMJ** (2009) Jasmonate signaling in plant interactions with resistance-inducing beneficial microbes. *Phytochemistry* **70**: 1581-1588
- van Loon LC** (2007) Plant responses to plant growth-promoting rhizobacteria. *European Journal of Plant Pathology* **119**: 243-254
- Van Wees SCM, Van der Ent S, Pieterse CMJ** (2008) Plant immune responses triggered by beneficial microbes. *Current Opinion in Plant Biology* **11**: 443-448
- Vanpeer R, Niemann GJ, Schippers B** (1991) Induced resistance and phytoalexin accumulation in biological control of Fusarium wilt of carnation by *Pseudomonas* sp strain WCS417R. *Phytopathology* **81**: 728-734
- Vidhyasekaran P, Muthamilan M** (1999) Evaluation of a powder formulation of *Pseudomonas fluorescens* Pfl for control of rice sheath blight. *Biocontrol Science and Technology* **9**: 67-74
- Vidhyasekaran P, Rabindran R, Muthamilan M, Nayar K, Rajappan K, Subramanian N, Vasumathi K** (1997) Development of a powder formulation of *Pseudomonas fluorescens* for control of rice blast. *Plant Pathology* **46**: 291-297
- Vitullo D, Di Pietro A, Romano A, Lanzotti V, Lima G** (2012) Role of new bacterial surfactins in the antifungal interaction between *Bacillus amyloliquefaciens* and *Fusarium oxysporum*. *Plant Pathology* **61**: 689-699

- Voisard C, Keel C, Haas D, Defago G** (1989) Cyanide production by *Pseudomonas fluorescens* helps suppress black root rot of tobacco under gnotobiotic conditions. *Embo Journal* **8**: 351-358
- Wahlund TM, Madigan MT** (1995) Genetic transfer by conjugation in the thermophilic green sulfur bacterium *Chlorobium tepidum*. *Journal of Bacteriology* **177**: 2583-2588
- Wang Q, Garrity GM, Tiedje JM, Cole JR** (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology* **73**: 5261-5267
- Wickham H** (2009) ggplot2: Elegant Graphics for Data Analysis. *Ggplot2: Elegant Graphics for Data Analysis*: 1-212
- Wilson RA, Talbot NJ** (2009) Under pressure: investigating the biology of plant infection by *Magnaporthe oryzae*. *Nature Reviews Microbiology* **7**: 185-195
- Wiwattanapatapee R, Pengnoo A, Kanjanamaneesathian M, Matchavanich W, Nilratana L, Jantharangsri A** (2004) Floating pellets containing bacterial antagonist for control sheath blight of rice: formulations, viability and bacterial release studies. *Journal of Controlled Release* **95**: 455-462
- Zeigler RS** (1998) Recombination in *Magnaporthe grisea*. *Annual Review of Phytopathology* **36**: 249-275
- Zlosnik JEA, Williams HD** (2004) Methods for assaying cyanide in bacterial culture supernatant. *Letters in Applied Microbiology* **38**: 360-365

ENDNOTES

² Modified from **Spence C, Alff E, Johnson C, Ramos C, Donofrio N, Sundaresan V, Bais H.** (2014) Natural rice rhizospheric microbes suppress rice blast infections. *BMC Plant Biology*. **14**:130 DOI: 10.1186/1471-2229-14-130

Chapter 3

GLOBAL GENE EXPRESSION IN RICE BLAST PATHOGEN *Magnaporthe oryzae* TREATED WITH SOIL ISOLATE EA105³

3.1 Abstract

The rhizospheric microbiome is comprised of many microbes, some of which reduce the virulence of their phytopathogenic neighbors; however, the mechanisms underlying these interactions are largely unknown. Rice soil isolate *Pseudomonas chlororaphis* EA105 strongly inhibits *Magnaporthe oryzae*'s in vitro growth by restricting fungal diameter as well as inhibiting the formation of the appressorium, required for penetration. The goal of this chapter was to elucidate *M. oryzae*'s response to EA105 treatment, and a microarray approach was used to obtain a global perspective of EA105-elicited changes in this pathogen. Based on this analysis, three genes of interest were knocked out in *M. oryzae* 70-15, and their sensitivity to EA105 treatment as well as their ability to infect rice was determined. Priming rice plants with EA105 prior to *M. oryzae* infection decreased lesion size, and the mutants were tested to see if this effect was retained. A null 70-15 mutant in a trichothecene biosynthesis gene showed less susceptibility to bacterial treatment, forming more appressoria than the parental type 70-15. A similar pattern was seen in a null mutant for a stress-inducible protein, MGG_03098. In addition, when this mutant was inoculated onto the leaves of EA105-primed rice plants, lesions were reduced to a greater extent than in 70-15, implicating the lack of this gene with an increased ISR response in rice. Understanding the global effect of biocontrol bacteria on

phytopathogens is key for developing successful and lasting solutions to crop loss caused by plant diseases and has the potential to greatly increase food supply.

3.2 Introduction

Exploiting the antifungal activities of natural soil microbes is currently being explored in many crop systems as a way to increase yields and reduce disease. In rice, (*Oryza sativa*), a staple food crop worldwide, crop protection could directly translate to increased food supply. There is evidence that sheath blight caused by *Rhizoctonia solani* may be diminished using multiple soil microbes including *Bacillus subtilis* (Mousivand *et al.*, 2012) as well as mycorrhizal fungi from orchids (Mosquera-Espinosa *et al.*, 2013). Bacterial diseases of rice such as seedling rot and seedling blight may be controllable through the use of bacteriophages (Adachi *et al.*, 2012) while leaf blight caused by the bacterial pathogen *Xanthomonas oryzae* could be reduced using biocontrol strains of *Bacillus* (Chithrashree *et al.*, 2011). The most devastating pathogen of rice, *Magnaporthe oryzae*, is the causal agent of fungal blast, and results in the loss of 10-30% of rice yields (Skamnioti and Gurr, 2009). To control it, several microorganisms are currently being investigated, including *B. subtilis* (Leelasuphakul *et al.*, 2006), *B. methylotrophicus* (Shan *et al.*, 2013), *Streptomyces globisporus* (Li *et al.*, 2011), and *Pseudomonas fluorescens* (De Vleeschauwer *et al.*, 2008; Krishnamurthy and Gnanamanickam, 1998).

Although multiple microorganisms are being investigated for their potential to reduce blast symptoms caused by *M. oryzae*, the specific mechanisms by which they work are largely unknown. Biocontrol bacteria can work either through direct antagonism of the pathogen, or through a mechanism known as induced systemic resistance (ISR). With ISR, the bacteria initiate a response in plants through the

signaling of small molecules such as salicylic acid (SA), jasmonic acid (JA) or ethylene (ETH) which results in the plants being less susceptible to pathogens (De Vleeschauwer and Hofte, 2009). However, even when considering just a direct inhibition of the pathogen by biocontrol bacteria, there is still much to be discovered about the compounds which are playing a role in inhibition and their response on pathogens. The identity of some of the antimicrobial compounds have been determined, such as hydrogen cyanide (HCN) (Blumer and Haas, 2000), 2,4-Diacetylphloroglucinol (2,4-DAPG) (Yang and Cao, 2012), pyrrolnitrin, pyoluteorin (Dubuis *et al.*, 2007), surfactants, and β 1-3-glucanases (Leelasuphakul *et al.*, 2006), though there are many more to be discovered. Although these compounds have been shown to inhibit phytopathogens, the specific response that they elicit in the pathogens is not well understood. The pathogen's response to inhibition is a crucial part of the communications, and will impact the composition of compounds secreted by the pathogen into the rhizosphere, which can directly impact other microorganisms as well as the crop plant. Trichothecenes, for example, play a large role in fungal defense response and are potent eukaryotic toxins (Desjardins *et al.*, 1993). Fungi typically synthesize trichothecenes in response to stress, such as inhibition, and these toxins can accumulate in crop plants, rendering them unsafe for consumption (Desjardins *et al.*, 1993; Jonkers *et al.*, 2012). Thus, it is important to understand the broad impact and secondary effects of using biocontrol strains to antagonize phytopathogens.

The transcriptional response of *M. oryzae* treated with chemical fungicides had revealed a glimpse of how the fungus is reacting to fungicide treatment. Some of the gene expression changes are general responses to stress, while others are specific to

the treatment. One of the most well studied transcriptional responses is the up-regulation of trichothecene biosynthesis genes. As previously mentioned, trichothecenes are extremely toxic to eukaryotes and function to inhibit protein synthesis at the ribosome. In plants, these compounds impede defense responses by interfering with cell wall fortification and callose deposition (Jansen *et al.*, 2005). Specific environmental conditions, such as the presence of sodium bicarbonate, can reduce the low basal expression of trichothecene synthesis (*TRI*) genes (Roinestad *et al.*, 1994), while the high levels of reactive oxygen species (ROS) or the presence of azole fungicides at sub-lethal levels, greatly increase the expression of *TRI* genes (Audenaert *et al.*, 2012; Kulik *et al.*, 2012). When respiration is blocked by compounds such as cyanide, antimycin A, or the antifungal compound SSF-126, alternative respiration genes are induced. The alternate oxidase (*AOX*) gene was one of the first of these genes to be identified in *M. oryzae* and was shown to be induced by SSF-136 and hydrogen peroxide (Yukioka *et al.*, 1998). Both the mycelial growth and conidial germination depend on *AOX* genes to survive in the presence of fungicides that block the canonical respiration pathway (Avila-Adame and Koller, 2003). The detoxification of ROS is also associated with specific transcriptional events. In yeasts and filamentous fungi there is a conserved mechanism for coping with ROS which is mediated through the induction of specific genes to either detoxify ROS (catalase, superoxide dismutase) or to repair ROS damage and maintain cellular homeostasis. The process is coordinated by proteins in the AP-1 family of bZIP activating proteins (Liu *et al.*, 2005). Additionally, transporters also play a role in fungicide response/tolerance by promoting the efflux of toxic compounds out of fungal cells. There are two main classes of transporters, the ATP-binding cassette

(ABC) transporters which use energy generated by ATP hydrolysis, and the major facilitator superfamily (MFS) which use proton motive force to move substances across membranes. Some transporters have a broad range of substrates, such as Pdr5p which is induced by multiple fungicides, whereas others are more specific (Del Sorbo *et al.*, 2000). For example, Pdr12p transports only C1-C7 organic acids, such as benzoic, sorbic, or propionic acid. Likewise, the transporter encoded by *ABC2* is induced by some but not all fungicides. Azoles (including tricyclazole), pyroquilon, carpropamide, and benomyl all induce transcription of *ABC2* but little to no transcriptional effect is seen after treatment with other fungicides such as phthalide, isoprothiolane, or kasugamycin (Lee *et al.*, 2005). Additionally, *ABC1* transcription is also strongly induced in *M. oryzae* in response to azoles. Fungicides also typically induce genes in the HOG pathway, responsible for general and osmotic stress responses. This pathway is not present in mammals, which makes it a prime target for development of fungicides (Jiang *et al.*, 2011). At least one of the genes in the HOG pathway, which is induced by ROS as well as fungicides such as tricyclazole, also plays a role in trichothecene biosynthesis. As of yet, there are no genome wide examinations of *M. oryzae*'s response to fungicides.

Although *M. oryzae*'s broad transcriptional response to *in vitro* stresses has been examined previously (Mathioni *et al.*, 2011), this study, to our knowledge, is the first to examine the global transcriptional responses of *M. oryzae* to treatment with an antagonistic bacterium with biocontrol potential. In addition, EA105 was originally isolated directly from field-grown rice plants. Survival and assimilation into the rhizospheric microbiome is a crucial factor in the success of a biocontrol bacterium, and a natural rice soil isolate is more likely to thrive and perform than isolates which

are non-native to rice soil. Our lab has previously shown the ability of EA105 to directly inhibit *M. oryzae*'s growth and appressoria formation, as well as trigger ISR in rice to reduce blast lesions. The objective of this chapter is to examine the effect of EA105 treatment on the *M. oryzae*'s transcriptome. We also show the functional significance of some of the key genes in *M. oryzae* targeted by EA105. These results provide new insights into the importance of functional microbiome in suppressing plant diseases.

3.3 Materials and Methods

3.3.1 Fungal and bacterial strains and growth conditions

Wild-type *M. oryzae* 70-15, the sequenced reference strain, was used throughout the experiments. Knockout mutants were constructed in the 70-15 background. For vegetative growth, the fungi were placed on complete medium (CM) containing sucrose (10 g/L), casamino acids (6 g/L), yeast extract (6 g/L), and 1 mL of *Aspergillus nidulans* trace elements (Per 100 mL: 0.22 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.05 g KI, 0.02 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g H_3BO_4 , 0.1 mL concentrated H_2SO_4 , 0.008 g $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.007 g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$). Oatmeal agar consisting of ground oats (50 g/L) and agar (15 g/L) were used for sporulation. Plates were kept at 25°C with constant fluorescent light. Bacterial strain EA105, a *Pseudomonas chlororaphis*, was isolated from rice cultivar M-104 grown in the field by Dr. Venkatesan Sundaresan's lab from the University of California (Davis). Strain D5 is a cyanide biosynthetic mutant of EA105 (Spence *et al.*, 2014). *P. fluorescens* biocontrol strain CHAO was obtained from the Culture Collection of Switzerland. Strain CHA77, an *hcnABC* mutant in the CHAO background (Laville *et al.*, 1998) was also obtained from the Culture Collection of

Switzerland. Bacterial strains were grown in liquid or solid Luria Bertani (LB) medium at 28-30°C.

3.3.2 Plant material and growth conditions

Oryza sativa cultivar M-104 was donated by Dr. Venkatesan Sundaresan from the University of California (Davis). *O. sativa* Seraceltik, a cultivar of rice that is susceptible to *M. oryzae*, was maintained in Dr. Nicole Donofrio's lab at the University of Delaware. Seeds for *O. sativa* Nipponbare were obtained from the United States Department of Agriculture, Agricultural Research Service, from the Genetic Stocks-Oryzae collection. Rice plants were grown in Cornell mix rice soil with 16 hours of light (28°C, 80% relative humidity) and 8 hours of darkness (26°C, 80% relative humidity).

3.3.3 Sample preparation and on-color microarray

A 5 mm plug of *M. oryzae* 70-15 was placed on a CM agar plate, 4 cm from a 5 µL drop of LB broth or bacteria in LB (5×10^5 cells/mL). Co-inoculated plates were dried in a laminar flow hood to reduce the motility of EA105. If the bacterial droplet was not given adequate time to dry, EA105 showed enhanced motility, physically interacting with and killing 70-15. At 72 hours post co-inoculation, the fungal mass was scraped from the top of the agar and ground in liquid nitrogen. RNA was extracted using the Qiagen RNeasy Plant Mini Kit. RNA samples were sent on dry ice to Beckman Coulter Genomics where a one-color gene expression microarray was performed using Agilent Magnaporthe (V2) 4X44K slides. Each biological replicate contained tissue from 5 plates, and there were three biological replicates per treatment.

3.3.4 Microarray analysis

From the data set provided by Beckman Coulter Genomics, data was filtered and excluded from probes that produced intensity values of less than 100 as well as from those that had a p-value greater than 0.01. Additionally, fold changes were calculated for each sequence ID by comparing intensities over untreated *M. oryzae* samples. Fold changes smaller than 2 were also excluded. Heat maps were constructed using the University of Toronto Bar Heatmapper Tool (<http://bar.utoronto.ca/ntools/cgi>). The Gene Ontology (GO) terms were determined for the top 100 up and down-regulated genes in each treatment by using the Sequence ID to find the 60-mer probe sequence using a cross reference file available through NCBI. Then, the probe sequence was put through BLASTn (NCBI) to find the gene accession number for the gene corresponding to the probe. The gene accession number was used to search for previously annotated GO terms based on the version 5 *M. oryzae* genome sequence (Meng *et al.*, 2009). The data obtained from the microarray were deposited in the Gene Expression Omnibus (GEO) database at <http://ncbi.nlm.nih.gov/geo> under accession number GSE49597.

3.3.5 Creating gene knockout mutants in *M. oryzae*

Homologous recombination was used to replace genes of interest with a hygromycin resistance cassette through adaptamer mediated PCR (Reid *et al.*, 2002). For each gene to be knocked out, a 1.2 kb segment upstream of the 5' UTR and another 1.2 kb segment downstream of the 3' UTR was amplified, using primers that would add an adaptor to the 3' end of the first segment, as well as to the 5' end of the other segment. The hygromycin resistance cassette was amplified from plasmid pCB1003 using primers that had adaptors complementary to those used in amplifying

the upstream and downstream segments. All three segments were combined in a PCR reaction to make the full length constructs, approximately 3.3kb. Creation of protoplasts and transformations were conducted following traditional methods (Sweigard *et al.*, 1992). Primers are listed in Table 3.1.

Table 3.1 List of primers used to create *M. oryzae* 70-15 deletion mutants. Adaptor sequences are bolded.

	Sequence
MGG_03098 5' flanking forward	CGACATGGCCTACTGGATGA
MGG_03098 5' flanking reverse	CACGGCGCGCCTAGCAGCGGG CATAAAACGCAGCAAAGCG
MGG_03098 3' flanking forward	GCAGGGATGCGGCCGCTGAC ACTTGGCGACTTTGACCTCG
MGG_03098 3' flanking reverse	AGCAAGATATGGCGAACCGA
MGG_17822 5' flanking forward	TCCACTGCTATCGTCCCAAC
MGG_17822 5' flanking reverse	CACGGCGCGCCTAGCAGCGG CGCGAGTTTTGCGTCACATT
MGG_17822 3' flanking forward	GCAGGGATGCGGCCGCTGAC TAAAGAAGACCACGCCTGCC
MGG_17822 3' flanking reverse	GATTGGAGATGCGAGGTCCC
MGG_08440 5' flanking forward	GCGCTTGTTGTAGAAACCCG
MGG_08440 5' flanking reverse	CACGGCGCGCCTAGCAGCGG TACCCCAAGCCGAGAGATGA
MGG_08440 3' flanking forward	GCAGGGATGCGGCCGCTGAC CCCATCACCCCTCTCCCAGAT
MGG_08440 3' flanking reverse	ACTTGTGCTACTCGGCATAGG
hygromycin resistance cassette forward	CCGCTGCTAGGCGCGCCGTGGCTGGAGCTAGTGGAGGTCA
hygromycin resistance cassette reverse	GTCAGCGGCCGCATCCCTGCGGTCGGCATCTACTCTATTC

3.3.6 Diffusible and volatile *in-vitro* inhibition assays

For the diffusible assays, a 5 mm fungal plug was placed 4 cm from a 5 μ L drop of bacteria (5×10^5 cells/mL in LB) on a solid CM plate. For the volatile assay, compartmentalized plates were used which had 4 distinct quadrants. The fungal plug was placed in the quadrant opposite of the bacterial drop. The diameter of fungal growth was measured every 24 hours over the course of 7 days. Three biological replicates of this experiment were performed, each consisting of 5 plates per treatment.

3.3.7 Spore germination and appressoria formation assays

Plastic coverslips were sterilized in ethanol and used as hydrophobic surfaces to encourage the germination of conidiospores and subsequent appressoria formation. The 70-15 spores were grown on oatmeal agar for 10 days prior to being scraped into CM broth and filtered through Miracloth. Each coverslip was inoculated with a 50 μ L drop containing a final concentration of 10^5 spores/mL, and 10^5 bacterial cells/mL. Spores were suspended in CM and bacteria were in LB. Controls contained 10^5 spores/mL with equal amounts of CM and LB. These experiments were repeated in water with similar results. The coverslips were placed in petri dishes with wet filter discs in the center to promote humidity. Plates were sealed and placed in the dark. Germination percentages were calculated after 3 hours incubation, and appressoria formation was determined after 24 hours. Coverslips were imaged using a Zeiss Axioscope2 light microscope. Five images were taken per coverslip, and 5 coverslips were used per treatment. Two biological replicates were examined.

3.3.8 *In-planta* infection assays

Rice plants of cultivars M-104, Nipponbare, and Seraceltik were grown in soil for three weeks. To check the effect of bacterial priming, overnight cultures of bacteria were washed in water and re-suspended to 0.5 OD. For each plant, 2 mL of bacteria were dispensed onto the soil surface at the base of the plant. At 24 hours post bacterial treatment, the second youngest leaf was cut and affixed to a large 15 cm diameter petri dish, on top of moistened paper towels and treated with 70-15 spores. Spores were grown on oatmeal agar for 10 days, and were subsequently scraped into sterile water with .02% gelatin. Spore concentration was adjusted to 10^5 spores/mL. On each cut leaf, a total of 4-30 μ L droplets of spores were placed along the length of the leaf. Plates were incubated in the dark for 24 hours at 25°C, after which time the spore droplets were wicked away. Plates were then kept in cycles of 16 hr light/8 hr darkness for 5 days at 25°C. On the 5th day, the length and width of lesions was measured. A minimum of 8 leaves with 4 droplets per leaf were included per replicate. Three biological replicates were completed.

3.3.9 Sample preparation and confocal imaging of *M. oryzae* on rice

Rice plants were grown in soil for three weeks. To test the effect of bacterial treatment on the plants, some plants were primed as described above, 24 hours prior to removing the leaf sheath. The innermost leaf sheath was then removed and cut into an approximately 6 cm segment. The shape of the leaf sheath created a tube into which approximately 200 μ L of spores (10^5 cells/mL in water) were pipetted. The leaf sheaths were kept in a container with moist paper towels in the dark at 25°C for 18 hours. The leaf sheaths were then further dissected to obtain a thin layer (around 2-3 cells thick) of the leaf sheath epidermis as per the previously published protocol

(Kankanala *et al.*, 2007). Samples were fixed in 4% paraformaldehyde. Unstained samples were visualized using a Zeiss 510 NLO multiphoton confocal microscope with a C-Apochromat 40x water objective. Samples were excited with an argon laser at 488 nm. At least 4 plants were examined per replicate, and three biological replicates were completed.

3.3.10 Statistical analysis

Statistical analyses of the results were performed using the statistical software JMP10. To compare across treatments, the Tukey's HSD test was used and results were considered to be statistically different when $p < 0.05$.

3.4 Results

3.4.1 Global transcriptional changes in *M. oryzae* 70-15 following treatment with soil bacteria

Previously, we have shown that *M. oryzae* 70-15 (hereafter 70-15) is drastically inhibited *in vitro* by rice soil isolate EA105, a strain of *Pseudomonas chlororaphis* isolated from field grown *O. sativa* cv. M-104 (Spence *et al.*, 2014). To further examine the effects of EA105 treatment on 70-15, we used microarray analysis to examine transcriptional changes in 70-15 at 72 hours post EA105 treatment. The bacterium was placed 4 cm from the fungal plug, and plates were kept dry to prevent the bacteria from spreading and overtaking the fungal plug. For comparison, we also included known biocontrol agent *P. fluorescens* CHAO, as well as the corresponding *hcnABC* mutant CHA77, to determine the contribution of cyanide to transcriptional changes in 70-15. We have also previously shown that both CHAO and CHA77 inhibit 70-15 but to a lesser extent than EA105. Approximately half of *M. oryzae*'s

genes were significantly down-regulated after treatment with EA105, compared to untreated controls (Table 3.2). Treatment with CHAO or CHA77 led to down-regulation of only 463 or 80 genes, respectively. EA105 only significantly up-regulated 44 genes, less than the 133 and 124 genes which were seen, respectively, with CHAO or CHA77 treatments (Table 3.2). When considering the 44 genes that are up-regulated with EA105 treatment, 36 were also up-regulated with CHAO treatment, 7 were up-regulated by CHAO and CHA77 treatment, and only one gene was uniquely up-regulated with EA105 treatment (Figure 3.1). This gene, MGG_04034, is a putative NAD dependent formate dehydrogenase. Due to the large number of 70-15 genes down-regulated with EA105 treatment, the top 100 down-regulated genes from each treatment were considered. Of these, EA105 and CHAO treatments resulted in the unique down-regulation of approximately 30 genes, whereas CHA77 was able to uniquely down-regulate twice as many. Only 14 of these genes were commonly down-regulated with all three bacterial treatments (Figure 3.1).

Table 3.2. Gene expression changes in *M. oryzae*. The total number significantly up- and down- regulated genes in *M. oryzae* at 72 hours post treatment with EA105, CHAO, or CHA77. Genes were included only if they had an intensity value of at least 100, $p < 0.01$, and a fold change of at least 2. Three biological replicates were included for each treatment .

Bacterial strains	Up-regulated	Down-regulated
EA105	44	5819
CHAO	133	463
CHA77	124	80

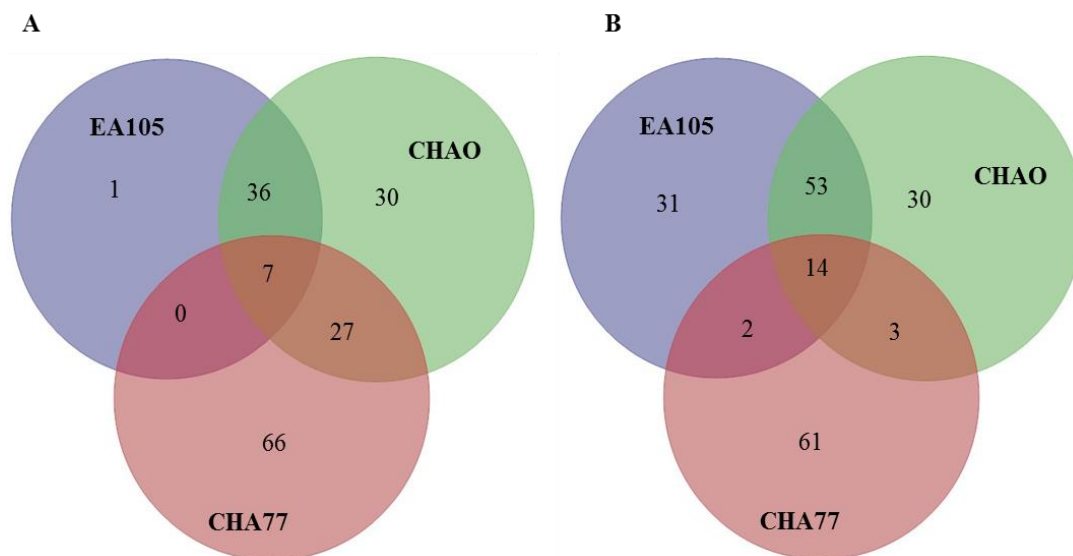


Figure 3.1. **Differentially regulated *M. oryzae* genes.** Venn diagrams of the top 100 genes that were **A)** up- or **B)** down-regulated with EA105, CHAO, or CHA77 treatment. Each treatment was done in biological triplicate, and differentially expressed genes had intensity values of at least 100, fold changes of at least 2, and a p value less than 0.01.

The vast majority of the most highly differentially expressed genes were of unknown function, based on GO term annotations (Figures 3.2 and 3.3). However, we examined several GO categories and generated heat maps to visualize the expression patterns seen with the different bacterial treatments (Figure 3.4). When looking at genes involved in mycelium development, there are several that show a similar expression pattern with EA105 or CHAO treatment, but little change with CHA77 treatment. There are about 6 of these genes that are down-regulated with EA105 treatment, and to a lesser extent CHAO treatment, but not with CHA77 treatment. Genes related to pathogenesis were largely unaffected by any of the bacterial treatments, except MGG_10315 which was significantly down-regulated after EA105 and CHAO treatment (Figure 3.4). Similarly, in the transporter category that included

both ABC and MFS transporters, only one gene, MGG_06794, showed significant gene expression change. This gene was significantly down-regulated by both EA105 and CHAO treatment, but not CHA77 treatment. Genes involved in detoxification of ROS were unchanged with all three treatments. As expected, genes involved in cyanide detoxification were up-regulated by cyanide producers EA105 and CHAO, but not induced by cyanide mutant CHA77. A major fungal defense response is the production of trichothecenes, compounds that are toxic to eukaryotic organisms (Bennett and Klich, 2003). Genes involved in trichothecene biosynthesis were largely unaffected by EA105 treatment, while the same genes were induced by CHAO and CHA77 treatment (Figure 3.4). A selection of 13 genes from multiple categories with varying expression patterns were examined using RT-PCR and expression patterns were similar to data obtained in the microarray (Figure 3.5).

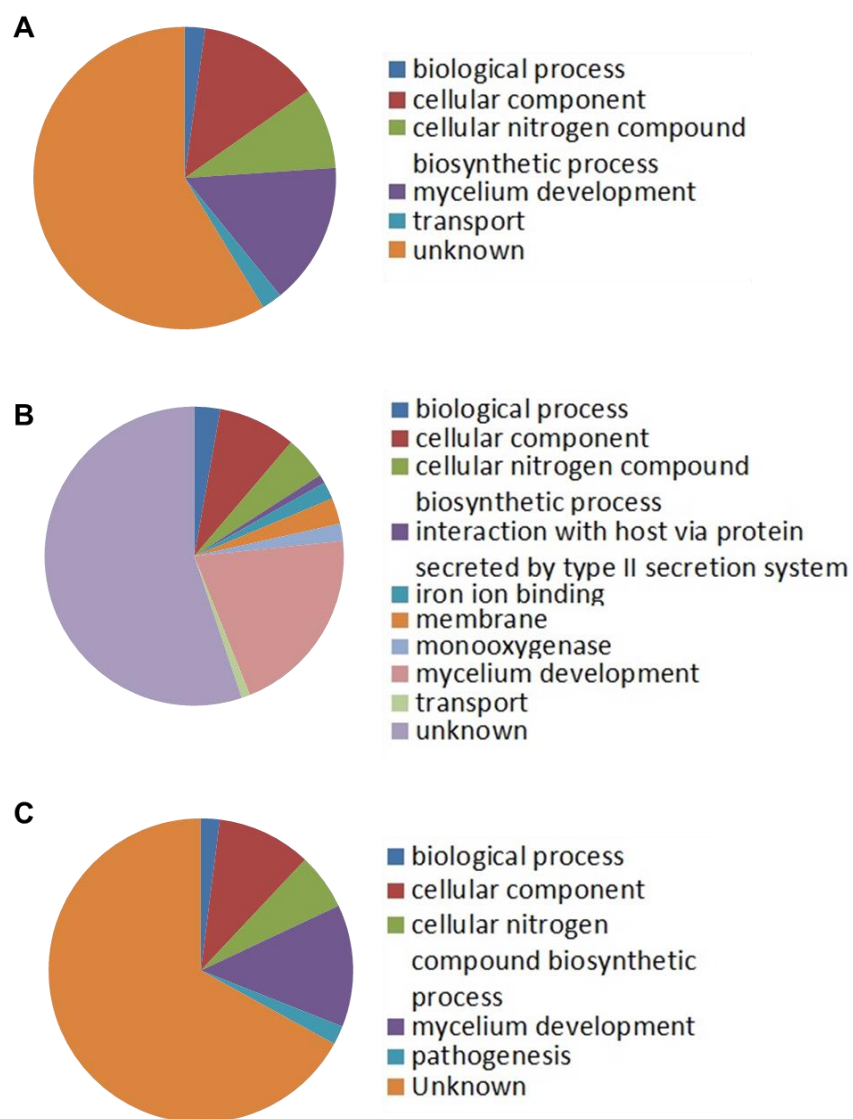


Figure 3.2. **Up-regulated *M. oryzae* genes.** Pie charts were constructed to visualize the distribution of the top 100 up-regulated *M. oryzae* genes with **A)** EA105, **B)** CHAO, and **C)** CHA77 treatments into GO term categories.

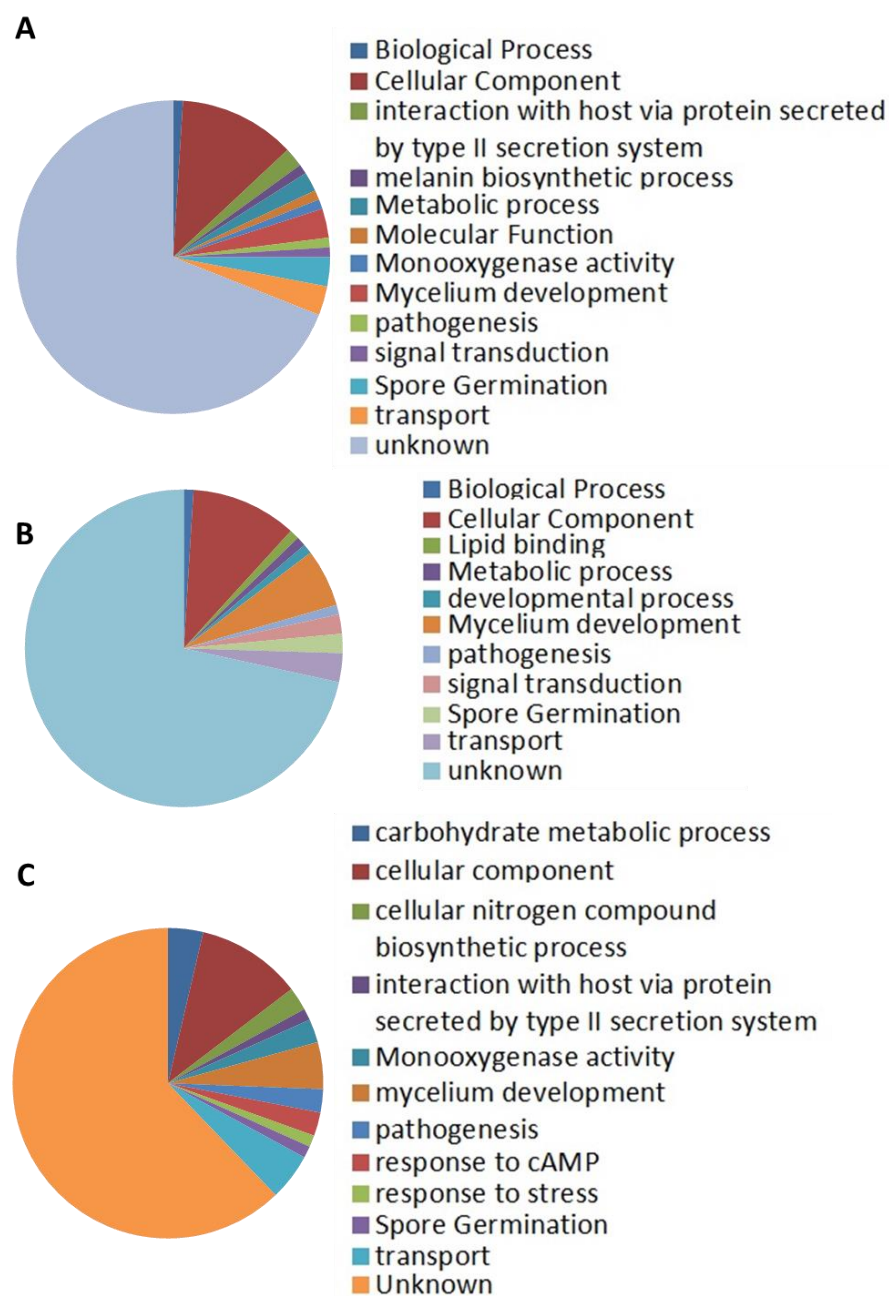


Figure 3.3. **Down-regulated *M. oryzae* genes.** Pie charts were constructed to visualize the distribution of the top 100 down-regulated *M. oryzae* genes with **A)** EA105, **B)** CHAO, and **C)** CHA77 treatments into GO term categories.

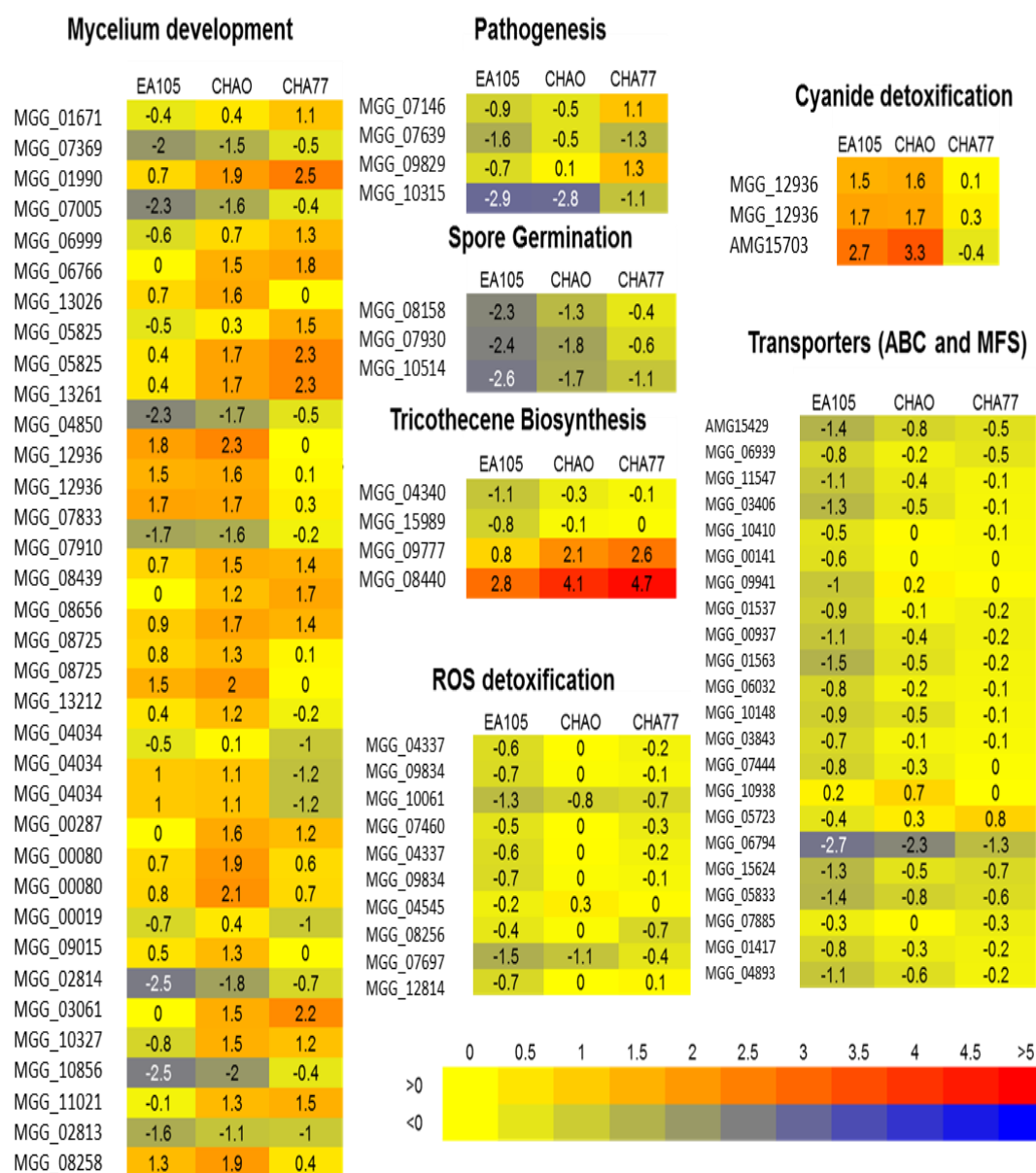


Figure 3.4. **Heat maps for functional categories of genes.** *M. oryzae* genes were categorized and heat maps were generated to visualize expression patterns. Numbers indicate $\log_2$ of fold changes for each treatment compared to untreated samples. Each treatment was done in biological triplicate, and differentially expressed genes had intensity values of at least 100, fold changes of at least 2, and a P value less than 0.01.

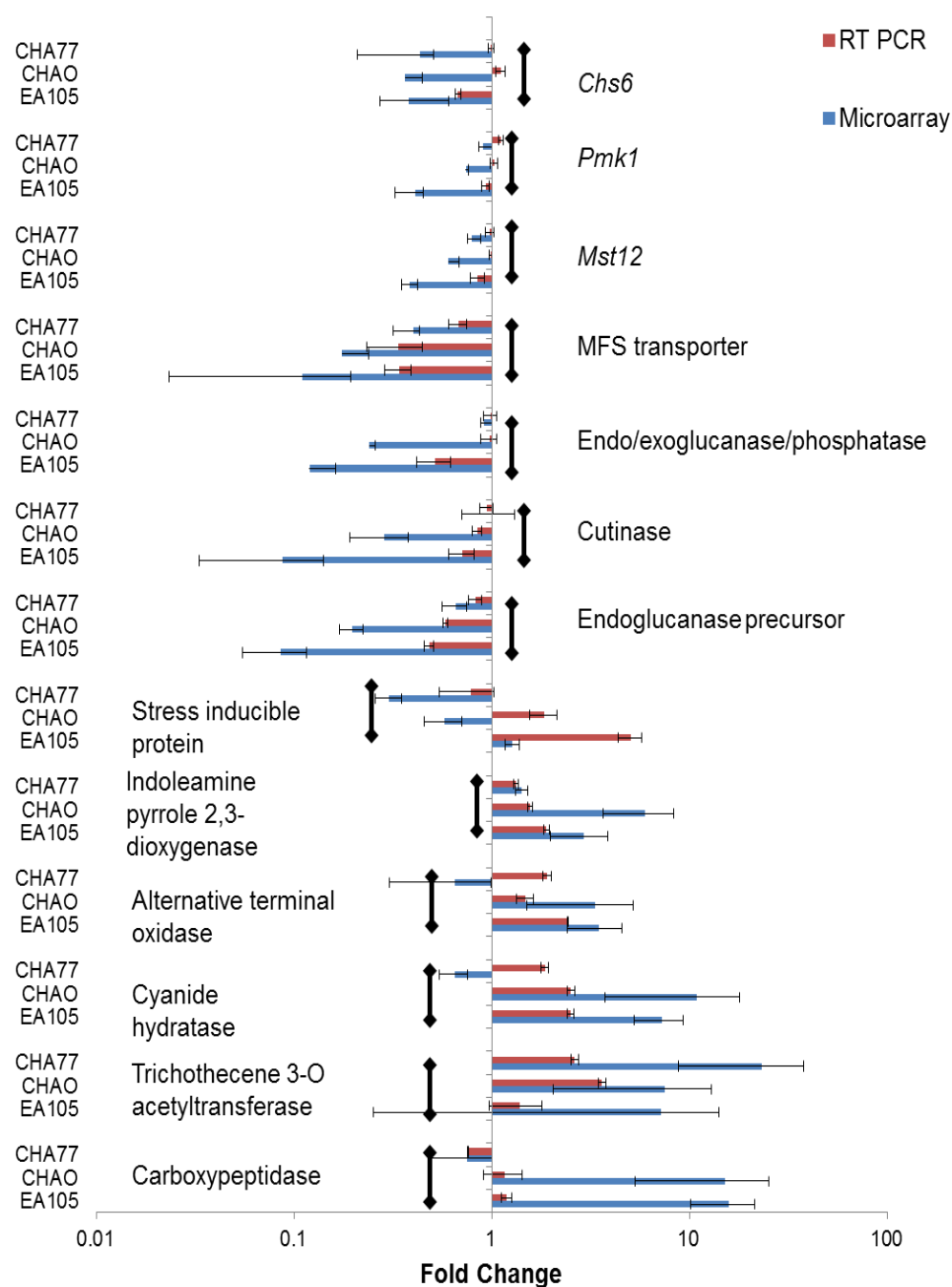


Figure 3.5. **RT-PCR validation.** RT-PCR was used to validate microarray data for 13 genes of various categories with various expression patterns. Error bars indicate standard error.

3.4.2 *In-vitro* characterization of *M. oryzae* deletion mutants

Based on the microarray data analysis, several genes of interest were chosen for further examination. Genes of interest included those which had a unique expression pattern with EA105 treatment compared to the other bacterial treatments, as well as genes with large expression changes with all three bacterial treatments. MGG_08440 was the only gene involved in trichothecene biosynthesis which was up-regulated by EA105 treatment. It was also the most highly up-regulated trichothecene biosynthesis gene with CHAO and CHA77 treatments. When EA105 treatment was compared to CHAO treatment, only one gene, MGG_03098, was significantly (2.2 fold) up-regulated. This gene codes for a stress inducible protein which is highly conserved across kingdoms. Compared to untreated *M. oryzae*, it is actually down-regulated with CHAO and CHA77 treatments and only very slightly up-regulated with EA105 treatment. A third gene of interest, MGG_17822, codes for a carboxypeptidase and is very highly up-regulated in 70-15 treated with EA105 and CHAO, but not CHA77 (Figure 3.6). For each of these genes, full deletion mutants were created in the 70-15 background. Mutants had vegetative growth rates equivalent to 70-15 (Figure 3.7), appeared normal when grown on CM plates, and retained the ability to germinate and form appressoria *in planta* (Figure 3.8). Some of the appressoria of 70-15ΔMGG_08440 appeared to be elongated, particularly on M-104 leaves (Figure 3.8) but the majority of appressoria appeared normal. Spore germination and appressoria formation on plastic hydrophobic coverslips was similar between the mutants and 70-15, with a slight reduction in spore germination with 70-15ΔMGG_08440 (Figure 3.9). Additionally, all three mutants retained the ability to form lesions in rice cultivars M-104, Seraceltik, and Nipponbare (Figure 3.10).

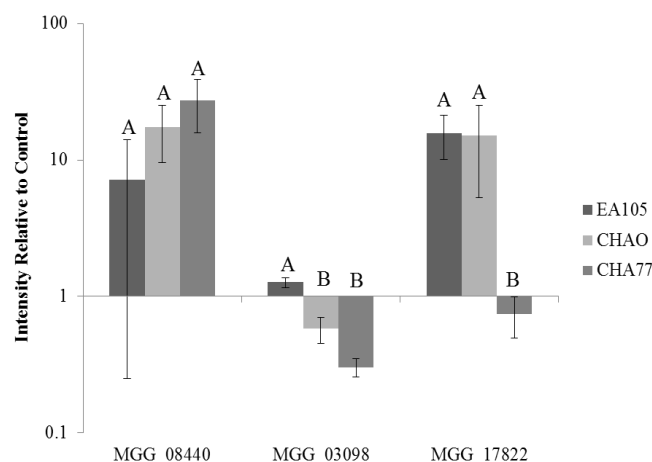


Figure 3.6. **Expression patterns of genes of interest.** Microarray fold change data for three genes of interest which were individually knocked out in *M. oryzae* 70-15. Error bars indicate standard error. A means comparison was done within each gene with Tukey's HSD test. Means with the same letter are not significantly different ($p < 0.05$).

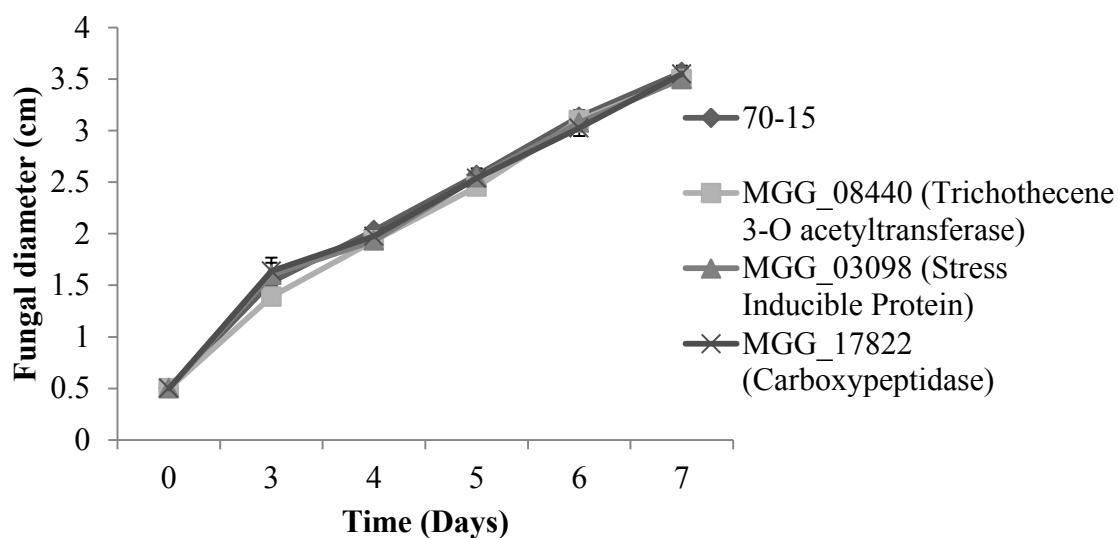


Figure 3.7. **Growth rate of mutants.** The growth rate of the mutants was compared to that of wild type 70-15 over the course of 7 days on CM plates. Error bars indicate standard error.

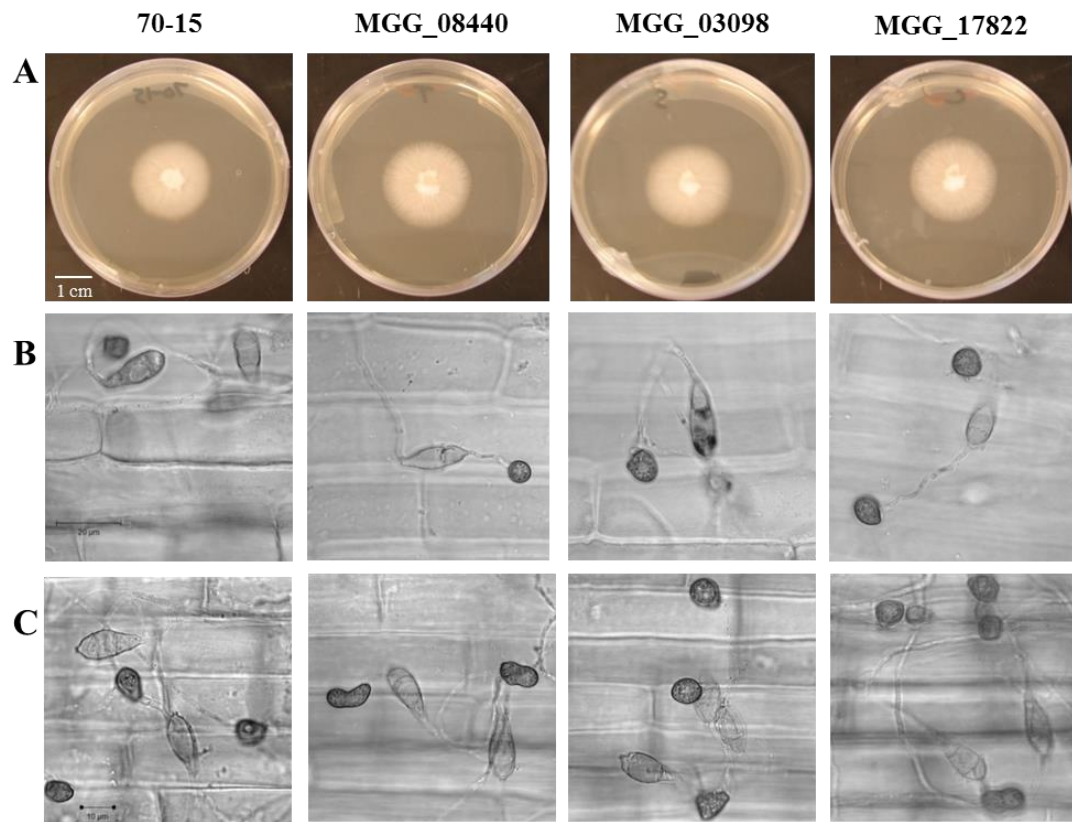


Figure 3.8. **Morphology of mutants.** Three knock-out mutants were created in *Magnaporthe oryzae* 70-15 background and growth and morphology was examined on CM plates **A**). Fungal strains were also tested *in planta* for their abilities to germinate and subsequently form normal appressoria on rice cultivars **B**) Seraceltik and **C**) M-104.

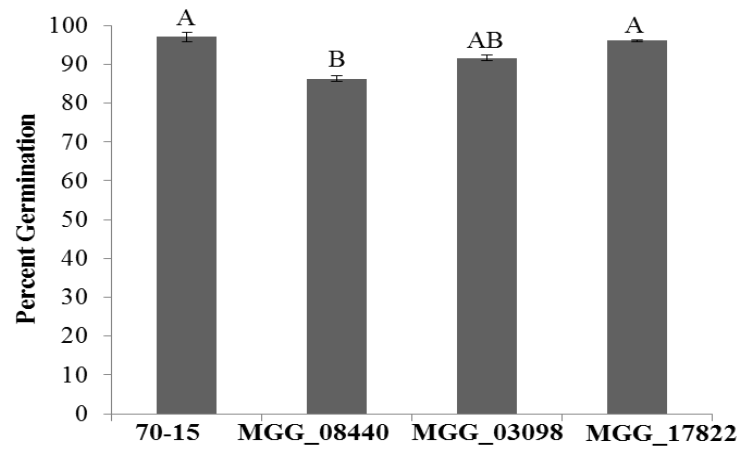
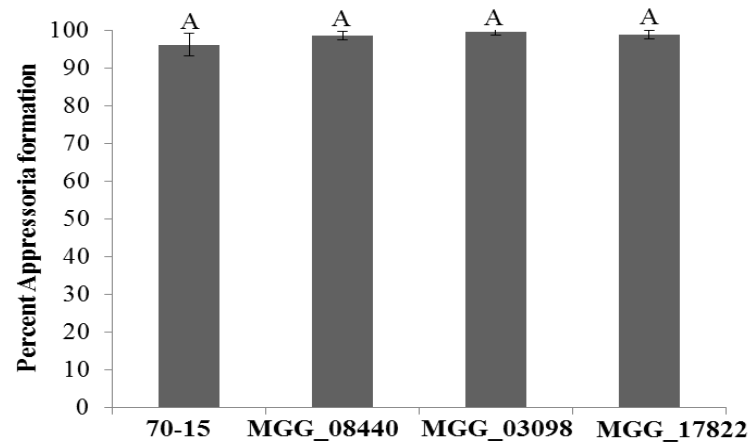
A**B**

Figure 3.9. **Mutant germination and appressoria formation.** **A)** The germination percentage and **B)** appressoria formation percentage of untreated *M. oryzae* 70-15 and mutants were tested. Error bars indicate standard error. A means comparison was done for all pairs with Tukey's HSD test. Means with the same letter are not significantly different ($p < 0.05$).

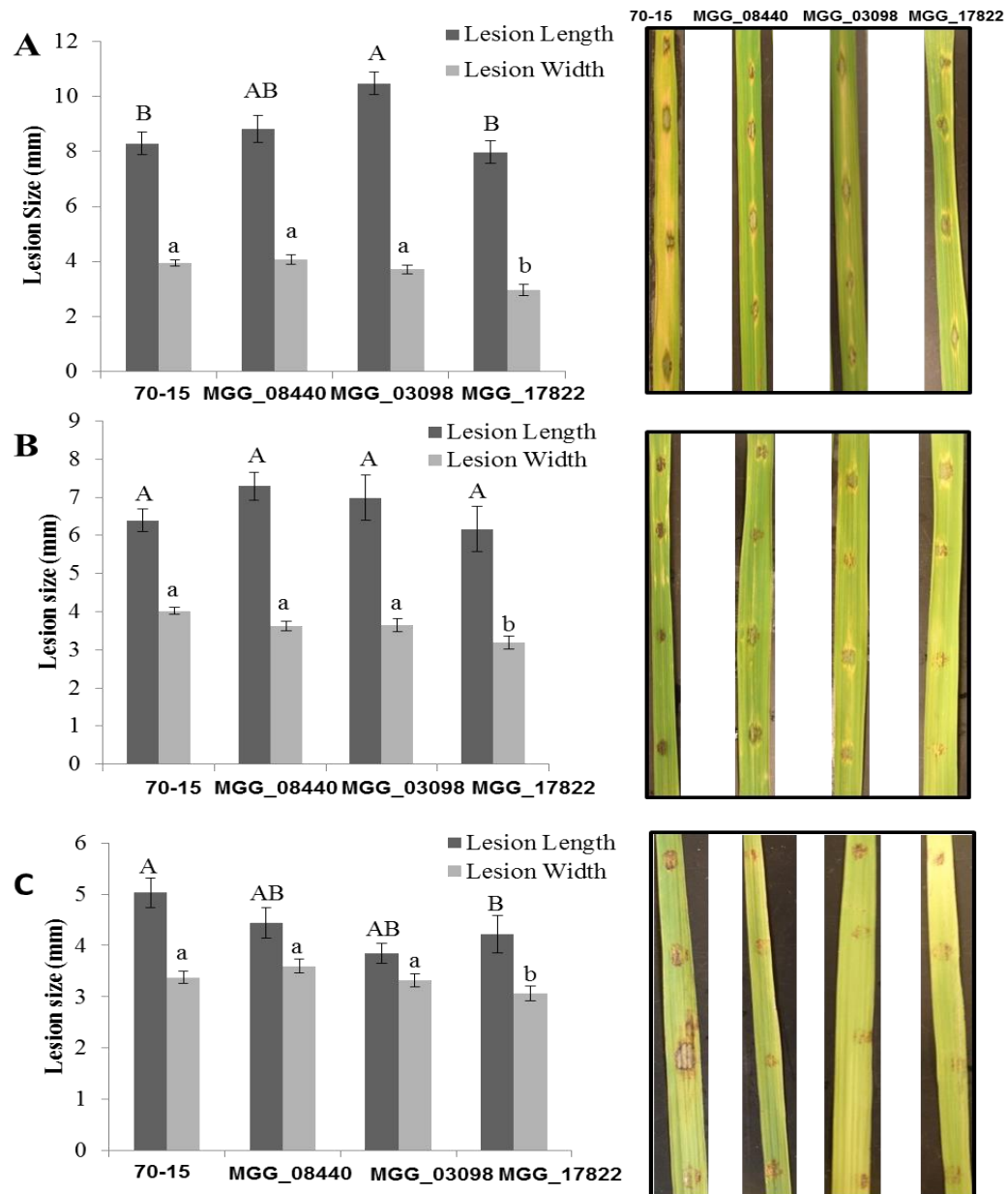


Figure 3.10. **Mutant lesions on rice.** *M. oryzae* 70-15 and the three mutants were tested for their ability to form lesions in **A)** Seraceltik, **B)** M-104, and **C)** Nipponbare. Error bars indicate standard error. A means comparison was done within lesion length and within lesion width for all pairs with Tukey's HSD test. Means with the same letter are not significantly different ($p < 0.05$). Twelve leaves were examined per treatment, in each of three biological replicates.

The mutants were tested for their response to bacterial treatments in an *in vitro* diffusible assay in which a fungal plug and bacterial droplet were co-inoculated on a CM plate, 4 cm apart. Susceptibility to EA105, naturally isolated from *O. sativa* cv. M-104 soil, D5, a cyanide non-producing mutant in the EA105 background, and CHAO, a known *P. fluorescens* biocontrol strain, were tested. At 7 days post co-inoculation, the degree of mutant inhibition in response to EA105, D5, and CHAO was similar to that seen in 70-15. However, 70-15ΔMGG_17822 was significantly less susceptible to D5 treatment compared to EA105 treatment. In all the other *M. oryzae* strains there was no significant difference in the inhibition by EA105 and D5 (Figure 3.11). A similar assay was set up to test the contribution of bacterial volatile compounds to the inhibition of *M. oryzae*. In this assay, compartmentalized plates were used so that the fungal plug and bacterial droplet could only share headspace within the dish, but could not exchange diffusible compounds through the media. In 70-15 and all three mutants, EA105 and D5 have equivalent inhibitory activities, which are stronger than that seen with CHAO (Figure 3.11). However, mutants 70-15ΔMGG_03098 and 7015Δ17822 were more susceptible to CHAO treatment than wild-type 70-15 (Figure 3.11).

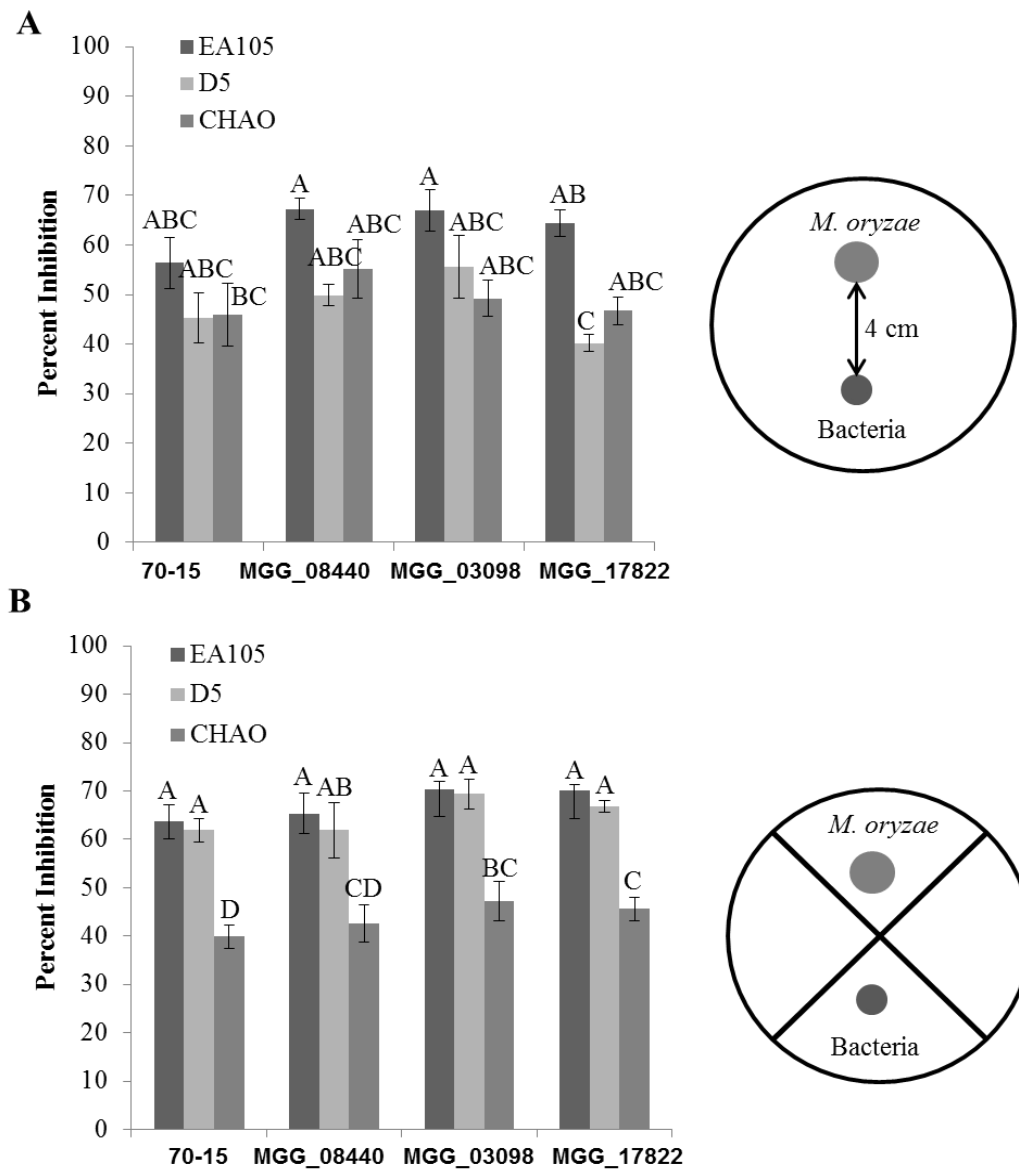


Figure 3.11. **Mutant susceptibility to bacterial treatment.** Susceptibility of fungal strains to EA105, D5, and CHAO was tested using a diffusible plate assay in which a 5 mm fungal plug was placed 4 cm from a droplet of bacteria on a solid plate (**A**), as well as a volatile component only assay in which quadrant plates were used to physically separate the media on which the bacterium and fungus were grown (**B**). Error bars indicate standard error. A means comparison was done for all pairs with Tukey's HSD test. Means with the same letter are not significantly different ($p < 0.05$).

The effect of bacterial treatment on the *M. oryzae* mutants' abilities to germinate and form appressoria was also examined. Bacterial treatment with EA105, D5, and CHAO had no effect on the germination percentage of 70-15 or 70-15ΔMGG_17822, however, EA105 was able to slightly reduce germination in 70-15ΔMGG_08440, while D5 marginally reduced germination in 70-15ΔMGG_03098 (Figure 3.12). Appressoria formation is almost completely halted in 70-15 treated with EA105 or D5. The *M. oryzae* deletion mutants also show a similar pattern, although the reduction in appressoria formation is not as drastic as the parental 70-15. With 70-15ΔMGG_03098, D5 was not able to restrict appressoria formation to the same extent as EA105, as was the case with all the other *M. oryzae* strains (Figure 3.12). CHAO also reduces appressoria formation in 70-15, and to a lesser extent in 70-15ΔMGG_17822. However, CHAO was unable to significantly reduce appressoria formation in mutants 70-15Δ08440 and 70-15Δ03098 (Figure 3.12).

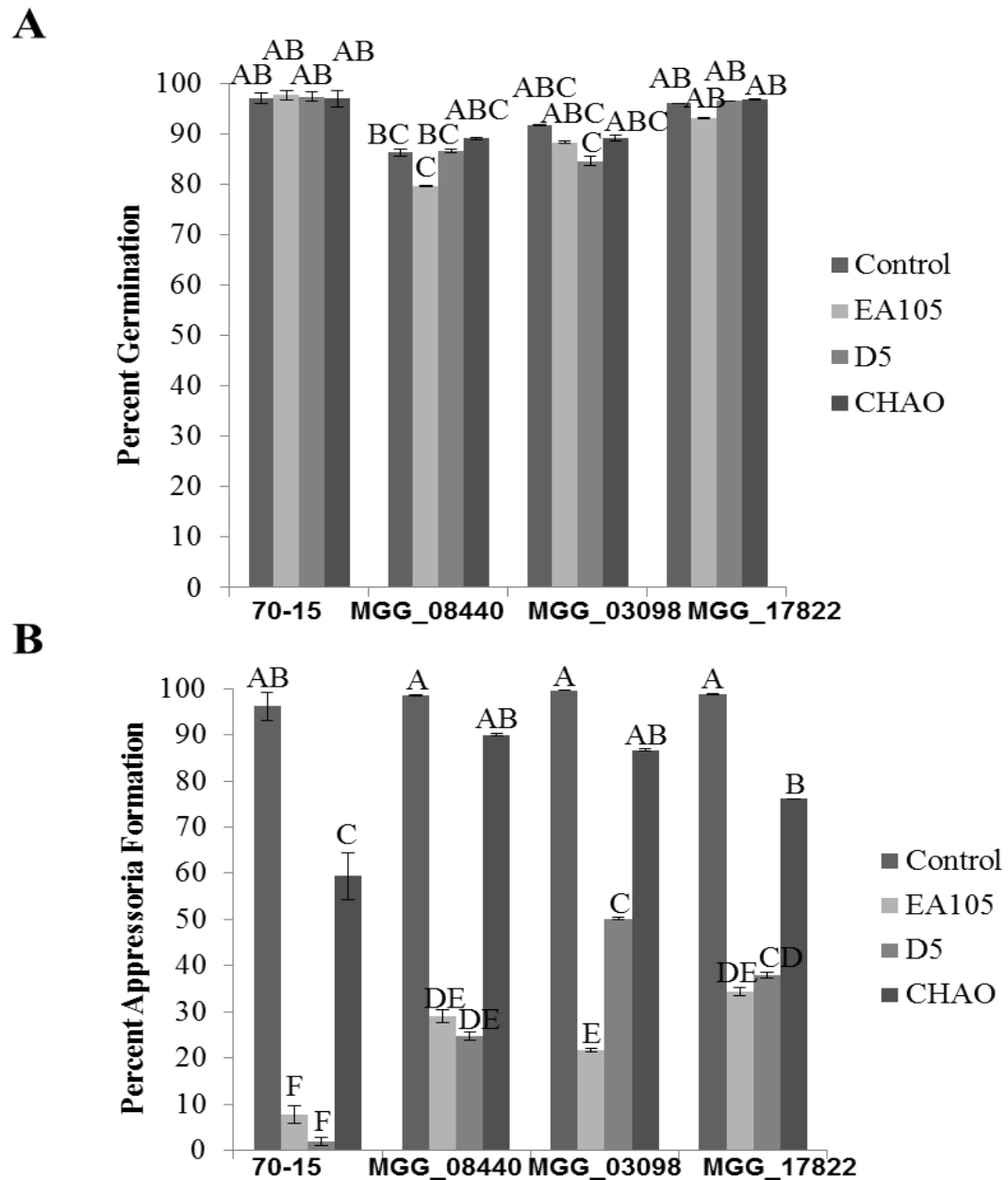


Figure 3.12. **Germination and appressoria formation of mutants with bacterial treatment.** Fungal strains were treated with different bacteria and **A)** percent germination and **B)** appressoria formation was quantified. Error bars indicate standard error. A means comparison was done for all pairs with Tukey's HSD test. Means with the same letter are not significantly different ($p < 0.05$).

Fluorescent pseudomonads such as EA105, D5, and CHAO produce multiple antimicrobial compounds that play a role in inhibiting phytopathogens in the soil. The production of cyanide is common amongst these biocontrol bacteria and has a broad range of action. At soil pH, cyanide is typically present as HCN, a gaseous form (Blumer and Haas, 2000). HCN interferes with cellular respiration by binding and inhibiting the activity of an enzyme of the electron transport chain, cytochrome C oxidase, interfering with the transfer of electrons to oxygen, and therefore preventing the cell from aerobically producing ATP (Blumer and Haas, 2000). When 70-15 and the mutants were treated in the diffusible and volatile compartment plate assays with cyanide at concentrations up to 500 μ M, fungal growth was not significantly inhibited (Figure 3.13). Another antibiotic compound frequently produced by biocontrol pseudomonads is 2,4-diacetylphloroglucinol (2,4-DAPG) which is a diffusible compound. When tested against the *M. oryzae* strains, concentrations of 25 μ M and above significantly inhibited vegetative growth (Figure 3.13). However, there were no differences in the degree of inhibition between wild type 70-15 and the mutant strains.

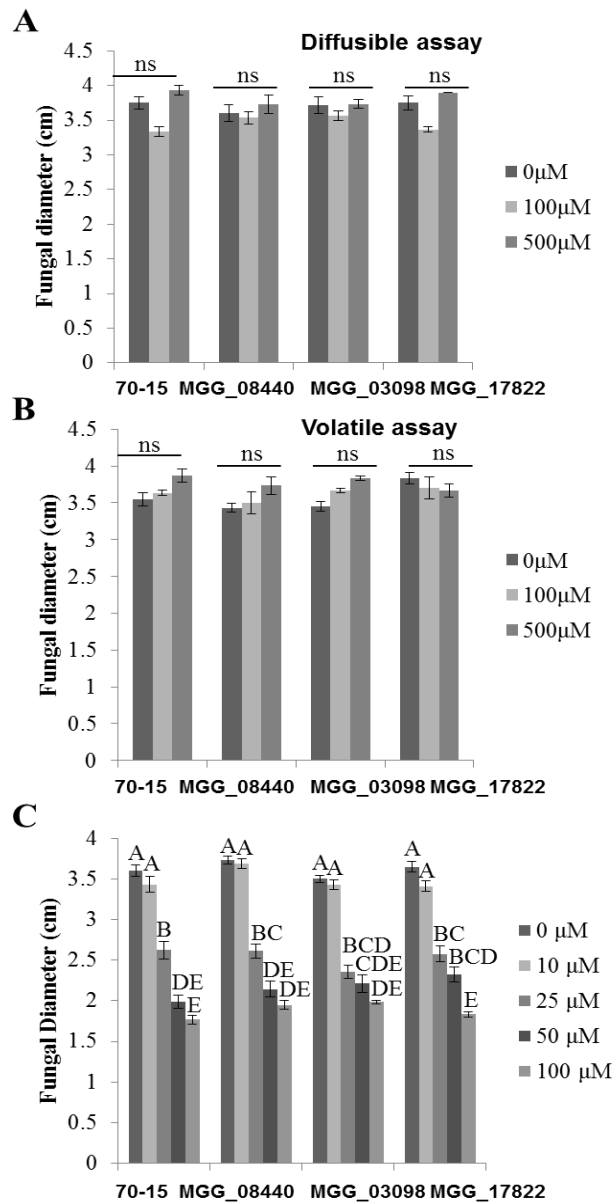


Figure 3.13. **Mutant response to cyanide.** The effect of cyanide on the *M. oryzae* strains was tested using a diffusible **A)** and volatile only **B)** assay. A Tukey's HSD test was done within each strain, ns = not significant. The effect of various concentrations of 2,4-DAPG was examined in the diffusible assay **C)**. Error bars indicate standard error. A means comparison was done for all pairs with Tukey's HSD test. Means with the same letter are not significantly different ($p < 0.05$).

3.4.3 Ability of rhizobacteria to indirectly protect rice against wild-type and mutant *M. oryzae* infection

The ability of the *M. oryzae* mutants to form lesions on rice leaves was examined in rice cultivars M-104, Seraceltik, and Nipponbare. All mutants were capable of forming lesions in these cultivars. However, 70-15 Δ MGG_17822 formed lesions that were slightly but significantly smaller in width than 70-15 lesions in cultivars M-104, Seraceltik, and Nipponbare (Figure 3.10). Previously we have shown that pre-treating rice roots with EA105 prior to *M. oryzae* infection reduces the number of lesions on rice plants (Spence *et al.*, 2014). Here, we show that the size of lesions is also reduced when plants were primed with EA105. When M-104 plants were primed with EA105, the size of lesions from the *M. oryzae* mutants was reduced, except for 70-15 Δ MGG_17822. For this mutant, priming with EA105 significantly reduced lesion width, but lesion length was not significantly reduced in cultivar M-104 (Figure 3.14). In Seraceltik, lesion length was modestly but significantly reduced, but there was no change in lesion width (Figure 3.15) while EA105 priming had no effect on the length or width of lesions that this mutant made on Nipponbare leaves (Figure 3.16). In all three rice cultivars there is a clear reduction in the ability of EA105 to reduce the size of lesions.

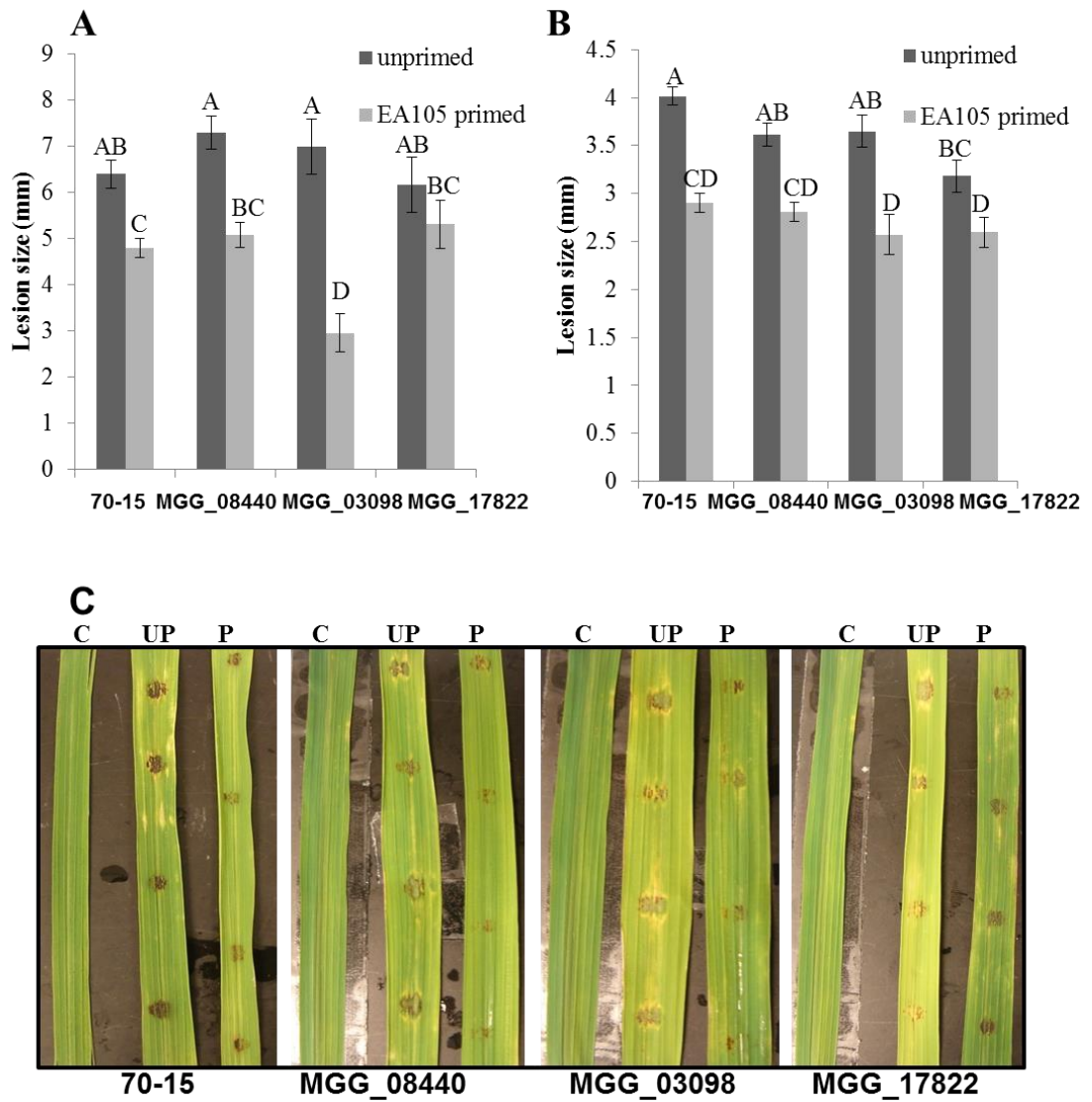


Figure 3.14. **Mutant lesions on primed M-104 rice plants.** Rice plants of cultivar M-104 were primed with EA105 and subsequently infected with the *M. oryzae* strains. **A)** Lesion length and **B)** lesion width were measured at 5 days post infection and **C)** images were taken post priming with EA105 and *M. oryzae* treatments. C=Control; UP=Unprimed; P=Primed. Error bars indicate standard error. A means comparison was done for all pairs with Tukey's HSD test. Means with the same letter are not significantly different ($p < 0.05$).

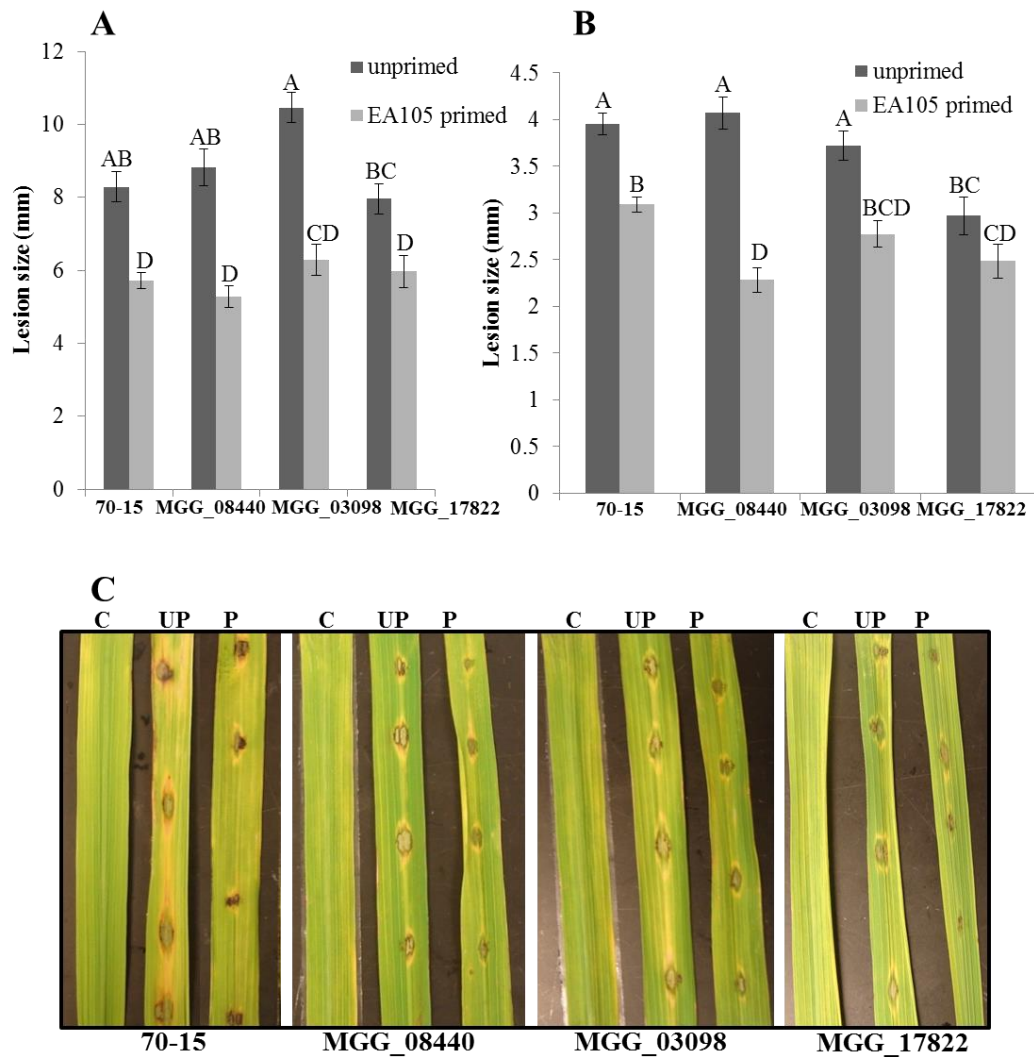


Figure 3.15. **Mutant lesions on primed Seraceltik rice plants.** Rice plants of cultivar Seraceltik were primed with EA105 and subsequently infected with the *M. oryzae* strains. **A)** Lesion length and **B)** lesion width were measured at 5 days post infection and **C)** images were taken. C=Control; UP=Unprimed; P=Primed. Error bars indicate standard error. A means comparison was done for all pairs with Tukey's HSD test. Means with the same letter are not significantly different ($p < 0.05$).

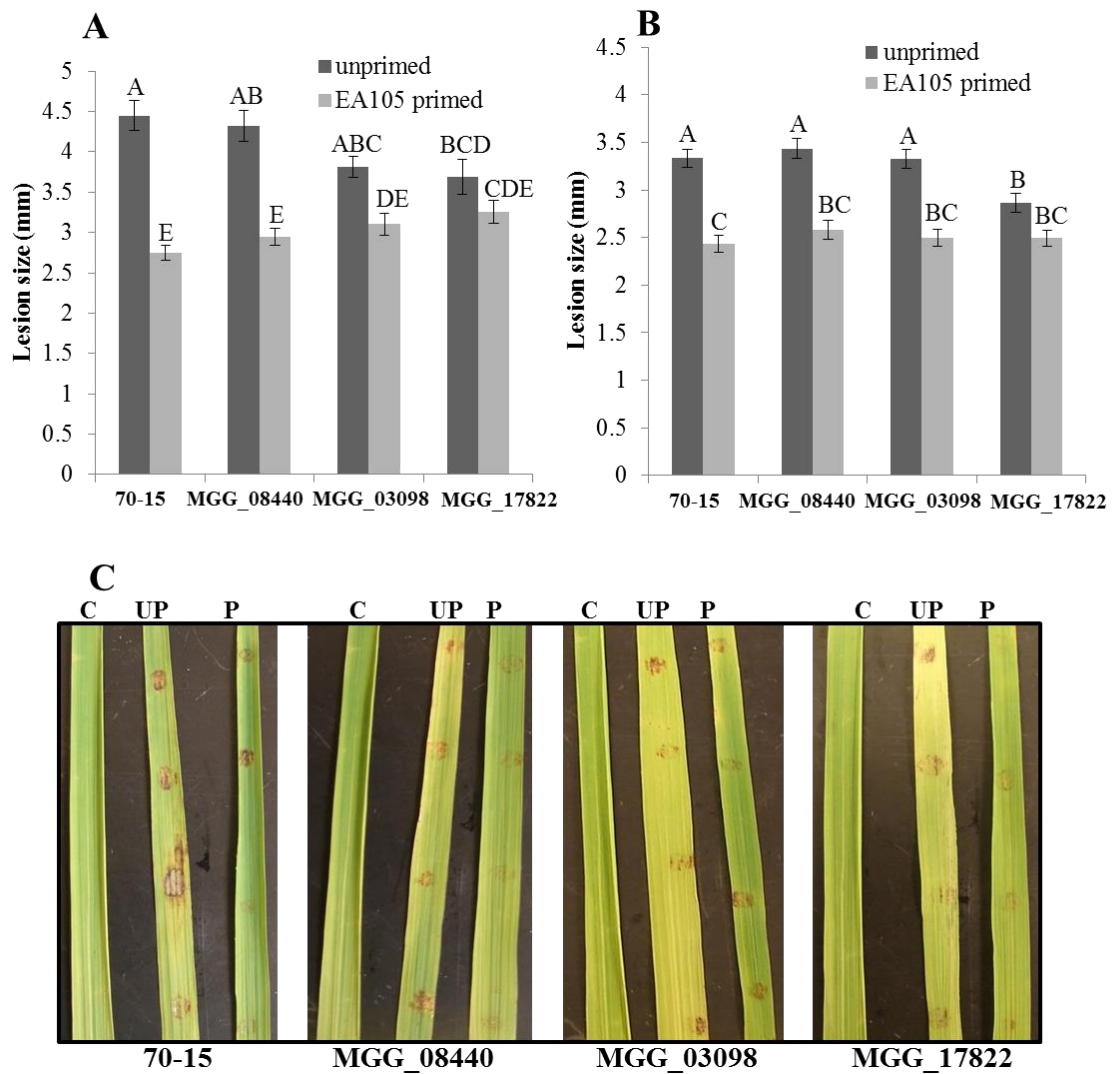


Figure 3.16. **Mutant lesions on primed Nipponbare rice plants.** Rice plants of cultivar Nipponbare were primed with EA105 and subsequently infected with the *M. oryzae* strains. **A)** Lesion length and **B)** lesion width were measured at 5 days post infection and **C)** images were taken. C=Control; UP=Unprimed; P=Primed. Error bars indicate standard error. A means comparison was done for all pairs with Tukey's HSD test. Means with the same letter are not significantly different ($p < 0.05$).

3.5 Discussion

Rice blast infections caused by *M. oryzae* ruin 10-30% of rice crops (Skamnioti and Gurr, 2009), which poses a threat to the food security of a staple crop consumed worldwide. This devastating plant pathogen has natural resistance to many fungicides, and can evolve resistance to newly encountered fungicides in as quickly as two growing seasons (Hamada *et al.*, 1967; Sakurai *et al.*, 1976; Takagaki *et al.*, 2004). Further, many of these fungicidal chemicals also pose a risk to the environment and human health (Nomoto and Mori, 1997; Yoon *et al.*, 2011) and may also reduce soil fertility by harming beneficial microbes (Aktar *et al.*, 2009). More recently, soil microbes are being evaluated as a more sustainable and effective method to control diseases in many plants, including rice, but none are currently being used which control *M. oryzae*. We have isolated a bacterium, EA105, from rice soil which shows direct *in vitro* inhibition of *M. oryzae* vegetative growth as well as an ability to interfere with the formation of appressoria, a structure that is critical during *M. oryzae*'s invasion of rice. When rice roots were pre-treated with EA105 prior to *M. oryzae* infection, the length and width of blast lesions were reduced. However, the mechanisms by which EA105 can directly antagonize *M. oryzae*, as well as reduce blast lesion size in rice, are unknown. We have used global transcriptional profiling to better understand *M. oryzae*'s response to EA105 treatment.

The most striking aspect of the transcriptional analysis is the down-regulation of nearly 6,000 *M. oryzae* genes following EA105 treatment. Likely, the decrease in expression of many of these genes is a secondary effect of the drastic growth inhibition that is seen with EA105 treatment. Although EA105, CHAO, and CHA77 all significantly restrict *M. oryzae* growth, only 14 of the 100 most highly down-regulated genes were common to all three treatments, which could be indicative either

of EA105's stronger antifungal activity that leads to down-regulation of a larger number of genes, or of a different mode of action for these bacteria against the fungal pathogen. Genes involved in routine cellular processes such as growth and metabolism were amongst those generally down-regulated. Few *M. oryzae* genes were significantly up-regulated following EA105 treatment, with only one being uniquely up-regulated by EA105. The putative function of this gene, MGG_04034, is a NAD dependent formate dehydrogenase. Typically these enzymes catalyze the oxidation of formate to bicarbonate, transferring electrons to NAD^+ , and they are crucial for metabolizing C1 compounds (Popov and Lamzin, 1994). The up-regulation of this gene with EA105 treatment but not the other two bacterial treatments suggests that EA105 may be producing a one-carbon compound that is not produced by the other two bacteria.

There was similarity in the expression pattern of many genes following EA105 and CHAO treatment, which were unchanged with CHA77 treatment. With CHA77 being a non-producer of cyanide in CHAO background, these expression changes are likely a response to cyanide. As expected, multiple cyanide hydratases were up-regulated with EA105 and CHAO but not CHA77 treatment. A subset of genes involved in mycelium development were down-regulated in this pattern, as well as one transporter gene, MGG_06794, and one pathogenesis-related gene, MGG_10315. Aside from these examples, there was largely no change in the expression of genes involved in pathogenesis, transport, or ROS detoxification, contrary to what was expected. ATP-Binding Cassette (ABC) transporters are transmembrane proteins responsible not only for transferring substrates, including toxins, out of the cell, but also can function in DNA repair (Higgins, 1992). Additionally, multi-drug resistance

is typically the result of over expression of a variety of ABC transporters which remove antibiotics from within the cell. However, there was no change in expression of ABC transporter genes with any of the bacterial treatments. Similarly, there was no change in expression of Major Facilitator Superfamily (MFS) transporters, secondary carriers that move small solutes across chemiosmotic gradients (Del Sorbo *et al.*, 2000). There was also no change in the expression of peroxidases, catalases, and superoxide dismutases associated with ROS detoxification, suggesting that none of the bacterial treatments are leading to ROS accumulation in *M. oryzae*.

An integral part of the defense response in fungi is the production of potent toxins called trichothecenes. Knowledge regarding trichothecene production in *M. oryzae* is limited; however the production of these compounds in other fungi such as *Fusarium graminearum* has been studied extensively (Desjardins *et al.*, 1993; Havrankova and Ovesna, 2012; Merhej *et al.*, 2011). Secretion of trichothecenes is linked to increased virulence in some phytopathogenic fungi (Desjardins *et al.*, 1996), and increased trichothecene biosynthesis occurs when fungi are challenged with antifungal compounds at sub-lethal levels (Kulik *et al.*, 2012). Interestingly, CHAO and CHA77 treatment increased transcripts of trichothecene biosynthesis gene MGG_09777 but EA105 did not. Similarly, AMG07075 was induced nearly twice as much with CHAO and CHA77 treatment as compared to EA105. CHAO and CHA77 may be producing an antifungal that is triggering higher up-regulation of these biosynthetic genes; however these two bacteria are not able to inhibit *M. oryzae* to the extent that EA105 does. EA105 may possess a mechanism for partially evading detection by *M. oryzae*, which prevents defense responses such as trichothecene biosynthesis.

To further examine the role of trichothecene biosynthetic gene MGG_08440, a knockout mutant was created in 70-15. This mutant showed a slight defect in germination, but was otherwise similar to 70-15 in morphology and growth. There were no differences in the mutant's susceptibility to EA105, D5 (a cyanide mutant in EA105), or CHAO treatment in regards to vegetative growth. However, EA105 was able to reduce spore germination in 70-15 Δ MGG_08440 but not in 70-15. Spore germination is a critical process that only occurs under favorable conditions (Osheroov and May, 2001). It is possible that in 70-15 Δ MGG_08440, may lack a gene in antibacterial defense, EA105 is at an advantage and is able to create an environment which is unfavorable for spore germination. Contrastingly, EA105 and D5 were less effective in reducing appressorium formation in this mutant as compared to 70-15, and CHAO also failed to inhibit appressorium formation in this mutant. The response of 70-15 Δ MGG_08440 to cyanide and 2,4-DAPG did not differ from 70-15, and are not likely to be the bacterially produced compounds that target induction of this gene in 70-15. There was no difference in this mutant's ability to form lesions on rice leaves, and EA105 priming reduced 70-15 Δ MGG_08440 lesion size to a similar extent as 70-15, indicating that this gene is unlikely to be involved in EA105's mechanism of inducing ISR. This finding is particularly interesting, as this gene of interest had been identified through a direct antagonistic interaction between the bacteria and fungus yet it may also be playing a role in a process where the bacterium indirectly reduces disease symptoms, mediated through the plant.

Another gene involved in *M. oryzae* defense response is MGG_03098, which codes for a stress inducible protein. Interestingly, this was the only gene that was significantly up-regulated with EA105 treatment compared to CHAO treatment,

suggesting that it is induced by a compound produced by EA105 but not CHAO. This gene is highly conserved in both prokaryotes and eukaryotes, and is involved in thiazole biosynthesis. Thiazole is commercially used as a fungicide, but is also necessary for the synthesis of thiamine. Thiamine accumulation in plants has been associated with an ISR-like defense response and disease reduction in soybean (Abdel-Monaim, 2011), pearl millet (Pushpalatha *et al.*, 2011), tobacco (Malamy *et al.*, 1996), *Arabidopsis thaliana* (Ahn *et al.*, 2005; Ahn *et al.*, 2007), and rice (Wang *et al.*, 2006). The *A. thaliana* ortholog, *THI1*, has been implicated in DNA repair (Machado *et al.*, 1996), but there are no reports of fungal homologs with this function. The *Fusarium oxysporum* homolog, *STI35*, is responsive to oxidative stress specifically, (Ruiz-Roldan *et al.*, 2008) yet there was no change in expression of ROS detoxification genes after EA105 treatment. To examine the importance of this gene in *M. oryzae*'s response to EA105, a knock-out mutant was created. This mutant, 70-15ΔMGG_03098, does not appear to have any phenotypic differences from 70-15 nor growth defects. It also does not differ in its susceptibility to EA105, D5, or CHAO treatment in either the diffusible or volatile vegetative growth assays. Additionally, 70-15ΔMGG_03098 germinates and forms appressoria similarly to 70-15. As with 70-15, bacterial treatment had no effect on spore germination, but CHAO was unable to inhibit appressoria formation in this mutant, and D5 was impaired in its ability to reduce appressoria formation, which may indicate a role of this gene as a target for bacteria to evade recognition by *M. oryzae*. When this gene is missing, perhaps bacteria are recognized sooner, and have less of a detrimental effect on appressoria formation. EA105 still reduces 70-15ΔMGG_03098 appressoria formation to almost the same extent seen with 70-15, perhaps due to an alternative strategy involving a

combination of cyanide and other bacterial components which are not present in CHAO. There were no differences in the susceptibility of this mutant to cyanide or 2,4-DAPG, again indicating that these are not likely to be the bacterial products targeting this gene. *In planta*, 70-15ΔMGG_03098 results in similar sized lesions as 70-15, but there is a more drastic reduction in lesion length following priming with EA105. Reduced lesion size has previously been correlated with reduced blast disease severity and reduced virulence (Cacique *et al.*, 2012; Mentlak *et al.*, 2012; Tredway *et al.*, 2003).

One of the most highly up-regulated *M. oryzae* genes following EA105 and CHAO treatment was MGG_17822, which encodes a putative carboxypeptidase. There have not been any reports of the function of this gene in *M. oryzae*. A BLASTn search revealed similarity to zinc carboxypeptidases, Type A digestive carboxypeptidases, and soluble (rather than anchored) carboxypeptidases. Additionally, it was similar to carboxypeptidases which must be cleaved to become active, and those which are involved in proteolysis. Due to the lack of information regarding this gene, it is difficult to speculate on its function in *M. oryzae* and further functional investigations are needed. One explanation is that it may be involved in the proteolysis of a bacterially-produced antifungal protein, resulting in its up-regulation with EA105 and CHAO treatment. However, this gene also appears to be responsive to cyanide, since it was highly up-regulated in the presence of EA105 and CHAO, but not with CHAO's cyanide deficient mutant, CHA77. A full deletion mutant for this gene, 70-15ΔMGG_17822 showed no phenotypic changes or growth defects. In addition, conidiospores germinated and formed appressoria normally. This mutant was significantly less susceptible to D5 treatment than EA105 in the diffusible assay,

but not the volatile assay, implicating a role for diffusible cyanide in the inhibition. However, there were no differences in this mutant's susceptibility to cyanide or 2,4-DAPG. Bacterial treatments did not affect 70-15ΔMGG_17822 germination, but led to a similar but smaller reduction in appressoria formation than in 70-15. *In planta*, 70-15ΔMGG_17822 lesions were not as wide as 70-15 lesions, and lesion length was not significantly decreased with EA105 treatment. Surprisingly, this gene which is targeted by EA105 in a direct interaction between bacteria and fungus, also appears to be playing a critical role in ISR since the lack of this gene in *M. oryzae* renders EA105 unable to reduce blast lesion length. Three rice cultivars were tested, with differing susceptibilities to rice blast. Even so, the trends seen with EA105 priming as well as the ability of the mutant *M. oryzae* strains to infect were similar across cultivars.

The antagonistic interaction between EA105 and *M. oryzae* is a complicated two-way communication, which becomes more complex when examining their indirect communications mediated through rice. However, the details of these complex communications are important for understanding the ways in which biocontrol bacteria effectively reduce disease. Using microbes to combat plant disease has many advantages over chemical fungicides including sustainability and reduced environmental impact, but most importantly this method promotes a lasting effectiveness due to the natural ability of microbes to co-evolve with their phytopathogen counterparts. Another important consideration in examining bacteria for biocontrol potential is their viability in the field. EA105 was isolated from rice soil, and is therefore more likely to thrive in rice field conditions than non-native bacteria. Thus far, investigations have mainly focused on the compounds secreted by biocontrol bacteria, but little is known about their transcriptional and functional effects

in the pathogen. Global transcriptional profiling provides insight into the pathogen's response to treatment, complementing studies which identify bacterially-produced antifungal compounds. The studies pertaining to understanding the systemic defense response in plants subjected to biocontrol bacteria will also give insights to transcriptional changes in plants associated with beneficial microbiome. Biocontrol of plant diseases is a promising alternative to traditional disease management strategies, but there is much to discover about the relationships and communications that occur between biocontrol bacteria, pathogens, and other residents of the plant microbiome.

3.6 Acknowledgements

Support from National Science Foundation Award PGPR-0923806; Dr. Venkatesan Sundaresan and his lab for the M-104 seeds and for the soil and root samples from which the isolates were obtained; Dr. Sandra Mathioni for her advice on microarray analysis.

3.7 References

- Abdel-Monaim MF** (2011) Role of riboflavin and thiamine in induced resistance against charcoal rot disease of soybean. *African Journal of Biotechnology* **10**: 10842-10855
- Adachi N, Tsukamoto S, Inoue Y, Azegami K** (2012) Control of bacterial seedling rot and seedling blight of rice by bacteriophage. *Plant Disease* **96**: 1033-1036
- Ahn IP, Kim S, Lee YH** (2005) Vitamin B-1 functions as an activator of plant disease resistance. *Plant Physiology* **138**: 1505-1515
- Ahn IP, Kim S, Lee YH, Suh SC** (2007) Vitamin B-1-induced priming is dependent on hydrogen peroxide and the *NPR1* gene in *Arabidopsis*. *Plant Physiology* **143**: 838-848
- Aktar W, Sengupta D, Chowdhury A** (2009) Impact of pesticides use in agriculture: their benefits and hazards. *Interdisciplinary toxicology* **2**: 1-12
- Audenaert K, Monbaliu S, Deschuyffeleer N, Maene P, Vekeman F, Haesaert G, De Saeger S, Eeckhout M** (2012) Neutralized electrolyzed water efficiently reduces *Fusarium* spp. in vitro and on wheat kernels but can trigger deoxynivalenol (DON) biosynthesis. *Food Control* **23**: 515-521
- Avila-Adame C, Koller W** (2003) Impact of alternative respiration and target-site mutations on responses of germinating conidia of *Magnaporthe grisea* to Qo-inhibiting fungicides. *Pest Management Science* **59**: 303-309
- Bennett JW, Klich M** (2003) Mycotoxins. *Clinical Microbiology Reviews* **16**: 497
- Blumer C, Haas D** (2000) Mechanism, regulation, and ecological role of bacterial cyanide biosynthesis. *Archives of Microbiology* **173**: 170-177
- Cacique IS, Domiciano GP, Rodrigues FA, do Vale FXR** (2012) Silicon and manganese on rice resistance to blast. *Bragantia* **71**: 239-244
- Chithrashree, Udayashankar AC, Nayaka SC, Reddy MS, Srinivas C** (2011) Plant growth-promoting rhizobacteria mediate induced systemic resistance in rice against bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae*. *Biological Control* **59**: 114-122

- De Vleesschauwer D, Djavaheeri M, Bakker P, Hofte M** (2008) *Pseudomonas fluorescens* WCS374r-induced systemic resistance in rice against *Magnaporthe oryzae* is based on pseudobactin-mediated priming for a salicylic acid-repressible multifaceted defense response. *Plant Physiology* **148**: 1996-2012
- De Vleesschauwer D, Hofte M** (2009) Rhizobacteria-induced systemic resistance. In LC VanLoon, ed, *Plant Innate Immunity*, Vol 51. Academic Press Ltd-Elsevier Science Ltd, London, pp 223-281
- Del Sorbo G, Schoonbeek HJ, De Waard MA** (2000) Fungal transporters involved in efflux of natural toxic compounds and fungicides. *Fungal Genetics and Biology* **30**: 1-15
- Desjardins AE, Hohn TM, McCormick SP** (1993) Tricothecene biosynthesis in *Fusarium* species – chemistry, genetics, and significance. *Microbiological Reviews* **57**: 595-604
- Desjardins AE, Proctor RH, Bai GH, McCormick SP, Shaner G, Buechley G, Hohn TM** (1996) Reduced virulence of tricothecene-nonproducing mutants of *Gibberella zeae* in wheat field tests. *Molecular Plant-Microbe Interactions* **9**: 775-781
- Dubuis C, Keel C, Haas D** (2007) Dialogues of root-colonizing biocontrol pseudomonads. *European Journal of Plant Pathology* **119**: 311-328
- Hamada M, Hashimoto T, Takahashi S, Yoneyama M, Miyake T, Takeuchi Y, Okami Y, Umezawa H** (1967) Antifungal activity of kasugamycin. *Journal of Antibiotics*. **20**: 424-426
- Havrankova H, Ovesna J** (2012) Genes of tricothecene biosynthesis in the *Fusarium* genus. *Chemicke Listy* **106**: 818-825
- Higgins CF** (1992) ABC transporters – from microorganisms to man. *Annual Review of Cell Biology* **8**: 67-113
- Jansen C, von Wettstein D, Schafer W, Kogel KH, Felk A, Maier FJ** (2005) Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted *Fusarium graminearum*. *Proceedings of the National Academy of Sciences of the United States of America* **102**: 16892-16897

- Jiang JH, Yun YZ, Fu J, Shim WB, Ma ZH** (2011) Involvement of a putative response regulator FgRrg-1 in osmotic stress response, fungicide resistance and virulence in *Fusarium graminearum*. *Molecular Plant Pathology* **12**: 425-436
- Jonkers W, Dong YH, Broz K, Kistler HC** (2012) The Wor1-like protein Fgp1 regulates pathogenicity, toxin synthesis and reproduction in the phytopathogenic fungus *Fusarium graminearum*. *Plos Pathogens* **8**
- Kankanala P, Czymmek K, Valent B** (2007) Roles for rice membrane dynamics and plasmodesmata during biotrophic invasion by the blast fungus. *Plant Cell* **19**: 706-724
- Krishnamurthy K, Gnanamanickam SS** (1998) Biological control of rice blast by *Pseudomonas fluorescens* strain Pf7-14: Evaluation of a marker gene and formulations. *Biological Control* **13**: 158-165
- Kulik T, Lojko M, Jestoi M, Perkowski J** (2012) Sublethal concentrations of azoles induce tri transcript levels and trichothecene production in *Fusarium graminearum*. *Fems Microbiology Letters* **335**: 58-67
- Laville J, Blumer C, Von Schroetter C, Gaia V, Defago G, Keel C, Haas D** (1998) Characterization of the hcnABC gene cluster encoding hydrogen cyanide synthase and anaerobic regulation by ANR in the strictly aerobic biocontrol agent *Pseudomonas fluorescens* CHAO. *Journal of Bacteriology* **180**: 3187-3196
- Lee YJ, Yamamoto K, Hamamoto H, Nakaune R, Hibi T** (2005) A novel ABC transporter gene ABC2 involved in multidrug susceptibility but not pathogenicity in rice blast fungus, *Magnaporthe grisea*. *Pesticide Biochemistry and Physiology* **81**: 13-23
- Leelasuphakul W, Sivanunsakul P, Phongpaichit S** (2006) Purification, characterization and synergistic activity of beta-1,3-glucanase and antibiotic extract from an antagonistic *Bacillus subtilis* NSRS 89-24 against rice blast and sheath blight. *Enzyme and Microbial Technology* **38**: 990-997
- Li QL, Jiang YH, Ning P, Zheng L, Huang JB, Li GQ, Jiang DH, Hsiang T** (2011) Suppression of *Magnaporthe oryzae* by culture filtrates of *Streptomyces globisporus* JK-1. *Biological Control* **58**: 139-148
- Liu HJ, Colavitti R, Rovira II, Finkel T** (2005) Redox-dependent transcriptional regulation. *Circulation Research* **97**: 967-974

- Machado CR, deOliveira RLC, Boiteux S, Praekelt UM, Meacock PA, Lenck CFM** (1996) Thi1, a thiamine biosynthetic gene in *Arabidopsis thaliana*, complements bacterial defects in DNA repair. *Plant Molecular Biology* **31**: 585-593
- Malamy J, SanchezCasas P, Hennig J, Guo AL, Klessig DF** (1996) Dissection of the salicylic acid signaling pathway in tobacco. *Molecular Plant-Microbe Interactions* **9**: 474-482
- Mathioni SM, Belo A, Rizzo CJ, Dean RA, Donofrio NM** (2011) Transcriptome profiling of the rice blast fungus during invasive plant infection and in vitro stresses. *Bmc Genomics* **12**
- Meng SW, Brown DE, Ebbole DJ, Torto-Alalibo T, Oh YY, Deng JX, Mitchell TK, Dean RA** (2009) Gene Ontology annotation of the rice blast fungus, *Magnaporthe oryzae*. *Bmc Microbiology* **9**
- Mentlak TA, Kombrink A, Shinya T, Ryder LS, Otomo I, Saitoh H, Terauchi R, Nishizawa Y, Shibuya N, Thomma B, Talbot NJ** (2012) Effector-mediated suppression of chitin-triggered immunity by *Magnaporthe oryzae* is necessary for rice blast disease. *Plant Cell* **24**: 322-335
- Merhej J, Richard-Forget F, Barreau C** (2011) Regulation of trichothecene biosynthesis in *Fusarium*: recent advances and new insights. *Applied Microbiology and Biotechnology* **91**: 519-528
- Mosquera-Espinosa AT, Bayman P, Prado GA, Gomez-Carabali A, Otero JT** (2013) The double life of *Ceratobasidium*: orchid mycorrhizal fungi and their potential for biocontrol of *Rhizoctonia solani* sheath blight of rice. *Mycologia* **105**: 141-150
- Mousivand M, Jouzani GS, Monazah M, Kowsari M** (2012) Characterization and antagonistic potential of some native biofilm-forming and surfactant-producing *Bacillus subtilis* strains against six pathotypes of *Rhizoctonia solani*. *Journal of Plant Pathology* **94**: 171-180
- Nomoto S, Mori K** (1997) Synthetic microbial chemistry .30. Synthesis of acetophthalidin, a fungal metabolite which inhibits the progression of the mammalian cell cycle. *Liebigs Annalen-Recueil*: 721-723
- Osheroov N, May GS** (2001) The molecular mechanisms of conidial germination. *Fems Microbiology Letters* **199**: 153-160

- Popov VO, Lamzin VS** (1994) NAD(+)-dependent formate dehydrogenase. *Biochemical Journal* **301**: 625-643
- Pushpalatha HG, Sudisha J, Geetha NP, Amruthesh KN, Shetty HS** (2011) Thiamine seed treatment enhances LOX expression, promotes growth and induces downy mildew disease resistance in pearl millet. *Biologia Plantarum* **55**: 522-527
- Reid RJD, Sunjevaric I, Kedacche M, Rothstein R** (2002) Efficient PCR-based gene disruption in *Saccharomyces* strains using intergenic primers. *Yeast* **19**: 319-328
- Roinestad KS, Montville TJ, Rosen JD** (1994) Mechanism for sodium-bicarbonate inhibition of trichothecene biosynthesis in *Fusarium-tricinatum*. *Journal of Agricultural and Food Chemistry* **42**: 2025-2028
- Ruiz-Roldan C, Puerto-Galan L, Roa J, Castro A, Di Pietro A, Roncero MIG, Hera C** (2008) The *Fusarium oxysporum* sti35 gene functions in thiamine biosynthesis and oxidative stress response. *Fungal Genetics and Biology* **45**: 6-16
- Sakurai H, Naito H, Fujita S** (1976) Sensitivity distribution of phytopathogenic bacteria and fungi to antibiotics. *Journal of Antibiotics* **29**: 1230-1236
- Shan HY, Zhao MM, Chen DX, Cheng JL, Li J, Feng ZZ, Ma ZY, An DR** (2013) Biocontrol of rice blast by the phenaminomethylacetic acid producer of *Bacillus methylotrophicus* strain BC79. *Crop Protection* **44**: 29-37
- Skamnioti P, Gurr SJ** (2009) Against the grain: safeguarding rice from rice blast disease. *Trends in Biotechnology* **27**: 141-150
- Spence C, Alff E, Johnson C, Ramos C, Donofrio N, Sundaresan V, Bais H.** (2014) Natural rice rhizospheric microbes suppress rice blast infections. *BMC Plant Biology* **14**:130 DOI: 10.1186/1471-2229-14-130
- Sweigard JA, Chumley FG, Valent B** (1992) Disruption of a *Magnaporthe-grisea* cutinase gene. *Molecular & General Genetics* **232**: 183-190
- Takagaki M, Kaku K, Watanabe S, Kawai K, Shimizu T, Sawada H, Kumakura K, Nagayama K** (2004) Mechanism of resistance to carpropamid in *Magnaporthe grisea*. *Pest Management Science* **60**: 921-926

- Tredway LP, Stevenson KL, Burpee LL** (2003) Components of resistance to *Magnaporthe grisea* in 'Coyote' and 'Coronado' tall fescue. *Plant Disease* **87**: 906-912
- Wang GN, Ding XH, Yuan M, Qiu DY, Li XH, Xu CG, Wang SP** (2006) Dual function of rice OsDR8 gene in disease resistance and thiamine accumulation. *Plant Molecular Biology* **60**: 437-449
- Yang F, Cao YJ** (2012) Biosynthesis of phloroglucinol compounds in microorganisms-review. *Applied Microbiology and Biotechnology* **93**: 487-495
- Yoon MY, Kim YS, Ryu SY, Choi GJ, Choi YH, Jang KS, Cha B, Han SS, Kim JC** (2011) In vitro and in vivo antifungal activities of decursin and decursinol angelate isolated from *Angelica gigas* against *Magnaporthe oryzae*, the causal agent of rice blast. *Pesticide Biochemistry and Physiology* **101**: 118-124
- Yukioka H, Inagaki S, Tanaka R, Katoh K, Miki N, Mizutani A, Masuko M** (1998) Transcriptional activation of the alternative oxidase gene of the fungus *Magnaporthe grisea* by a respiratory-inhibiting fungicide and hydrogen peroxide. *Biochimica Et Biophysica Acta-Gene Structure and Expression* **1442**: 161-169

ENDNOTES

³ Modified from **Spence CA, Raman V, Donofrio NM, Bais HP.** (2014)
Global gene expression in rice blast pathogen *Magnaporthe oryzae* treated with a
natural rice soil isolate. *Planta* **239**: 171-185

Chapter 4

UNDERGROUND SOLUTION TO AN ABOVE GROUND PROBLEM: RICE ISOLATE DISARMS ABA-MEDIATED VIRULENCE OF BLAST PATHOGEN *Magnaporthe oryzae* TO INDUCE RESISTANCE

4.1 Abstract

Rice suffers dramatic yield losses due to blast caused by *Magnaporthe oryzae*. *Pseudomonas chlororaphis* EA105 was isolated from the rhizospheric soil of field-grown rice and has previously shown substantial inhibition of *M. oryzae* growth. Pre-treatment of rice plants with EA105 reduced the size of blast lesions through JA- and ETH-mediated ISR. Abscisic acid (ABA) acts antagonistically towards SA, JA, and ETH signaling, impeding plant defense responses and increasing susceptibility to pathogens. EA105 may be reducing the virulence of *M. oryzae* by preventing the pathogen from up-regulating key ABA biosynthetic gene *NCED3* in rice roots, as well as a beta glucosidase likely involved in activating conjugated inactive forms of ABA. However, changes in total ABA concentrations were not apparent, provoking the question of whether ABA concentration is an indicator of ABA signaling and response. In the rice-*M. oryzae* interaction, ABA plays a dual role in disease severity by not only increasing plant susceptibility but also by potentially accelerating pathogenesis in the fungus itself. ABA was detected in *M. oryzae* spores and mycelia, confirming that the fungus can synthesize ABA. Further, exogenous application of ABA increased spore germination and appressoria formation, distinct from other plant growth regulators. EA105, which inhibits appressoria formation, counteracted the

virulence-promoting effects of ABA on *M. oryzae*. Therefore, it appears that EA105 is invoking multiple strategies in its protection of rice from blast including direct mechanisms as well as those mediated through plant signaling. ABA is a molecule which is likely implicated in both tactics.

4.2 Introduction

Rice (*Oryza sativa*) is a staple food crop world-wide, providing about one fifth of the calories consumed by humans. One of the largest problems impacting rice production is crop loss due to blast disease, caused by the hemibiotrophic fungal pathogen, *Magnaporthe oryzae*. Previously, we isolated and characterized a natural rice rhizospheric bacterium, EA105, which shows a strong direct antagonism toward *M. oryzae* vegetative growth and pathogenesis. When EA105 is root-inoculated on rice plants, it also triggers Induced Systemic Resistance (ISR) resulting in smaller blast lesions (Spence *et al.*, 2014a), facilitated through jasmonic acid (JA) and ethylene (ETH) but not salicylic acid (SA) signaling (Spence *et al.* 2014b). Although SA, JA, and ETH are the three hormones that are most closely linked to plant defense, another important component is the stress responsive hormone, abscisic acid (ABA).

ABA is a small signaling molecule involved in multiple plant processes including seed dormancy, development, and response to abiotic and biotic stresses. Due to its involvement in numerous and overlapping processes, the activity of ABA is complex and tightly regulated at multiple steps. ABA is detected by a receptor complex, PYR/PYL/RCAR. The family of small soluble receptors termed PYR/PYL were identified using a synthetic ABA agonist, pyrabactin, and were named Pyrabactin Resistance 1 (PYR) and PYR1-Like (PYL). Within this family, some of the proteins were identified concurrently and also given the name Regulatory Components of ABA

Receptors, or RCAR (Santiago *et al.*, 2012). In the absence of ABA, PYR/PYL/RCAR receptors are found dimerized in the cytosol and nucleus. The binding of ABA results in dissociation of the dimers, and the monomer which is bound to ABA undergoes a conformational change forming a binding site for Phosphatase type 2 Cs (PP2Cs) (Santiago *et al.*, 2012). The PP2Cs are inactivated when bound to the ABA-receptor complex. In the absence of ABA, PP2Cs inactivate SNF-1 Related Kinases (SnRK2s) which are positive regulators of ABA signaling (Szostkiewicz *et al.*, 2010). When SnRK2s are no longer inhibited by PP2Cs, they enter the nucleus and phosphorylate, thus activating, transcription factors which positively influence expression of stress/ABA-responsive genes (Kulik *et al.*, 2011). These genes contain sequences within their promoters called ABA Responsive Elements (ABREs) which the transcription factors, referred to more specifically as ABRE-binding proteins (AREBs) or ABRE binding factors (ABFs), recognize (Fujita *et al.*, 2013) (Figure 1.1).

The amount of ABA in a particular location within a plant tissue depends on biosynthesis, catabolism, transport, and compartmentalization (Ye *et al.*, 2012). In plants, ABA is synthesized through the cleavage of carotenoids in a multi-step pathway (Schwartz *et al.*, 1997; Schwartz *et al.*, 2003). The key regulatory enzyme in ABA biosynthesis is coded for by *NCED3* (Nine-cis-epoxycarotenoid dioxygenase 3) (Liotenberg *et al.*, 1999; Qin and Zeevaart, 1999). The first committed step in ABA catabolism is hydroxylation of the methyl group at carbon eight. ABA contains three methyl groups which can be hydroxylated, with carbon eight hydroxylation being most closely linked to catabolism. Hydroxylation does not inactivate ABA, but it can flag ABA for conversion into phaseic acid (PA) and subsequently dihydrophaseic acid

(Ye *et al.*, 2012). In rice, there are three enzymes with differing expression patterns which hydroxylate ABA, *OsABA8ox1*, 2, and 3 (Ye *et al.*, 2012) which are homologs of *Arabidopsis thaliana* *CYP707A* (Nambara and Marion-Poll, 2005). *OsABA8ox1* is primarily responsible for ABA catabolism following drought stress, and is negatively regulated by ethylene (Saika *et al.*, 2007). Additionally, ABA can be reversibly inactivated through conjugation, primarily to glucosyl esters forming ABA-GE which can be stored in vacuoles or apoplasts. The addition of glucosyl esters to ABA is catalyzed by an ABA glucosyl transferase (Xu *et al.*, 2002) and the conjugate can be subsequently removed through hydrolysis by a β -glucosidase such as *AtBGL* (Lee *et al.*, 2006). Glucosyl transferases and β -glucosidases involved in the inactivation and activation of ABA have not previously been characterized in rice.

While ABA is a crucial molecule for regulating plant growth, development, and stress response, some phytopathogens have evolved mechanisms to stimulate overproduction of ABA in plants, resulting in the suppression of Systemic Acquired Resistance (SAR), while also causing a reduction in growth, transpiration, and photosynthesis (Loake and Grant, 2007). SAR is typically mediated through SA signaling, and ABA acts antagonistically to SA signaling, blocking the SAR response (Jiang *et al.*, 2010; Xu *et al.*, 2013; Meguro and Sato, 2014). Elevated ABA levels in rice plants are associated with increased disease severity of rice blast caused by *M. oryzae* (Koga *et al.*, 2004; Jiang *et al.*, 2010; Yazawa *et al.*, 2012) as well as bacterial blight caused by *Xanthomonas oryzae* (Xu *et al.*, 2013). The reverse experiment highlighted the same point; knocking down ABA levels reduces susceptibility to blast by impairing the ability of *M. oryzae* to penetrate host cells, ultimately resulting in reduced disease symptoms (Yazawa *et al.*, 2012).

In addition to increasing plant susceptibility to disease, we hypothesize that high levels of ABA may also directly promote virulence in *M. oryzae*. ABA biosynthesis and signaling is likely to be an ancient process found in early unicellular eukaryotes which has followed divergent evolution (Hauser *et al.*, 2011). Several phytopathogens retain the ability to synthesize ABA, though the role of fungal-derived ABA is still unclear and there is evidence that most fungal-produced ABA is secreted (Hartung, 2010). The fungal ABA biosynthesis pathway is distinct from that of plants. The ABA biosynthesis gene cluster in fungi was first identified in *Botrytis cinerea* (Siewers *et al.*, 2006) and has been named the “direct” or “mevalonate” pathway in contrast to plants which cannot use mevalonic acid as a precursor to ABA biosynthesis (Hirai *et al.*, 2000). ABA perception and signaling mechanisms have also diverged, with differences arising even between monocots and dicots (Hauser *et al.*, 2011). ABA production has been documented in *M. oryzae* races 007.0 and 102.0 (Jiang *et al.*, 2010) though it has not previously been shown in the sequenced reference strain, 70-15. We have examined the role of ABA in a three way communication between rice, a fungal pathogen, and a natural rice beneficial isolate with the potential to provide a sustainable solution to blast, with the goal of further elucidating the mechanisms by which this bacterium can directly antagonize *M. oryzae* and trigger ISR in rice to protect against blast.

4.3 Materials and Methods

4.3.1 Fungal and bacterial strains and growth conditions

Wild type *M. oryzae* 70-15, the sequenced reference strain, was used throughout the experiments. For vegetative growth, the fungi were placed on

complete medium (CM) containing sucrose (10 g/L), casamino acids (6 g/L), yeast extract (6 g/L), and 1 mL of *Aspergillus nidulans* trace elements (Per 100 mL: 0.22 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.05 g KI, 0.02 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g H_3BO_4 , 0.1 mL concentrated H_2SO_4 , 0.008 g $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.007 g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$). Oatmeal agar consisting of ground oats (50 g/L) and agar (15 g/L) was used for sporulation. Plates were kept at 25°C with constant fluorescent light. Bacterial strains EA105, EA106, and EA201 were isolated from rhizospheric soil surrounding the roots of rice cultivar M-104 grown in the field by Dr. Venkatesan Sundaresan's lab from the University of California (Davis). The bacteria were cultured in liquid or solid Luria Bertani (LB) medium at 28-30°C.

4.3.2 Plant materials and growth conditions

Oryza sativa cultivar M-104 was donated by Dr. Venkatesan Sundaresan from the University of California (Davis). Rice seeds were dehusked and sterilized using ethanol and bleach. Ten day old seedlings were transferred to sterile clear plastic boxes containing 50 mL of Hoagland's medium, pH 5.7 and placed on a shaker at 150 rpm. Treatments were done at 14 days.

4.3.3 Rice treatments

Exponential phase bacterial cultures were washed in water and resuspended to approximately 1×10^9 cells per mL. In each clear box containing rice seedlings, 50 μL of bacteria were added to the media, for a final concentration of 1×10^6 cells per mL. For 70-15 treatment, spores were grown on oatmeal, then scraped into sterile water and filtered through a sterile cloth. Rice leaves were dipped in a spore suspension containing 0.2% gelatin and 1×10^5 spores per mL for 5 minutes. For

growth hormone treatments, each was added into the liquid media at the following concentrations: ABA (100 μ M), IAA (20 μ M), IBA (1 μ M), Kinetin (100 μ M), GA (50 μ M). Kinetin stock was prepared in 1N NaOH, while ABA, IAA, IBA, and GA stocks were prepared in methanol. All were filter sterilized. Controls were treated with an equal amount (1 μ l per mL) of methanol. Each treatment was done in biological triplicate, and each biological replicate contained five plants.

4.3.4 Spore germination and appressoria formation assays

Plastic coverslips were sterilized with ethanol and UV, and used as a hydrophobic surface to encourage spore germination and appressoria formation. *M. oryzae* spores were grown on oatmeal agar for ten days prior to being scraped into water and filtered through a cloth. Each coverslip was inoculated with a 50 μ L drop containing a final concentration of 10^5 spores/mL, and/or 10^5 bacterial cells/mL (also in water). The coverslips were placed in petri dishes with wet filter discs in the center to promote humidity. Plates were sealed and placed in the dark. Germination percentages were calculated after two hours incubation, and appressoria formation was determined after six hours. Coverslips were imaged using a Zeiss Axioscope2 light microscope. Three images were taken per coverslip, and five coverslips were used per treatment. Three biological replicates were examined.

4.3.5 ABA quantification

Plants and spores were treated as described above. Roots, shoots, and fungal tissue were ground in liquid nitrogen. For plant samples, approximately 100 mg of tissue was used. For fungal samples, approximately 250 mg of tissue was used. ABA was extracted from each sample in 1 mL of 90% methanol containing 10 mg of

butylated hydroxytoluene and 20 mL of glacial acetic acid per liter. The extraction was done at 4°C for 24 hours. The samples were then used with the Phytodetek Absciscic Acid ELISA-based kit, per the directions. Plant samples were run at the following dilutions (in TBS buffer): 1:10, 1:20, 1:40, 1:80, and 1:160. Fungal samples were run at the following dilutions: 1:10, 1:100, 1:200, and 1:400. ABA content was calculated per gram of tissue. Each biological replicate was five plants, and the experiment was done in biological triplicate.

4.3.6 Gene expression

Tissue was ground in liquid nitrogen and RNA was extracted using the EZ-10 Total RNA Mini-prep kit (BioBasic). Samples were treated with DNaseI (Thermo Scientific) and cDNA was synthesized from 500 ng of RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Standard Taq polymerase (New England Biolabs) was used for PCR, and products were run on a 1.4% agarose gel. Gene-specific primers are listed in Table 4.1. Band intensities were quantified using ImageJ, and normalized to ubiquitin expression. Each biological replicate was pooled from five plants, and each treatment was done in biological triplicate.

4.3.7 Statistical analysis

Statistical analyses of the results were performed using the statistical software JMP 10. To compare across treatments, the Tukey's HSD test was used and results were considered to be statistically different when $p < 0.05$.

Table 4.1. Primer sequences.

Gene Name	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')	Amplicon Size
<i>Ubiquitin</i>	ACCACTTCGACCGCC ACTACT	ACGCCTAAGCCTGCT GGTT	69
<i>OsPR1a</i>	TCGTATGCTATGCTA CGTGTTT	CACTAAGCAAATAC GGCTGACA	154
<i>OsWRKY77</i>	CTGTGTCCAGCTACC TCTCC	TGAAGAGAGCGATC ACCTC	780
<i>JAR1</i>	TCTCCCCAGCCTTAA CCGTA	CTAAACGCGACGAC AAACCC	287
<i>WRKY30</i>	ACTTCTTGAGTCGCC GGTTT	GCTTCTGGGATGCTC ACTGT	499
<i>EIL1</i>	ATCACCAGCGCCATA TCGTT	CACGGTTGTTTCAGCA TCAGC	256
<i>ERF1</i>	CATATCACCTTGACG CCCCA	ACCCTCACAAACTCA CTCGG	422
<i>NCED3</i>	CTTCGCCATCACCGA GAACT	CAGAGGTGGAAGCA GAAGCA	198
<i>SalT</i>	GCTTCTGCACGAGGA AATTTAAGC	GCCTAACAGCTTCTT GGGTG	121
<i>RCAR5</i>	TGGACGTGCCTGATG GAAAT	CGAGGTGGGTGACT GAACTG	122
<i>OsABA8ox1</i>	CGTCCCGGTTTCGAGG TG	ACCTGTACTTGGTTG CGAGG	135
<i>Beta glucosidase</i>	ATCCCCAACAGCACA TCCATC	GAGCCTCTGCCGAAC AACTT	351
<i>Glucosyl transferase</i>	GGGAAACCCAACCA GCGAAT	TAGCTGCCTGCGAGA TAAGG	184
<i>OsMPK1</i>	GCCCTTGCACATCCT TACCT	ATCACTGTCGGCGAC CTTTT	245
<i>Rab25</i>	CACGAGAAGAAGGG CATCGT	CATGCATGGACAGTT GCACG	122

4.4 Results

4.4.1 Expression of SA- and JA-responsive genes remains elevated in 70-15-infected plants pre-treated with EA105

Previously, we showed that when *P. chlororaphis* EA105 was inoculated onto uninfected rice plants, there was approximately a ten-fold increase in the ETH responsive genes *EIL1* and *ERF1* as well an approximately three-fold increase in the JA responsive genes *JAR1* and *WRKY30* at 24 hours post treatment (Spence *et al.* 2014b). However, the SA responsive genes *PR1* and *WRKY77* were minimally affected (Spence *et al.* 2014b). To examine rice signaling in the tritrophic interaction, plants were root-treated with EA105 and then leaf-infected with spores after 24 hours. Samples were collected after an additional 24 hours (a total of 48 hours after EA105 treatment). Gene expression was calculated in terms of fold change over untreated plants, and compared with plants which were treated with either bacteria or fungus alone. The same experiment was repeated with two other rice rhizosphere-isolated bacteria, *Pantoea agglomerans* EA106 and *Arthrobacter oxydans* EA201, the latter of which is not capable of inducing ISR. Spores alone were able to up-regulate the SA-responsive *WRKY77* but not *PR1*. In the tritrophic interaction with EA105, *WRKY77* remained elevated (Figure 4.1), in contrast to the tritrophic interaction where EA105 was substituted with EA106 or EA201 and *WRKY77* expression was not induced (Figure 4.2).

Spores and EA105 can independently induce the JA responsive genes *JAR1* and *WRKY30*, and these genes remain elevated in the tritrophic interaction (Figure 4.1). However, when EA105 is substituted with EA106 or EA201, the JA responsive genes are no longer up-regulated in plants treated with spores and bacteria together (Figure 4.2). Independently, EA106 can elevate these genes slightly, but EA201

cannot (Figure 4.2). There were no significant changes in ETH responsive genes, even with EA105 alone at 48 hours post treatment (Figure 4.2), in contrast to the 24 hour time-point, at which EA105 did up-regulate ETH responsive genes (Spence *et al.*, 2014b). The up-regulation in ETH and JA responsive genes with EA105 and EA106 treatment peaks around 24 hours before receding (Figure 4.3). Overall, EA105 affects the plant differently than EA106 or EA201, in a way that allows SA and JA signaling to remain elevated when the bacteria and spores are both present on rice.

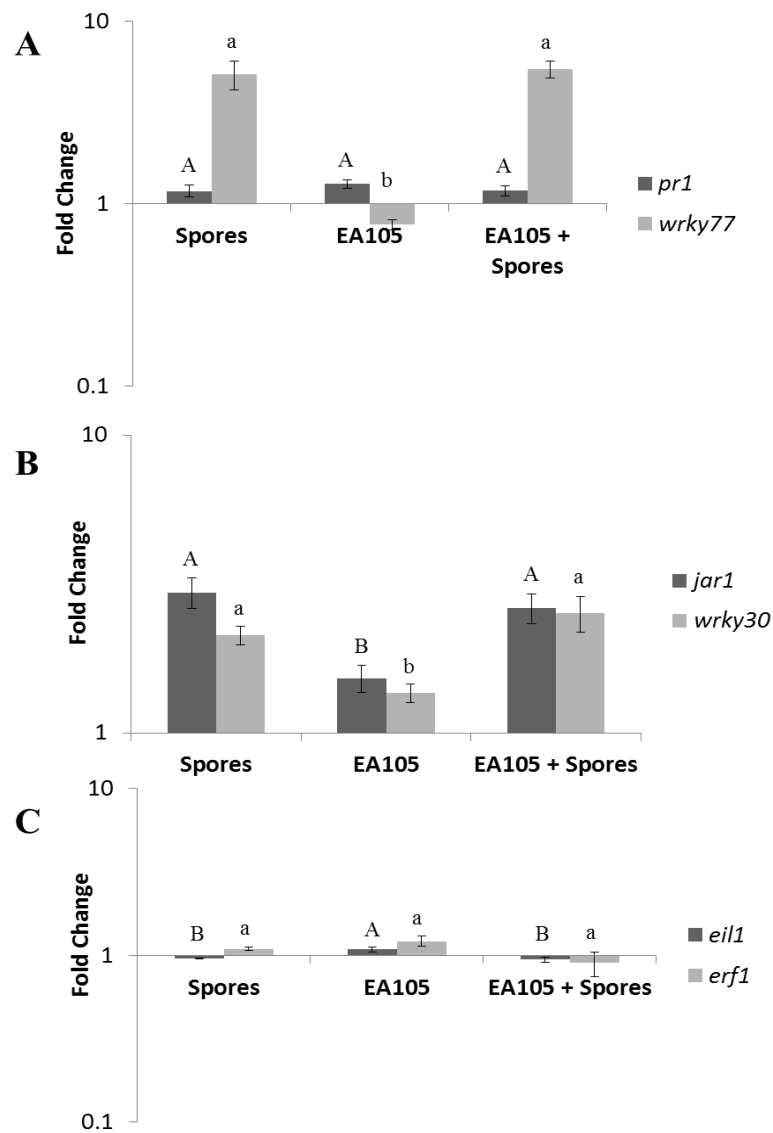


Figure 4.1. **Expression of SA, JA, and ETH responsive genes in rice plants treated with 70-15 spores, EA105, and both.** Rice plants were root inoculated with EA105 24 hours prior to dipping shoots into a solution of 70-15 spores. Shoot gene expression was examined 24 hours after spore treatment using primers for **A)** SA responsive genes *PR1* and *WRKY77*, **B)** JA responsive genes *JAR1* and *WRKY30*, and **C)** ETH responsive genes *EIL1* and *ERF1*. Error bars indicate standard error based on three biological replicates, each including five plants. Different letters represent a statistically significant difference based on the Tukey-Kramer test, $p < 0.05$.

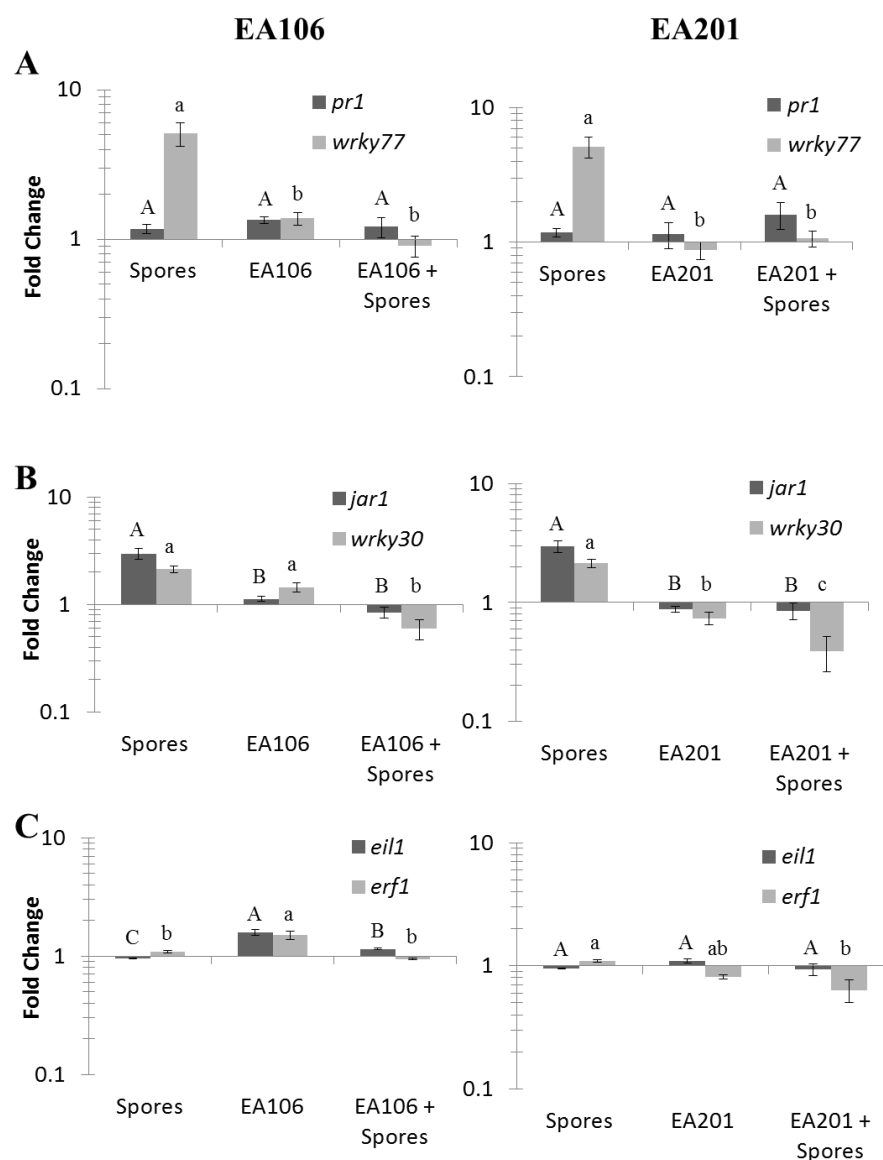


Figure 4.2. **Expression of SA, JA, and ETH responsive genes in rice plants treated with 70-15 spores, rhizospheric bacteria, and both.** Rice plants were root inoculated with either EA106 or EA201 24 hours prior to dipping the shoots into a solution of 70-15 spores. After 24 hours, shoot gene expression was examined using primers for **A)** SA responsive genes *PR1* and *WRKY77*, **B)** JA responsive genes *JAR1* and *WRKY30*, and **C)** ETH responsive genes *EIL1* and *ERF1*. Error bars indicate standard error based on three biological replicates, each including five plants. Different letters represent a statistically significant difference based on the Tukey-Kramer test when $p < 0.05$.

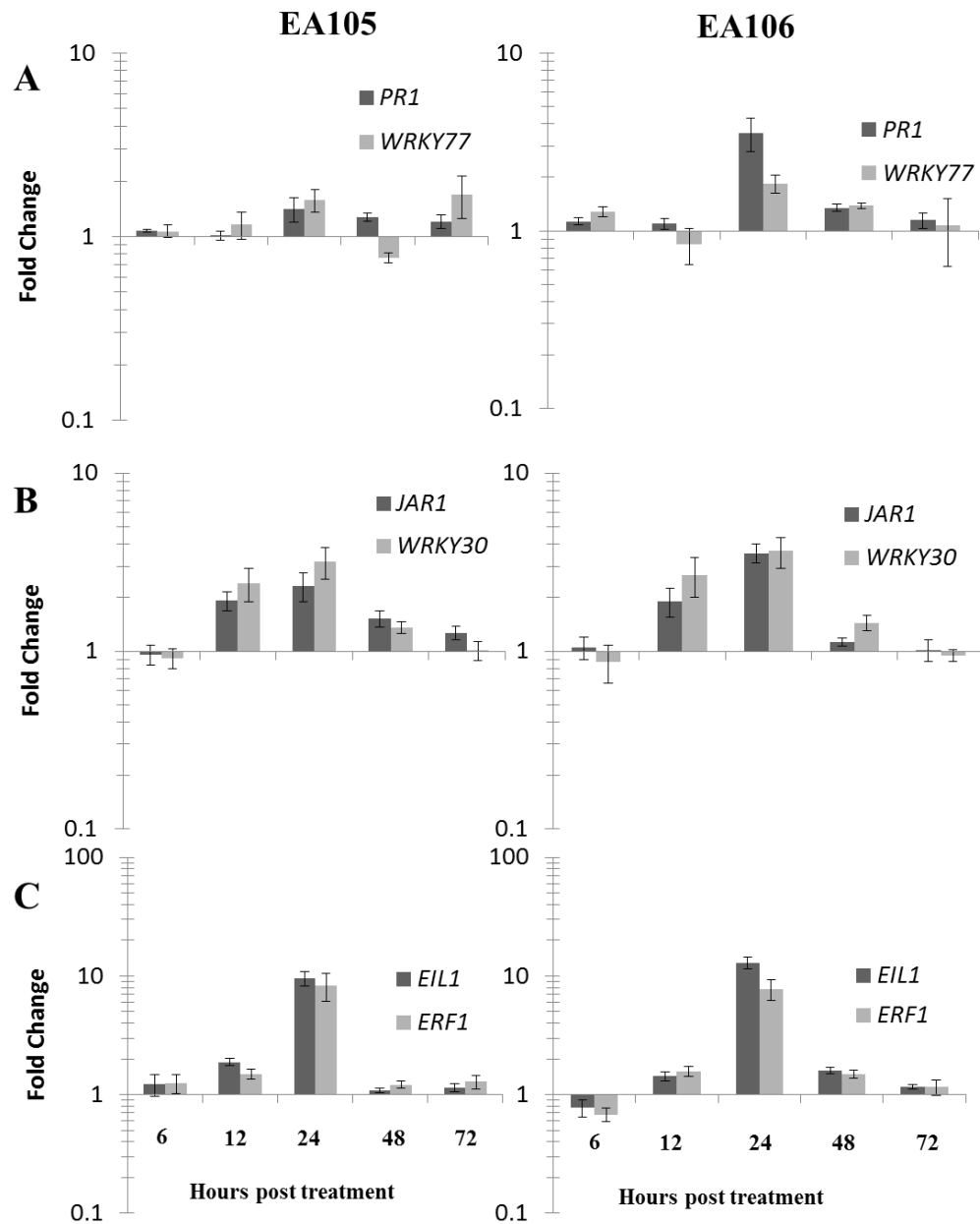


Figure 4.3. **Gene expression in rice shoots following bacterial treatment.** Rice plants were root-treated with EA105 (left) or EA106 (right) and shoot tissue was examined for the expression of genes involved in **A)** SA, **B)** JA, and **C)** ETH signaling at 6, 12, 24, 48, and 72 hours following bacterial treatment. Error bars indicate standard error based on three biological replicates, each including five plants.

4.4.2 ABA is antagonistic to SA, JA, and ETH signaling

Jiang *et al.* showed that ABA reduced SA signaling in comparison to untreated rice plants as well as plants treated with the SAR-inducing compound, benzathiodiazole (BTH) (Jiang *et al.*, 2010). Although we did not see antagonism of SA in un-elicited plants, we did find that ABA down-regulated the expression of the JA responsive genes *JAR1* and *WRKY30*. In plants treated with both ABA and EA105, *JAR1* is down-regulated to a similar degree as with ABA treatment alone. However, *WRKY30* is up-regulated almost to the level of EA105 treatment alone (Figure 4.4). ABA did not affect ETH responsive genes in un-elicited plants, but when plants were treated with ABA and EA105 together, there was a reduction in the amount of up-regulation of *EIL1* and *ERF1* that was seen with EA105 treatment alone (Figure 4.4). Therefore, ABA has shown antagonism toward SA, JA, and ETH signaling pathways, all three of which are important to plant defenses.

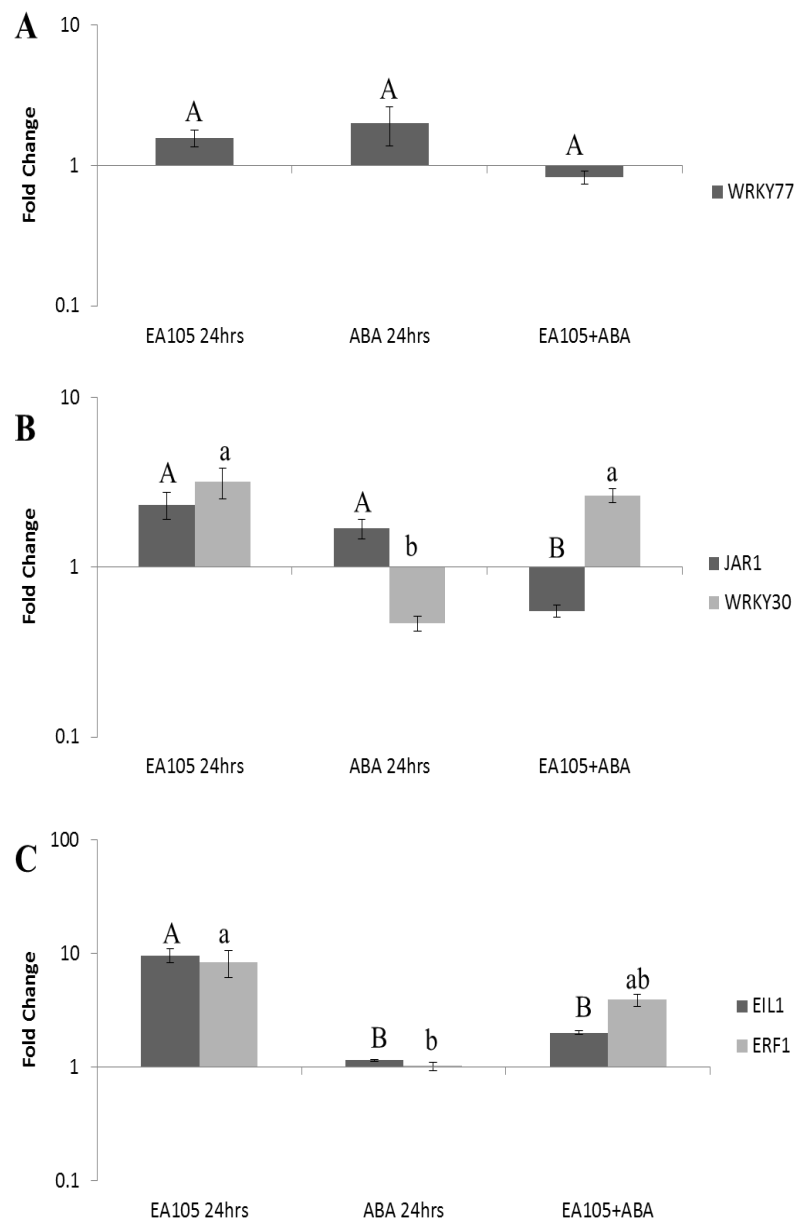


Figure 4.4. **ABA is antagonistic to JA signaling.** Plants were treated with EA105, 100 μ M ABA, or both. Shoot expression of **A)** SA-, **B)** JA-, and **C)** ETH-responsive genes was examined. Error bars indicate standard error based on three biological replicates, each including five plants. Different letters represent a statistically significant difference based on the Tukey-Kramer test, $p < 0.05$.

4.4.3 EA105 may prevent 70-15 spores from increasing ABA biosynthesis and signaling in rice

Since elevated ABA levels are associated with increased susceptibility, we examined the expression of *NCED3*, the rate limiting enzyme involved in ABA biosynthesis, in roots. 70-15 spores up-regulated *NCED3* while EA105 did not affect its expression. Interestingly, 70-15 spores were unable to induce *NCED3* expression in plants which were pre-treated with EA105 (Figure 4.5). Pretreatment with rice isolates EA106 and EA201 did not prevent 70-15 from up-regulating *NCED3* (Figure 4.5). To see if *NCED3* up-regulation coincided with higher ABA levels, total ABA concentrations in roots and shoots treated with bacteria, fungus, or both were examined. However, there were no significant differences in the ABA content (Figure 4.6). In all treatments, there was approximately 2000-2500 picomoles of ABA per gram of plant tissue. ABA content was checked at the same time-point used for expression analysis as well as 24 hours later, and still no differences were apparent (Figure 4.6). At the second time-point, there was actually a slight increase in ABA levels in plants which were treated with both EA105 and spores (Figure 4.6). ABA concentrations were also determined in 70-15 spores and mycelia. We found that ABA is produced by 70-15, with mycelia producing around 200 picomoles per gram and spores producing more than 400 picomoles per gram (Figure 4.6).

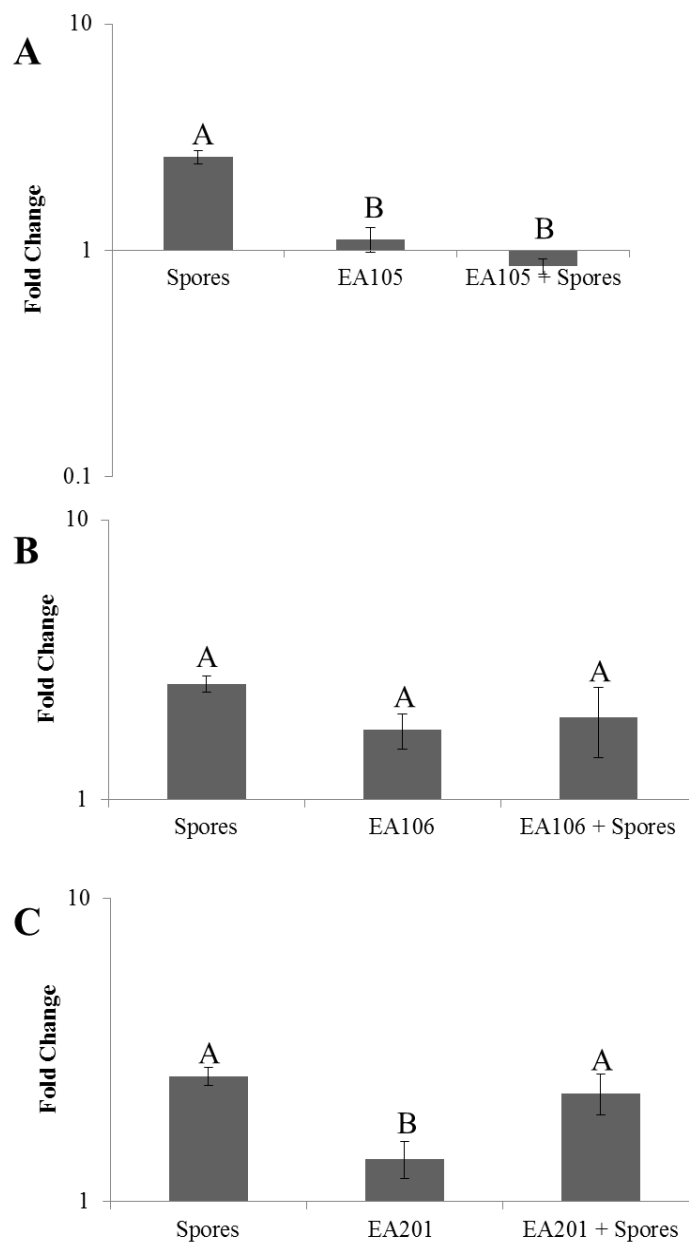


Figure 4.5. **Expression of ABA biosynthesis gene, *NCED3*.** Root samples were collected from plants which were treated with bacteria and/or spores. Roots were inoculated with either **A)** EA105, **B)** EA106, or **C)** EA201, 24 hours prior to being exposed to spores. Error bars indicate standard error based on three biological replicates, each including five plants. Different letters represent a statistically significant difference based on the Tukey-Kramer test, $p < 0.05$.

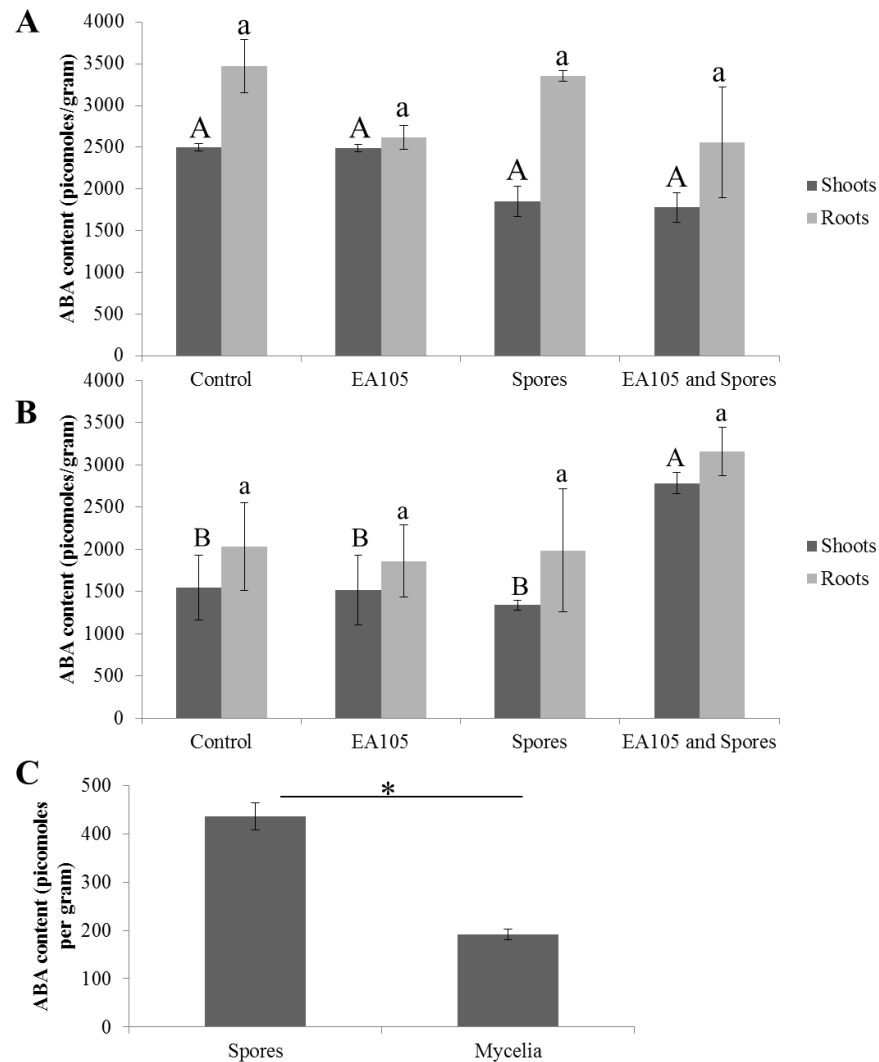


Figure 4.6. **Quantification of ABA content.** ABA concentrations were quantified in rice roots and shoots as well as 70-15 spores and mycelia using an ELISA-based kit. For plant treatments, plants were root inoculated with bacteria and then 24 hours later the leaves were dipped in 70-15 spores for five minutes. Root and shoot tissue was collected separately at **A**) 24 hours post 70-15 treatment and **B**) 48 hours post 70-15 treatment for ABA extraction. **C**) ABA content was quantified in untreated 70-15 spores and mycelia. Error bars indicate standard error based on three biological replicates, each pooled from five plants, or three plates of fungi. Each replicate was quantified in quadruplicate. Different letters represent a statistically significant difference based on the Tukey-Kramer test when $p < 0.05$. For spores and mycelia, mean ABA concentration is statistically significant, $p = 0.0036$, based on a t-Test.

Since the ABA concentrations did not match what was seen with *NCED3* expression patterns, it was necessary to examine other steps at which ABA signaling is regulated. We designed primers for multiple genes related to ABA including those involved in catabolism, perception, signaling, and response, to gain a better understanding of how EA105 and 70-15 spores are affecting ABA signals in rice. The expression of *OsABA8ox1*, involved in ABA catabolism in rice, was largely unaffected by any of the treatments; however there was slightly less of an induction of *OsABA8ox1* in plants treated with spores or exogenous ABA (Figures 4.7 and 4.8). Similarly, there were no significant expression changes in ABA receptor *RCAR5* (Figures 4.7 and 4.8).

ABA activity can also be modulated through inactivation and activation by adding or removing, respectively, glucosyl esters. In rice, the genes involved in this process had not previously been characterized. A BLAST search of the protein sequence for *AtBG1*, a gene implicated in the activation of ABA in *A. thaliana*, revealed a putative rice beta glucosidase. This gene was expressed more strongly in shoots than roots, and its expression in shoots was induced by ABA (Figure 4.8). When plants were treated with EA105 and ABA together, there was no longer up-regulation of this gene. Although the expression of this gene was weaker overall in roots, there was nearly twenty four fold up-regulation in plants infected with 70-15 spores (Figure 4.9). Similar to what was seen with *NCED3*, the presence of EA105 prevents spores from up-regulating this ABA-activating gene in roots. In *A. thaliana*, the inactivation of ABA is typically catalyzed by *UGT71B6*, a UDP glucosyl transferase. Using a protein BLAST of this gene, a putative rice glucosyl transferase was found. Expression of this gene in roots was very low compared to shoots.

Interestingly, shoot expression patterns mimicked those of the beta glucosidase, with ABA inducing this gene more than ten fold while EA105+ABA eliminated the induction of this gene (Figure 4.8).

Moving further through the ABA signaling pathway, the expression of Mitogen-activated protein (MAP)-kinase *OsMPK1* was also examined, but showed minimal expression changes (Figure 4.7). When ABA signaling is able to proceed, it culminates in the expression of genes containing ABREs in their promoter regions. One such gene is *Rab25*, although its expression was not affected by the treatments (Figure 4.7). Overall, the main expression changes were seen with NCED3 and the putative beta glucosidase in rice roots. Both of these genes would positively influence ABA signaling, and in both cases the presence of EA105 prevents spores from up-regulating the gene expression (Figure 4.10).

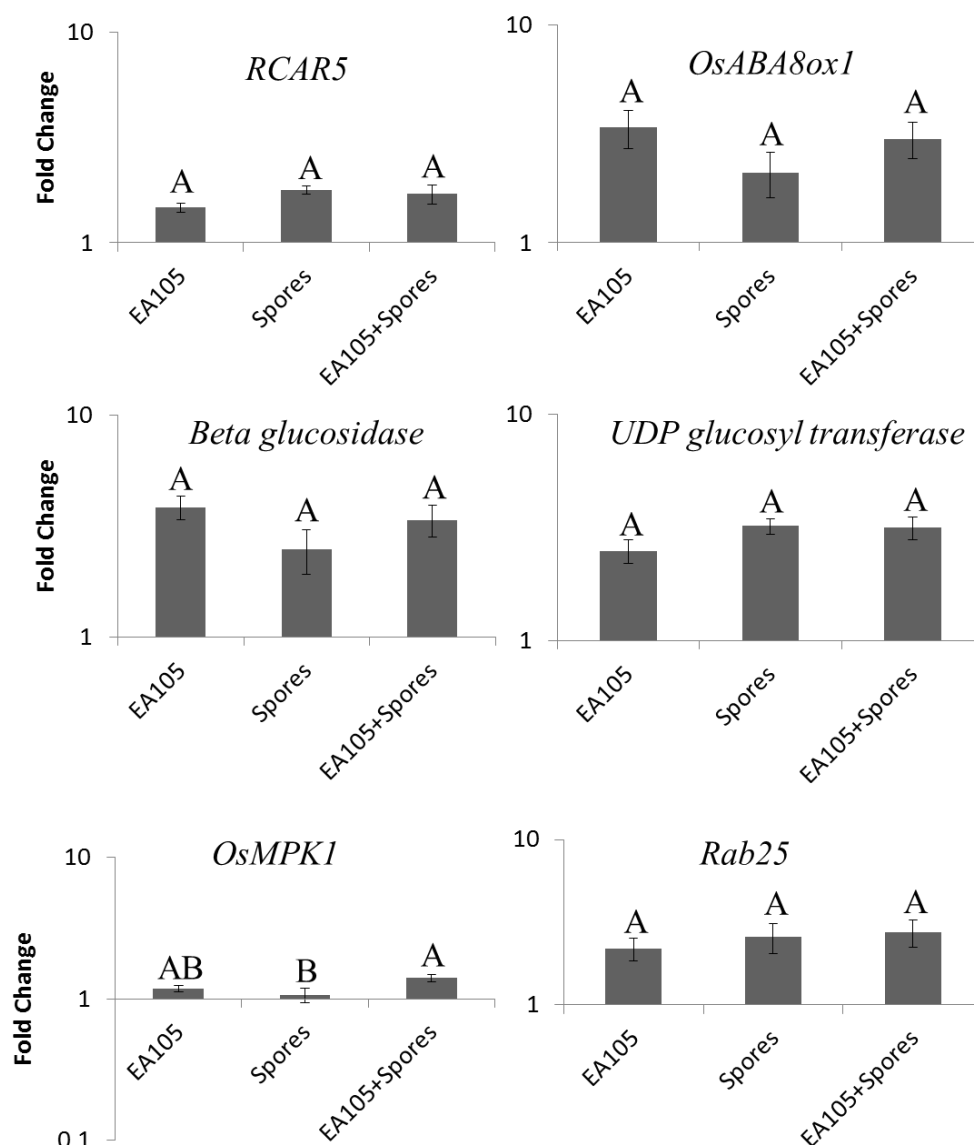


Figure 4.7. **Effect of EA105 and spores on ABA gene expression in rice.** Rice plants were root inoculated with EA105 then treated with spores after 24 hours. Shoot gene expression was examined an additional 24 hours after spore treatment using primers for ABA-related genes *RCAR5*, *OsABA8ox1*, beta glucosidase, UDP glucosyl transferase, *OsMPK1*, and *Rab25*. Error bars indicate standard error based on three biological replicates, each including five plants. Different letters represent a statistically significant difference based on the Tukey-Kramer test, $p < 0.05$.

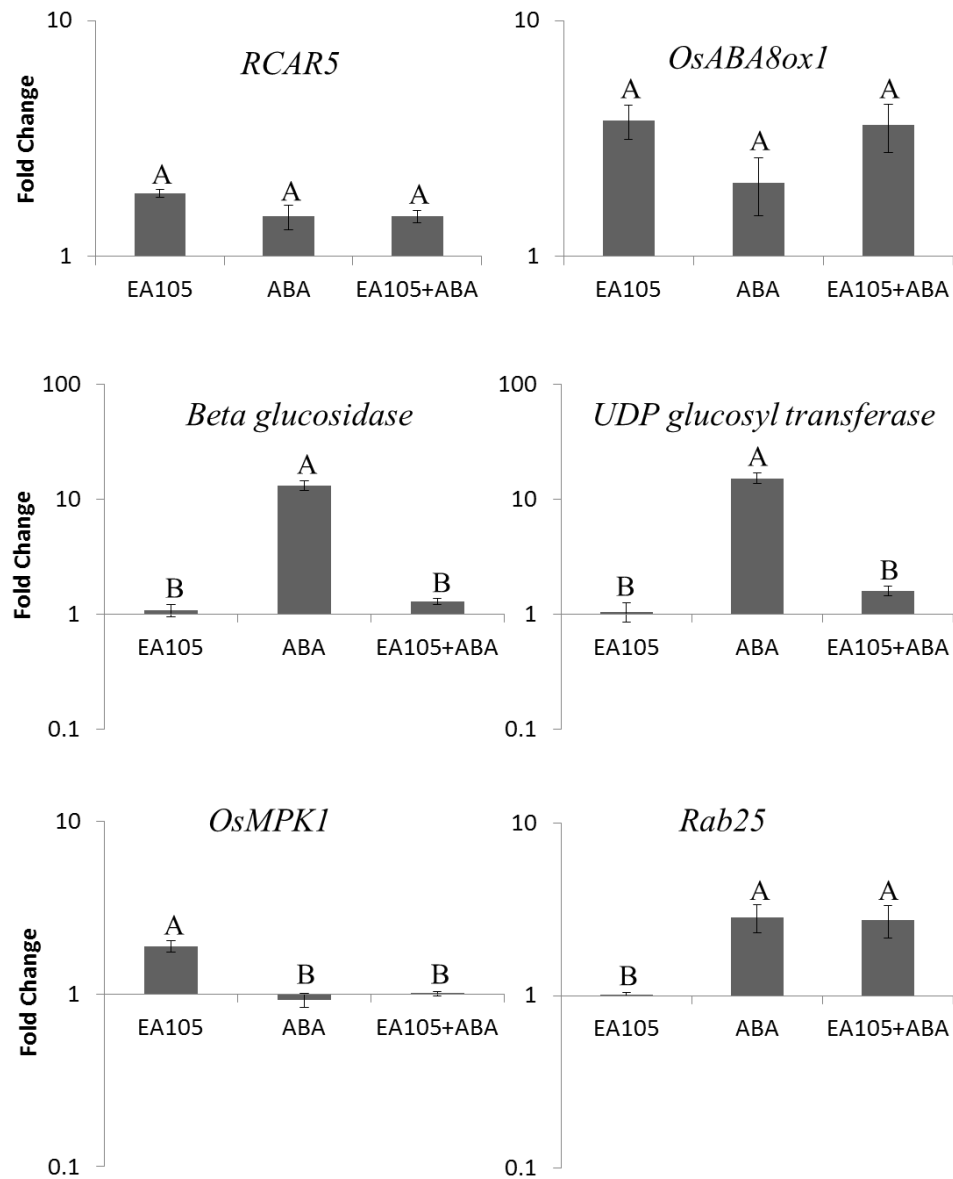


Figure 4.8. **Effect of EA105 and 100 μ M exogenous ABA on the expression of ABA-related genes in rice.** Rice plants were root inoculated with EA105 and treated with 100 μ M ABA. Shoot gene expression was examined at 24 hours using primers for ABA-related genes *RCAR5*, *OsABA8ox1*, beta glucosidase, UDP glucosyl transferase, *OsMPK1*, and *Rab25*. Error bars indicate standard error based on three biological replicates, each including five plants. Different letters represent a statistically significant difference based on the Tukey-Kramer test when $p < 0.05$.

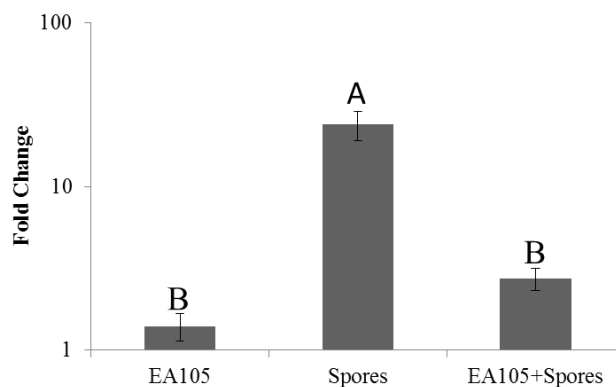


Figure 4.9. **Root expression of a putative rice beta glucosidase is highly induced by 70-15 spore treatment.** Primers were designed based on a putative rice gene which is orthologous to *AtBG1*, involved in activation of ABA by removing conjugated glucosyl esters. Root gene expression was examined 24 hours post-treatment. Error bars indicate standard error based on three biological replicates, each including five plants. Different letters represent a statistically significant difference based on the Tukey-Kramer test, $p < 0.05$.

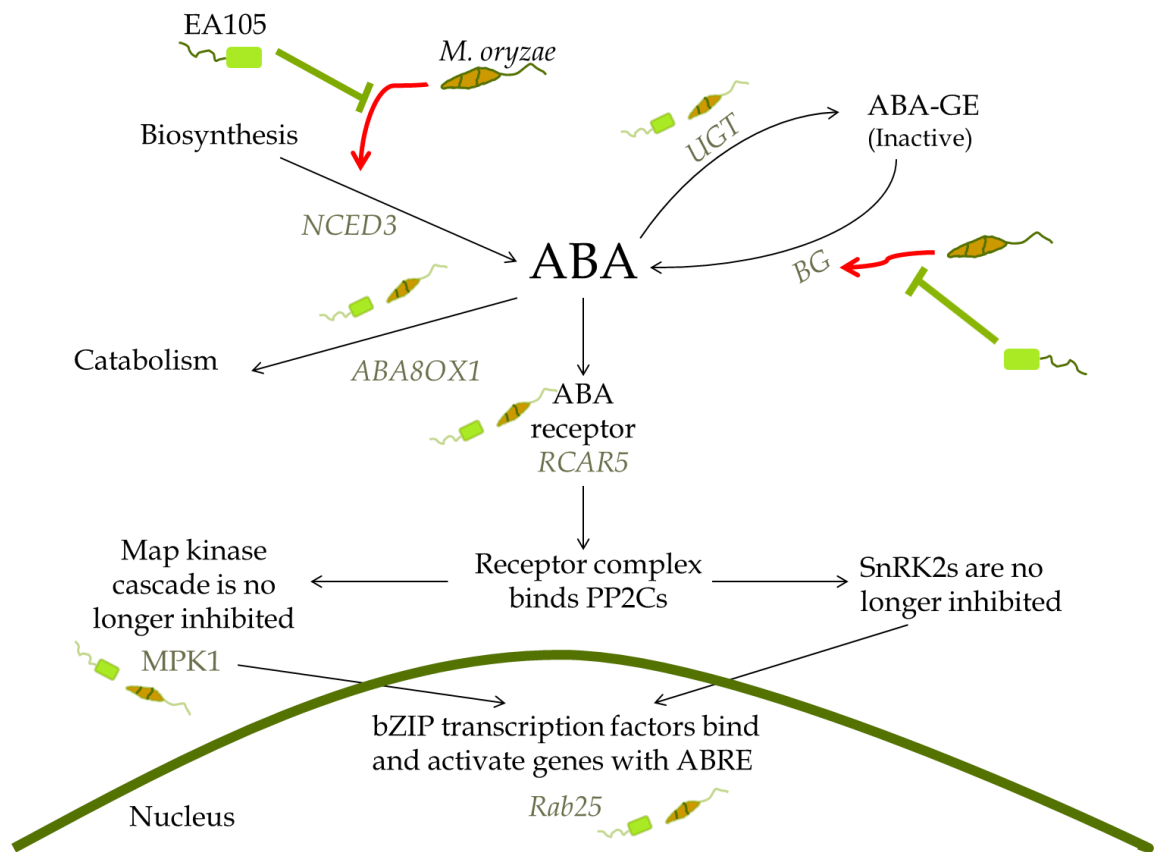


Figure 4.10. **Visualization of ABA-related gene expression data in rice.** *M. oryzae* spores up-regulated ABA biosynthesis gene *NCED3* and a putative beta glucosidase in rice roots, but not when plants were pre-treated with EA105. There were not any notable expression changes in *ABA8OX1*, a putative UDP glucosyl transferase, *RCAR 5*, *MPK1*, or *Rab25*.

4.4.4 ABA may be directly contributing to virulence in *M. oryzae*

Pathogenesis of *M. oryzae* begins with spore germination, and the crucial step in virulence is the formation of the appressorium, a specialized infection structure which accumulates high turgor pressure, ultimately forming a penetration peg which can enter the rice cuticle. To test whether ABA could enhance pathogenicity in *M. oryzae*, 70-15 spores were treated with ABA ranging from 10 to 100 μM . By 3 hours almost all spores germinated in the untreated controls, and by 24 hours most formed appressoria. To see if ABA was accelerating these processes, germination was examined at 2 hours and the initiation of appressoria formation at 6 hours. Spores which had been exposed to 50 or 100 μM ABA had a higher percent germination than those which were not exposed to ABA at 2 hours post treatment (Figure 4.11). Also, the percent of germinated spores which were forming appressoria at 6 hours post treatment was higher in the presence of 50 or 100 μM ABA (Figure 4.11). In addition to ABA, other plant growth regulators were tested including gibberellic acid (GA), the natural auxin indole-3-acetic acid (IAA), a synthetic auxin indole-3-butyric acid (IBA), and the cytokinin, kinetin. Aside from ABA, only GA was able to increase percent germination at 2 hours, but did not increase appressoria formation at 6 hours (Figure 4.12). Kinetin had no effect on germination, but increased appressoria formation at 6 hours. The natural and synthetic auxins had no effect on *M. oryzae* 70-15 spore germination or appressoria formation (Figure 4.12). The germination and appressoria formation was also quantified in spores treated with EA105 and ABA together. Previously, we have shown that EA105 had a minimal effect on spore germination at 3 hours, but almost completely abolished appressoria formation in *M. oryzae* at 24 hours (Spence *et al.* 2014b). At the earlier time points, EA105 behaves similarly. The percent germination is not statistically different from the control, while

appressoria formation is greatly reduced (Figure 4.13). When EA105 and ABA are co-treated on spores, the percent germination is about half way between what was seen with ABA or EA105 treatment alone. The percent of spores which were forming appressoria was decreased from about 84% with ABA treatment alone to about 23% when treated with both EA105 and ABA together (Figure 4.13).

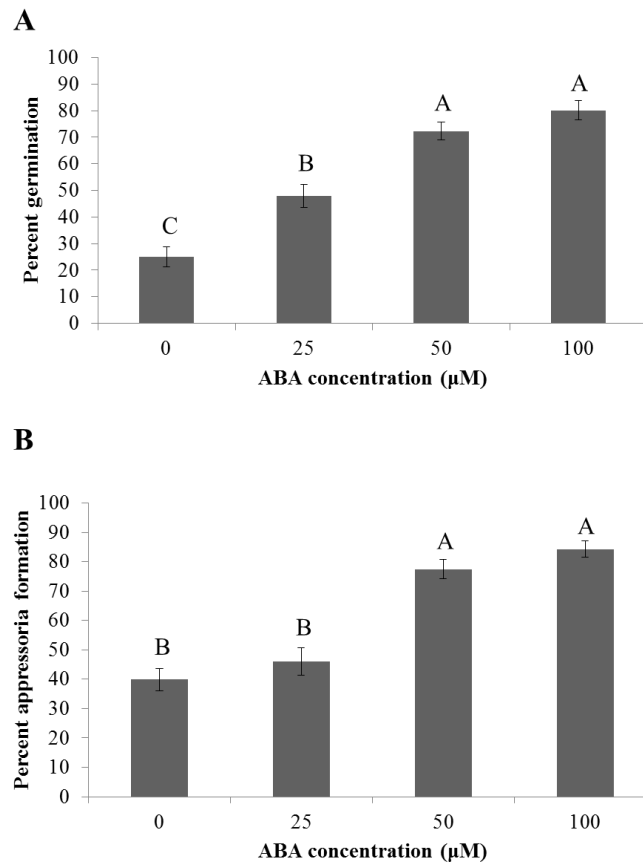


Figure 4.11. *M. oryzae* 70-15 spores treated with 0 to 100 μM ABA. Images were taken at 2 hours and 6 hours post-treatment and the percentage of spores **A)** germinating or **B)** forming appressoria were quantified. This experiment was repeated three times with 5 coverslips per treatment and 3 images per coverslip. Different letters represent statistical significance based on the Tukey-Kramer test ($p < 0.05$).

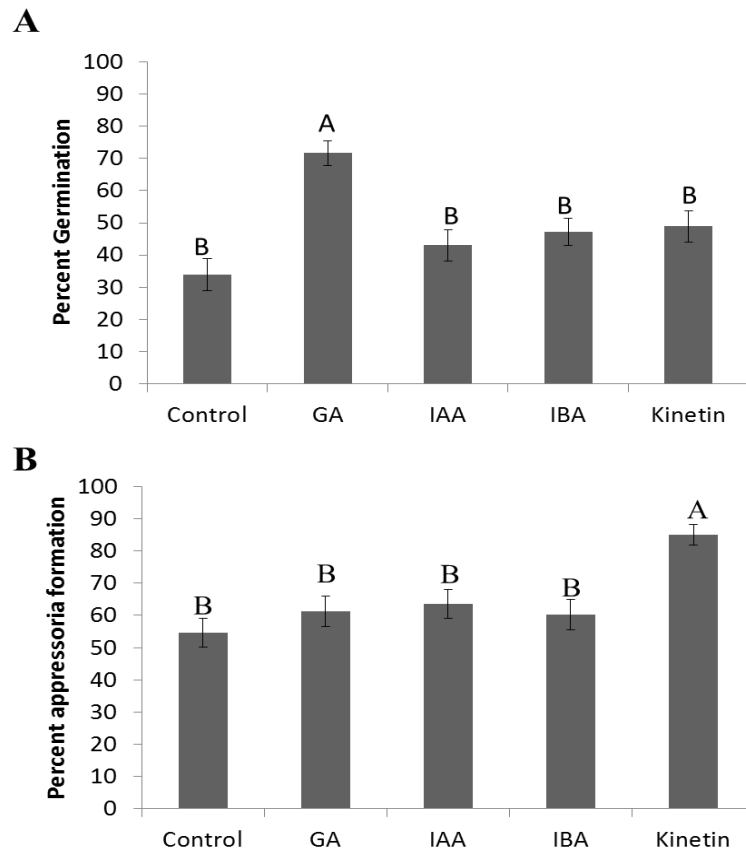


Figure 4.12. *M. oryzae* 70-15 spores treated with growth regulators GA, IAA, IBA, and kinetin. **A)** The percentage of germinating spores was quantified at 2 hours post-treatment and **B)** the percentage of spores forming appressoria was quantified at 6 hours post-treatment. This experiment was repeated three times with 5 coverslips per treatment and 3 images per coverslip. Different letters indicate a significant difference based on the Tukey-Kramer test ($p < 0.05$).

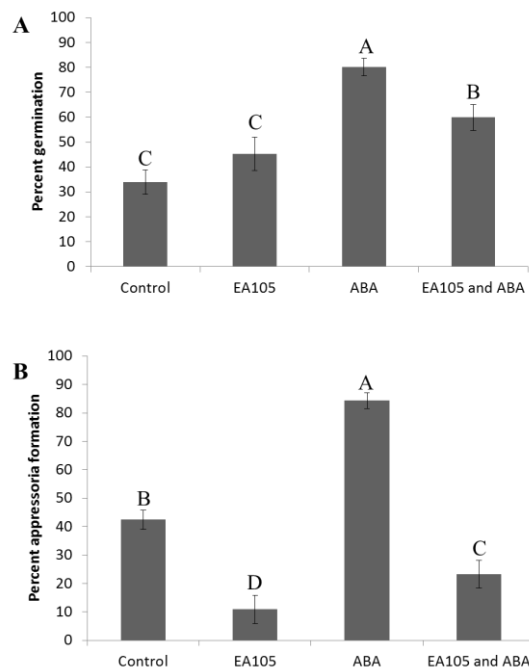


Figure 4.13. *M. oryzae* 70-15 spores treated with EA105 and/or exogenous ABA. A) Percent germination was quantified at 2 hours post treatment and B) percent appressoria formation was quantified at 6 hours post treatment. The experiment was repeated three times with 5 coverslips per treatment and 3 images per coverslip. Different letters represent statistical significance based on the Tukey-Kramer test ($p < 0.05$).

4.5 Discussion

With the global population expected to exceed 9 billion by 2050, developing an effective and sustainable method to reduce crop loss from rice blast could have a significant impact on food security. We have identified natural rice rhizospheric isolate EA105, which has the ability to reduce the symptoms of blast in rice, but another important aspect in the development of a biocontrol solution is understanding normal disease progression and how it is interrupted. The small signaling molecules SA, JA, and ETH have been studied for their involvement in SAR and ISR but they do not act independently within plants. Crosstalk from multiple other signals can

promote or antagonize the effects of these signaling molecules on plant resistance. It is becoming apparent that ABA is a critical factor involved in modulating plant defenses which may be manipulated in different ways by pathogens and beneficial microbes. The study of ABA as it relates to biotic stress is relatively new, and there is very little known about how biocontrol bacteria affect this important hormone. However, a crucial role for ABA was recently discovered in the growth promotion of tomato by *Bacillus megaterium* (Porcel *et al.*, 2014). Using the interaction between EA105, rice, and *M. oryzae* as a model system, we sought to investigate how the beneficial bacteria and the pathogenic fungus may be manipulating ABA to influence the susceptibility of rice to blast.

While elevated levels of ABA independently cause detriment to plants, antagonism toward SA (Jiang *et al.*, 2010), JA, and ETH signaling also compromise plant immunity. As a natural response to treatment with *M. oryzae* spores, rice plants initiate an SA-mediated defense response. *WRKY77*, a transcription factor involved in SA signaling which positively regulates *PR1* expression and is thought to directly bind its promoter (Lan *et al.*, 2013), was induced by 70-15 spores. However, *PR1* expression was not induced by the spores, potentially indicating that the spores are suppressing SAR by preventing the up-regulation of *PR1*. When EA105 and spores are both present on plants, similar expression patterns were seen, with *WRKY77* remaining elevated. The lack of *PR1* expression could be due to multiple factors by which the fungal pathogen is attempting to suppress plant defenses, with one possibility being through the provocation of ABA production in plants.

Since elevated ABA levels have been shown to increase rice susceptibility to *M. oryzae* and *X. oryzae* (Koga *et al.*, 2004; Jiang *et al.*, 2010; Yazawa *et al.*, 2012;

Xu *et al.*, 2013), the effect of EA105 and 70-15 on the expression of key ABA biosynthesis gene *NCED3* was examined. As expected, 70-15 treatment up-regulated *NCED3* in rice, while EA105 had very little effect. Interestingly, when rice plants were treated with EA105 prior to infection with spores, there was no longer an up-regulation of *NCED3*, potentially indicating that EA105 is preventing spores from increasing ABA biosynthesis in rice. When plants were treated with ABA, which also induces *NCED3* expression, and EA105, the up-regulation of *NCED3* persisted. EA105 was able to prevent spores but not exogenous ABA from up-regulating *NCED3* in rice, indicating that spores are affecting plants in ways which differ from ABA alone. In this aspect, EA105 was not able to counter the effects of ABA, yet it did attenuate the effects of spores.

In contrast to *NCED3* expression, ABA concentrations were examined in plants with the same treatments and no differences were found. Even in rice plants treated only with spores, the ABA concentration was not changed up to 48 hours following treatment. While it has been shown that elevated levels of ABA increase susceptibility to *M. oryzae* during very early stages of infection (Yazawa *et al.*, 2012), it is possible that ABA once again becomes important in later stages of disease progression. For instance, in sugar beet, ABA levels were slightly higher in plants infected with the hemibiotrophic pathogen *Cercospora beticola* on the first day, but then returned to control levels until day 5 when ABA started increasing once again. The highest levels of ABA were not seen until 15 days post-infection, when the fungal pathogen switched to the necrotic phase of infection (Schmidt *et al.*, 2008). *M. oryzae* is also a hemibiotrophic fungus, and it is possible that ABA levels in rice only reach their maxima during the necrotic stages of blast.

The discrepancy between *NCED3* expression and ABA concentration has been shown previously (Priest *et al.*, 2006; Ye *et al.*, 2012). Beyond biosynthesis, ABA concentrations are also influenced by catabolism, activation and inactivation, transport, compartmentalization, and inhibition of signal transduction. In rice, the genes involved in activation and inactivation of ABA have not been characterized. However, primers were designed based on putative genes and both were highly responsive to exogenous ABA treatment. Similarly to what was seen with *NCED3*, spores increased expression of the putative beta-glucosidase, potentially involved in activating ABA, in rice roots while the presence of EA105 prevented this. EA105 also prevented exogenous ABA from up-regulating the same gene in shoots. However, so far we have only seen EA105 prevent spore-induced gene expression changes in roots. Considering the limited number of genes examined, it is possible that shoot gene expression is also modulated, but root expression still holds importance as the primary location of ABA biosynthesis. It is logical to argue that any opposition between the beneficial and the pathogen involving ABA-mediated susceptibility/resistance would be initiated at the site of ABA biosynthesis.

Yet another factor to consider is the degree by which actual ABA concentrations contribute to ABA-mediated responses. ABA signaling pathways are intersected by multiple pathways including GA, SA, JA, and ETH (Robert-Seilaniantz *et al.*, 2011; Denance *et al.*, 2013; Yang *et al.*, 2013; Meguro and Sato, 2014), all of which can affect ABA-mediated responses without directly altering ABA concentrations. Regulation within ABA signaling, such as inactivation and binding of PP2Cs to the SnRK2s, also effect ABA-mediated responses, as does the availability of ABA receptors. Yazawa *et al.* used DEX-inducible lines to selectively overexpress

OsABA8ox1 and a dominant negative mutant form of *OsABI*, a PP2C, to show that decreasing ABA concentrations and interrupting ABA signaling reduces blast lesion number. However, they mentioned that in the mutant *OsABI* line, there were actually higher levels of ABA, possibly due to feedback mechanisms, though they still saw fewer lesions (Yazawa *et al.*, 2012). It is an important observation that lesions were reduced in plants which had elevated levels of ABA, but were impaired in a later step in ABA signaling, indicating that the actual ABA concentrations may not be as crucial as ABA perception and signaling in ABA-mediated susceptibility. EA105 may not be altering ABA concentration in rice plants during early stages of infection, but it could be interfering with ABA perception or signal transduction.

A hormone such as ABA which is involved in a multitude of normal developmental and stress-induced responses in plants must be tightly regulated. Each process for regulating the amount of active ABA, such as inactivation or catabolism, has multiple genes which can be triggered by different stimuli. ABA responses and ABA signal transduction can differ based on the type of stress, with particular differences having been found between abiotic and biotic stress (Kim, 2012; Ye *et al.*, 2012). ABA was originally studied for its role in abiotic stress response, and most of what is known has been determined through experimenting with drought, temperature, and salt stress (Lim *et al.*, 2012) while there is comparatively much less known about the role of ABA in biotic stress responses. There were only minimal expression changes to ABA-catabolism gene *OsABA8ox1*, ABA receptor *RCAR5*, *OsMPK1*, or *Rab25*. Though *OsABA8ox1* has been shown to be important in hydroxylation and ultimately catabolism of ABA following abiotic stress (Saika *et al.*, 2007) this has not been shown for biotic stress, and it is possible that one of the other 2 genes coding 8'

ABA hydroxylases in rice may play a larger role during biotic stress. Similarly, there are many ABA receptors which are induced by different situations, some of which negatively regulate ABA signaling (Szostkiewicz *et al.*, 2010; Kepka *et al.*, 2011). *RCAR5* positively regulates ABA signaling but has only been shown to do so during abiotic stress (Kim *et al.*, 2012; Kim *et al.*, 2014). Similarly, *Rab25*, a homolog of *Rab16a*, was identified for its role in ABA-mediated response to drought (RoyChoudhury *et al.*, 2007; Ganguly *et al.*, 2011) and may not play a role in ABA-mediated responses to biotic stress. In the case of *OsMPK1*, response to ABA can be transient, subsiding within 2 hours (Shi *et al.*, 2014) which may explain why minimal expression changes were seen at 24 hours post treatment.

Based on expression of *NCED3* and the putative rice beta glucosidase, it is possible that modulating ABA signaling in plants may be a contributing factor to how EA105 reduces blast lesions. It has been established that ABA plays a role in plant susceptibility to pathogens. However, ABA may be playing a dual role by not only increasing susceptibility in plants, but also by directly promoting virulence in *M. oryzae*. We have shown that 70-15 produced ABA during vegetative growth and during spore formation. Exogenous ABA accelerated both spore germination and appressoria formation in 70-15, both of which are necessary for virulence. *M. oryzae* may be producing its own ABA as well as utilizing the increased ABA produced in plants to enhance pathogenicity. ABA is a growth regulator in plants, and may also regulate growth in fungi. To determine if the effect of ABA on 70-15 germination and appressoria formation was specific, other growth regulators were also tested including auxins, cytokinin, and GA. Only ABA was able to accelerate both germination and appressoria formation. Previously, EA105 has been shown to almost completely

prevent the formation of appressoria (Spence *et al.* 2014b). When EA105 and ABA were added together on spores, there was a large reduction in the number of spores forming appressoria when compared to ABA treatment alone. Not only is EA105 interfering with ABA's ability to accelerate appressoria formation, but this also shows that high levels of ABA (100 μ M) are still unable to prevent EA105 from reducing appressoria formation.

4.6 Conclusion

ABA contributes to plant disease by increasing susceptibility and in the case of *M. oryzae* it may also directly promote virulence. Using isolate EA105, which is able to inhibit 70-15 vegetative growth and pathogenesis as well as reduce blast lesion size in rice, we have shown that during ABA biosynthesis and activation, EA105 may be able to prevent 70-15 from enhancing the ABA response in rice. We have also shown that 70-15 is capable of producing ABA, particularly at the spore stage, and that ABA accelerates spore germination and appressoria formation in 70-15. However, EA105 can almost completely prevent appressoria formation in 70-15, and even in the presence of ABA can drastically reduce the number of spores which are able to form this crucial infection structure. While the complete mechanism of how EA105 confers protection is still not clear, the modulation of ABA biosynthesis and activation may be a contributing factor. As the demand for staple food crops such as rice continues to increase with burgeoning population growth, it is imperative to develop and understand approaches to maximize production and yield.

4.7 Acknowledgments

Dr. Venkatesan Sundaresan and his lab for the M-104 seeds as well as the soil and root samples from which we obtained our isolates.

4.8 References

- Denance N, Sanchez-Vallet A, Goffner D, Molina A** (2013) Disease resistance or growth: the role of plant hormones in balancing immune responses and fitness costs. *Frontiers in Plant Science* **4**
- Fujita Y, Yoshida T, Yamaguchi-Shinozaki K** (2013) Pivotal role of the AREB/ABF-SnRK2 pathway in ABRE-mediated transcription in response to osmotic stress in plants. *Physiologia Plantarum* **147**: 15-27
- Ganguly M, Roychoudhury A, Sarkar SN, Sengupta DN, Datta SK, Datta K** (2011) Inducibility of three salinity/abscisic acid-regulated promoters in transgenic rice with gusA reporter gene. *Plant Cell Reports* **30**: 1617-1625
- Hartung W** (2010) The evolution of abscisic acid (ABA) and ABA function in lower plants, fungi and lichen. *Functional Plant Biology* **37**: 806-812
- Hauser F, Waadt R, Schroeder JI** (2011) Evolution of abscisic acid synthesis and signaling mechanisms. *Current Biology* **21**: R346-R355
- Hirai N, Yoshida R, Todoroki Y, Ohigashi H** (2000) Biosynthesis of abscisic acid by the non-mevalonate pathway in plants, and by the mevalonate pathway in fungi. *Bioscience Biotechnology and Biochemistry* **64**: 1448-1458
- Jiang CJ, Shimono M, Sugano S, Kojima M, Yazawa K, Yoshida R, Inoue H, Hayashi N, Sakakibara H, Takatsuji H** (2010) Abscisic acid interacts antagonistically with salicylic acid signaling pathway in rice-*Magnaporthe grisea* interaction. *Molecular Plant-Microbe Interactions* **23**: 791-798
- Kepka M, Benson CL, Gonugunta VK, Nelson KM, Christmann A, Grill E, Abrams SR** (2011) Action of natural abscisic acid precursors and catabolites on abscisic acid receptor complexes. *Plant Physiology* **157**: 2108-2119
- Kim H, Hwang H, Hong JW, Lee YN, Ahn IP, Yoon IS, Yoo SD, Lee S, Lee SC, Kim BG** (2012) A rice orthologue of the ABA receptor, OsPYL/RCAR5, is a positive regulator of the ABA signal transduction pathway in seed germination and early seedling growth. *Journal of Experimental Botany* **63**: 1013-1024
- Kim H, Lee K, Hwang H, Bhatnagar N, Kim DY, Yoon IS, Byun MO, Kim ST, Jung KH, Kim BG** (2014) Overexpression of PYL5 in rice enhances drought tolerance, inhibits growth, and modulates gene expression. *Journal of Experimental Botany* **65**: 453-464

- Kim TH** (2012) Plant stress surveillance monitored by ABA and disease signaling interactions. *Molecules and Cells* **33**: 1-7
- Koga H, Dohi K, Mori M** (2004) Absciscic acid and low temperatures suppress the whole plant-specific resistance reaction of rice plants to the infection of *Magnaporthe grisea*. *Physiological and Molecular Plant Pathology* **65**: 3-9
- Kulik A, Wawer I, Krzywinska E, Bucholc M, Dobrowolska G** (2011) SnRK2 protein kinases-key regulators of plant response to abiotic stresses. *Omics-a Journal of Integrative Biology* **15**: 859-872
- Lan A, Huang J, Zhao W, Peng Y, Chen Z, Kang D** (2013) A salicylic acid-induced rice (*Oryza sativa* L.) transcription factor OsWRKY77 is involved in disease resistance of *Arabidopsis thaliana*. *Plant Biology* **15**: 452-461
- Lee KH, Piao HL, Kim HY, Choi SM, Jiang F, Hartung W, Hwang I, Kwak JM, Lee IJ** (2006) Activation of glucosidase via stress-induced polymerization rapidly increases active pools of absciscic acid. *Cell* **126**: 1109-1120
- Lim CW, Baek W, Lim S, Lee SC** (2012) ABA signal transduction from ABA receptors to ion channels. *Genes & Genomics* **34**: 345-353
- Liotenberg S, North H, Marion-Poll A** (1999) Molecular biology and regulation of absciscic acid biosynthesis in plants. *Plant Physiology and Biochemistry* **37**: 341-350
- Loake G, Grant M** (2007) Salicylic acid in plant defence-the players and protagonists. *Current Opinion in Plant Biology* **10**: 466-472
- Meguro A, Sato Y** (2014) Salicylic acid antagonizes absciscic acid inhibition of shoot growth and cell cycle progression in rice. *Scientific Reports* **4**
- Nambara E, Marion-Poll A** (2005) Absciscic acid biosynthesis and catabolism. *Annual Review of Plant Biology* **56**: 165-185
- Porcel R, Zamarreno AM, Garcia-Mina JM, Aroca R** (2014) Involvement of plant endogenous ABA in *Bacillus megaterium* PGPR activity in tomato plants. *Bmc Plant Biology* **14**
- Priest DM, Ambrose SJ, Vaistij FE, Elias L, Higgins GS, Ross ARS, Abrams SR, Bowles DJ** (2006) Use of the glucosyltransferase UGT71B6 to disturb absciscic acid homeostasis in *Arabidopsis thaliana*. *Plant Journal* **46**: 492-502

- Qin XQ, Zeevaart JAD** (1999) The 9-cis-epoxycarotenoid cleavage reaction is the key regulatory step of abscisic acid biosynthesis in water-stressed bean. *Proceedings of the National Academy of Sciences of the United States of America* **96**: 15354-15361
- Robert-Seilanianz A, Grant M, Jones JDG** (2011) Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. *In* NK VanAlfen, G Bruening, JE Leach, eds, *Annual Review of Phytopathology*, Vol 49, Vol 49, pp 317-343
- RoyChoudhury A, Roy C, Sengupta DN** (2007) Transgenic tobacco plants overexpressing the heterologous lea gene Rab16A from rice during high salt and water deficit display enhanced tolerance to salinity stress. *Plant Cell Reports* **26**: 1839-1859
- Saika H, Okamoto M, Miyoshi K, Kushihiro T, Shinoda S, Jikumaru Y, Fujimoto M, Arikawa T, Takahashi H, Ando M, Arimura S, Miyao A, Hirochika H, Kamiya Y, Tsutsumi N, Nambara E, Nakazono M** (2007) Ethylene promotes submergence-induced expression of OsABA8ox1, a gene that encodes ABA 8'-hydroxylase in rice. *Plant and Cell Physiology* **48**: 287-298
- Santiago J, Dupeux F, Betz K, Antoni R, Gonzalez-Guzman M, Rodriguez L, Marquez JA, Rodriguez PL** (2012) Structural insights into PYR/PYL/RCAR ABA receptors and PP2Cs. *Plant Science* **182**: 3-11
- Schmidt K, Pflugmacher M, Klages S, Maser A, Mock A, Stahl DJ** (2008) Accumulation of the hormone abscisic acid (ABA) at the infection site of the fungus *Cercospora beticola* supports the role of ABA as a repressor of plant defence in sugar beet. *Molecular Plant Pathology* **9**: 661-673
- Schwartz SH, Qin XQ, Zeevaart JAD** (2003) Elucidation of the indirect pathway of abscisic acid biosynthesis by mutants, genes, and enzymes. *Plant Physiology* **131**: 1591-1601
- Schwartz SH, Tan BC, Gage DA, Zeevaart JAD, McCarty DR** (1997) Specific oxidative cleavage of carotenoids by VP14 of maize. *Science* **276**: 1872-1874
- Shi B, Ni L, Liu YP, Zhang AY, Tan MP, Jiang MY** (2014) OsDMI3-mediated activation of OsMPK1 regulates the activities of antioxidant enzymes in abscisic acid signalling in rice. *Plant Cell and Environment* **37**: 341-352
- Siewers V, Kokkelink L, Smedsgaard J, Tudzynski P** (2006) Identification of an abscisic acid gene cluster in the grey mold *Botrytis cinerea*. *Applied and Environmental Microbiology* **72**: 4619-4626

- Spence CA, Raman V, Donofrio NM, Bais HP** (2014) Global gene expression in rice blast pathogen *Magnaporthe oryzae* treated with a natural rice soil isolate. *Planta* **239**: 171-185
- Spence C, Alff E, Johnson C, Ramos C, Donofrio N, Sundaresan V, Bais H.** (2014) Natural rice rhizospheric microbes suppress rice blast infections. *BMC Plant Biology* **14**:130. DOI: 10.1186/1471-2229-14-130
- Szostkiewicz I, Richter K, Kepka M, Demmel S, Ma Y, Korte A, Assaad FF, Christmann A, Grill E** (2010) Closely related receptor complexes differ in their ABA selectivity and sensitivity. *Plant Journal* **61**: 25-35
- Xu J, Audenaert K, Hofte M, De Vleeschauwer D** (2013) Absciscic acid promotes susceptibility to the rice leaf blight pathogen *Xanthomonas oryzae* pv *oryzae* by suppressing salicylic acid-mediated defenses. *Plos One* **8**: 10
- Xu ZJ, Nakajima M, Suzuki Y, Yamaguchi I** (2002) Cloning and characterization of the abscisic acid-specific glucosyltransferase gene from adzuki bean seedlings. *Plant Physiology* **129**: 1285-1295
- Yang DL, Yang YN, He ZH** (2013) Roles of plant hormones and their interplay in rice immunity. *Molecular Plant* **6**: 675-685
- Yazawa K, Jiang CJ, Kojima M, Sakakibara H, Takatsuji H** (2012) Reduction of abscisic acid levels or inhibition of abscisic acid signaling in rice during the early phase of *Magnaporthe oryzae* infection decreases its susceptibility to the fungus. *Physiological and Molecular Plant Pathology* **78**: 1-7
- Ye NH, Jia LG, Zhang JH** (2012) ABA signal in rice under stress conditions. *Rice* **5**

Chapter 5

FINAL CONSIDERATIONS AND PROSPECTIVE RESEARCH

The objective of the research contained herein was to identify and evaluate the potential of rice rhizospheric bacteria to control blast disease caused by *M. oryzae*. Eleven distinct bacterial isolates were identified and characterized, with *P. chlororaphis* EA105 showing the strongest inhibition of *M. oryzae in-vitro*. EA105 inhibited mycelial growth through unknown diffusible and volatile compounds and almost completely prevented formation of the appressorium, a structure crucial to infection. Although many pseudomonads rely on cyanide production as a main component of their biocontrol arsenal, a cyanide non-producing mutant in the EA105 background showed that cyanide production plays little to no role in the direct antagonism of *M. oryzae* by EA105. In addition to direct antifungal activity, EA105 treatment also resulted in fewer and smaller blast lesions in plants which had been pre-treated at the root surface prior to infection. The reduction in lesions is mediated through a specific root-to-shoot signaling process known as ISR, and in the case of EA105 as well as other biocontrol bacteria it involves JA and ETH-dependent signaling (Figure 5.1).

The effect of EA105's direct antagonism was examined in *M. oryzae* using a global transcriptional approach. The severe growth restriction that EA105 imposes on *M. oryzae* led to the down-regulation of approximately half of *M. oryzae*'s genome. Knock-out mutants in *M. oryzae* revealed two potential targets of EA105's antifungal activity, a trichothecene biosynthesis gene (MGG_08440) as well as a stress-inducible

gene (MGG_03098). The lack of each gene corresponded to a reduction in the fungi's sensitivity to EA105 treatment during appressoria formation. Interestingly, the stress-inducible gene (MGG_03098), which was identified based on a direct interaction between the bacterium and fungus, also appears to play a role in ISR, though its effect was opposite from that of direct inhibition. When plants pre-treated with EA105 were infected with the null mutant for the stress-inducible gene (MGG_03098), lesions were reduced to an even greater extent, indicating that a lack of this gene enhances ISR.

EA105 may also use an ABA-mediated strategy to control blast. *M. oryzae* spores up-regulated the ABA-biosynthesis gene *NCED3* in rice roots, but pre-treatment of plants with EA105 prevented this from occurring. Similarly, EA105 prevented up-regulation of a putative rice β -glucosidase believed to be involved in activation of conjugated, inactive forms of ABA. However, differences in actual ABA content were not apparent. ABA shows antagonism toward defense signaling molecules SA, JA, and ETH, and enhanced ABA signaling is associated with increased susceptibility in plants. Further contributing to plant disease, ABA also accelerated the spore germination and appressoria formation in *M. oryzae*, though EA105 was able to largely negate these effects (Figure 5.1). It was determined that *M. oryzae* itself synthesizes ABA, with higher content seen in spores compared to vegetative mycelia.

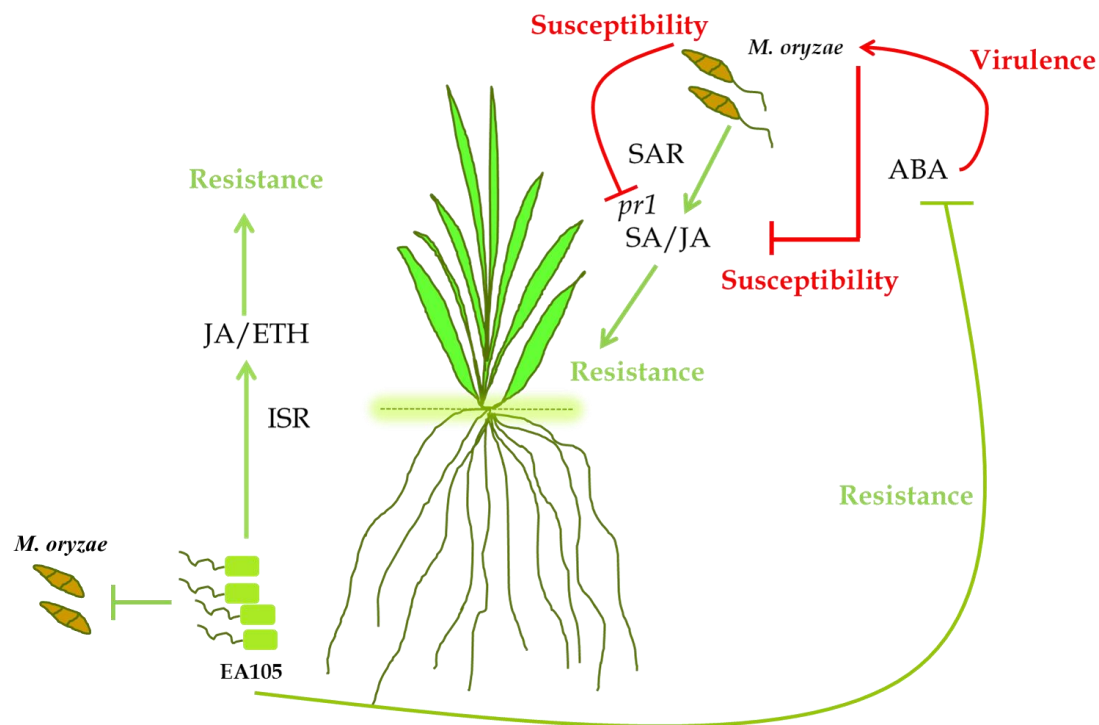


Figure 5.1. **Overview of the mechanisms by which EA105 confers protection against rice blast.** EA105 directly inhibited vegetative growth and appressoria formation in *M. oryzae*. When root-inoculated onto rice plants, EA105 also triggered ISR mediated through JA and ETH signaling, and reduced the number and size of blast lesions on pre-treated plants. Stimulation of ABA signaling in rice by *M. oryzae* may contribute to the disease strategy by decreasing resistance in plants and directly accelerating fungal virulence. However, EA105 interfered with both of these processes by preventing spores from up-regulating ABA signaling genes and by inhibiting the direct virulence-inducing effects of exogenous ABA on *M. oryzae*.

All in all, EA105 exhibits multiple mechanisms by which it can reduce blast symptoms. As a natural rice rhizospheric isolate, it is well suited to survive and thrive in rice soil, making it a promising candidate for the biocontrol of blast disease, which is one of the biggest threats to the global food supply. Field trials with EA105 will be crucial in determining how effectively the bacterium will persist in the soil during

typical conditions of rice cultivation, and amidst the microbes and environmental fluctuations that affect the rhizosphere in a natural system. The direct antifungal activity of EA105 could potentially antagonize *M. oryzae* which can inhabit the soil near rice plants. Preliminary experiments have shown that EA105 does not survive well on the aerial portions of rice plants where blast disease symptoms typically appear, so the more critical methods of protection will be mediated through rice signaling, which EA105 is capable of when inoculated on rice roots. The protective effects that are mediated through plant signaling include classical ISR as well as interruption of pathogen-induced ABA signaling. Although foliar application of EA105 is likely to be ineffective, several practical and more feasible methods for treating crops with EA105 include seed treatment, root drenching, or perhaps dipping seedlings prior to transplantation. Other important considerations will be the extent to which EA105 reduces disease symptoms in the field, and whether this minimizes the spread of infection and crop loss.

In the investigation of this isolate, a few questions still remain unanswered and the results within this dissertation have also generated additional scientific queries, particularly in regard to the role of ABA.

Though EA105's activity is not dependent on cyanide, the identities of the antifungal compounds which are required for antibiosis are yet to be determined. Sequencing of EA105 and comparison to other biocontrol pseudomonads may be helpful in revealing characteristics of EA105 which make it so effective against *M. oryzae*. It is likely that multiple compounds contribute to the activities shown in EA105, and that many of the compounds involved in direct antagonism will be distinct from those involved in the elicitation of ISR. Even if a cocktail of bioactive

compounds are identified, they may not be as potent as using the live bacterium. The complex communications that occur between the live fungus and bacterium, and also with rice in the case of the tritrophic interaction, may trigger production of additional compounds and facilitate more complex communication and antagonism.

Expanding on the importance of back-and-forth communication and microbe-microbe interactions, EA105 in isolation may not be as effective as multiple biocontrol isolates used collectively. A great deal of time and experimentation would be necessary to identify bacteria which may work collaboratively with EA105, and to determine ratios at which each should be used. It would be a difficult undertaking to study such a complex interaction. Additionally, the mode by which plants are treated with bacteria may affect the efficacy of lesion reduction. Spraying leaves with a bacterial suspension was effective, though not as effective as root inoculation. Seed treatment is another alternative that could be tested. The timing, frequency, concentration, and mode by which bacteria are introduced to the plant could all be fine-tuned to maximize the effects of isolate EA105.

Another avenue that is yet to be explored is the response of rice during this tri-trophic interaction. An analysis of transcriptional changes in rice which has been treated with EA105, *M. oryzae* spores, or both, could provide valuable insight into the process by which EA105 is able to reduce lesions. This would greatly expand the understanding of how the three organisms are communicating, and could elucidate on a genetic level the responses of rice to each microbe.

A complex but interesting field of study involves the molecule ABA. Classically regarded as a plant signaling molecule involved in drought tolerance, it has recently been implicated for its sometimes contradictory role in response to biotic

stress. This ancient molecule is not, however, exclusive to plants. Many fungi also produce ABA, including *M. oryzae*. The role of ABA in fungi remains an enigma. In the case of *M. oryzae*, it seems to be involved in promoting pathogenesis, though the mechanisms by which this occurs, and how it relates to ABA biosynthesis in plant hosts, is unclear.

Most importantly, however, is that a bacterium was identified with tremendous direct antifungal activity against a devastating pathogen of rice. EA105 also induces ISR and may impede ABA-mediated susceptibility. Further evaluation of this isolate as a potential biocontrol solution could provide a sustainable and effective remedy to the massive amount of food that is lost to blast each year. The implications of this could directly and rapidly provide relief from hunger and malnutrition, saving lives. Further, it would be a step towards meeting future demands for increased crop production as the population continues to expand exponentially and the need for efficient farming becomes more crucial. Current strategies for enhancing yields come with higher health and environmental risks as compared to using naturally isolate soil microbes. Isolate EA105 may be able to match or exceed the effectiveness of those strategies in a less harmful and more sustainable way, leading to a longer-term solution which will ultimately increase our food supply and provide relief to those who are suffering from malnutrition and hunger. The use of beneficial microbes to enhance plant productivity is an approach which can be cost-effectively implemented and deserves further examination so that solutions can be developed not only in rice but in in other agriculturally important food crops as well.

REFERENCES

- Abdel-Monaim MF** (2011) Role of riboflavin and thiamine in induced resistance against charcoal rot disease of soybean. *African Journal of Biotechnology* **10**: 10842-10855
- Adachi N, Tsukamoto S, Inoue Y, Azegami K** (2012) Control of bacterial seedling rot and seedling blight of rice by bacteriophage. *Plant Disease* **96**: 1033-1036
- Adie B, Chico JM, Rubio-Somoza I, Solano R** (2007) Modulation of plant defenses by ethylene. *Journal of Plant Growth Regulation* **26**: 160-177
- Ahmed M, Stal LJ, Hasnain S** (2010) Production of Indole-3-Acetic Acid by the *Cyanobacterium Arthrospira platensis* Strain MMG-9. *Journal of Microbiology and Biotechnology* **20**: 1259-1265
- Ahn IP, Kim S, Lee YH** (2005) Vitamin B-1 functions as an activator of plant disease resistance. *Plant Physiology* **138**: 1505-1515
- Ahn IP, Kim S, Lee YH, Suh SC** (2007) Vitamin B-1-induced priming is dependent on hydrogen peroxide and the *NPR1* gene in Arabidopsis. *Plant Physiology* **143**: 838-848
- Aktar W, Sengupta D, Chowdhury A** (2009) Impact of pesticides use in agriculture: their benefits and hazards. *Interdisciplinary toxicology* **2**: 1-12
- Andrews JH, Harris RF** (2000) The ecology and biogeography of microorganisms of plant surfaces. *Annual Review of Phytopathology* **38**: 145-180
- Arima K, Fukuta A, Imanaka H, Kousaka M, Tamura G** (1964) Pyrrolnitrin new antibiotic substance produced by *Pseudomonas*. *Agricultural and Biological Chemistry* **28**: 575-&
- Arnold AE, Mejia LC, Kylo D, Rojas EI, Maynard Z, Robbins N, Herre EA** (2003) Fungal endophytes limit pathogen damage in a tropical tree. *Proceedings of the National Academy of Sciences of the United States of America* **100**: 15649-15654

- Ashelford KE, Chuzhanova NA, Fry JC, Jones AJ, Weightman AJ** (2006) New screening software shows that most recent large 16S rRNA gene clone libraries contain chimeras. *Applied and Environmental Microbiology* **72**: 5734-5741
- Asselbergh B, Curvers K, Franca SC, Audenaert K, Vuylsteke M, Van Breusegem F, Hofte M** (2007) Resistance to *Botrytis cinerea* in sitiens, an abscisic acid-deficient tomato mutant, involves timely production of hydrogen peroxide and cell wall modifications in the epidermis. *Plant Physiology* **144**: 1863-1877
- Audenaert K, Monbaliu S, Deschuyffeleer N, Maene P, Vekeman F, Haesaert G, De Saeger S, Eeckhout M** (2012) Neutralized electrolyzed water efficiently reduces *Fusarium* spp. in vitro and on wheat kernels but can trigger deoxynivalenol (DON) biosynthesis. *Food Control* **23**: 515-521
- Avila-Adame C, Koller W** (2003) Impact of alternative respiration and target-site mutations on responses of germinating conidia of *Magnaporthe grisea* to Qo-inhibiting fungicides. *Pest Management Science* **59**: 303-309
- Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM** (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. *In Annual Review of Plant Biology*, Vol 57, pp 233-266
- Bechet M, Caradec T, Hussein W, Abderrahmani A, Chollet M, Leclere V, Dubois T, Lereclus D, Pupin M, Jacques P** (2012) Structure, biosynthesis, and properties of kurstakins, nonribosomal lipopeptides from *Bacillus* spp. *Applied Microbiology and Biotechnology* **95**: 593-600
- Bennett JW, Klich M** (2003) Mycotoxins. *Clinical Microbiology Reviews* **16**: 497
- Beringer JE** (1974) R factor transfer in *Rhizobia leguminosarum*. *Journal of General Microbiology* **84**: 188-198
- Berry CL, Nandi M, Manuel J, Brassinga AKC, Fernando WGD, Loewen PC, de Kievit TR** (2014) Characterization of the *Pseudomonas* sp DF41 quorum sensing locus and its role in fungal antagonism. *Biological Control* **69**: 82-89
- Bertani G** (1951) Studies on lysogenesis.1. The mode of phage liberation by lysogenic *Escherichia coli*. *Journal of Bacteriology* **62**: 293-300
- Bloemberg GV, Otoole GA, Lugtenberg BJJ, Kolter R** (1997) Green fluorescent protein as a marker for *Pseudomonas* spp. *Applied and Environmental Microbiology* **63**: 4543-4551

- Blumer C, Haas D** (2000) Mechanism, regulation, and ecological role of bacterial cyanide biosynthesis. *Archives of Microbiology* **173**: 170-177
- Bonman JM, Khush GS, Nelson RJ** (1992) Breeding rice for resistance to pests. *Annual Review of Phytopathology* **30**: 507-528
- Bordiec S, Paquis S, Lacroix H, Dhondt S, Barka EA, Kauffmann S, Jeandet P, Mazeyrat-Gourbeyre F, Clement C, Baillieul F, Dorey S** (2011) Comparative analysis of defence responses induced by the endophytic plant growth-promoting rhizobacterium *Burkholderia phytofirmans* strain PsJN and the non-host bacterium *Pseudomonas syringae* pv. pisi in grapevine cell suspensions. *Journal of Experimental Botany* **62**: 595-603
- Brencic A, Winans SC** (2005) Detection of and response to signals involved in host-microbe interactions by plant-associated bacteria. *Microbiology and Molecular Biology Reviews* **69**: 155-+
- Burd GI, Dixon DG, Glick BR** (2000) Plant growth-promoting bacteria that decrease heavy metal toxicity in plants. *Canadian Journal of Microbiology* **46**: 237-245
- Burgess JG, Jordan EM, Bregu M, Mearns-Spragg A, Boyd KG** (1999) Microbial antagonism: a neglected avenue of natural products research. *Journal of Biotechnology* **70**: 27-32
- Cacique IS, Domiciano GP, Rodrigues FA, do Vale FXR** (2012) Silicon and manganese on rice resistance to blast. *Bragantia* **71**: 239-244
- Cai RM, Lewis J, Yan SC, Liu HJ, Clarke CR, Campanile F, Almeida NF, Studholme DJ, Lindeberg M, Schneider D, Zaccardelli M, Setubal JC, Morales-Lizcano NP, Bernal A, Coaker G, Baker C, Bender CL, Leman S, Vinatzer BA** (2011) The plant pathogen *Pseudomonas syringae* pv. tomato is genetically monomorphic and under strong selection to evade tomato immunity. *Plos Pathogens* **7**
- Cani PD, Delzenne NM** (2011) The gut microbiome as therapeutic target. *Pharmacology & Therapeutics* **130**: 202-212
- Carroll G** (1988) Fungal endophytes in stems and leaves: From latent pathogen to mutualistic symbiont. *Ecology* **69**: 2-9
- Carroll G** (1995) Forest endophytes - pattern and process. *Canadian Journal of Botany-Revue Canadienne De Botanique* **73**: S1316-S1324

- Chisholm ST, Coaker G, Day B, Staskawicz BJ** (2006) Host-microbe interactions: Shaping the evolution of the plant immune response. *Cell* **124**: 803-814
- Chithrashree, Udayashankar AC, Nayaka SC, Reddy MS, Srinivas C** (2011) Plant growth-promoting rhizobacteria mediate induced systemic resistance in rice against bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae*. *Biological Control* **59**: 114-122
- Chuma I, Isobe C, Hotta Y, Ibaragi K, Futamata N, Kusaba M, Yoshida K, Terauchi R, Fujita Y, Nakayashiki H, Valent B, Tosa Y** (2011) Multiple translocation of the AVR-Pita effector gene among chromosomes of the rice blast fungus *Magnaporthe oryzae* and related species. *Plos Pathogens* **7**
- Clark TN, Ellsworth K, Li H, Johnson JA, Gray CA** (2013) Isolation of the plant hormone (+) - abscisic acid as an antimycobacterial constituent of the medicinal plant endophyte, *Nigrospora* sp. *Natural Product Communications* **8**: 1673-1674
- Coca M, Bortolotti C, Rufat M, Penas G, Eritja R, Tharreau D, del Pozo AM, Messeguer J, San Segundo B** (2004) Transgenic rice plants expressing the antifungal AFP protein from *Aspergillus giganteus* show enhanced resistance to the rice blast fungus *Magnaporthe grisea*. *Plant Molecular Biology* **54**: 245-259
- Colborn T, Saal FSV, Soto AM** (1993) Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environmental Health Perspectives* **101**: 378-384
- Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, Kulam-Syed-Mohideen AS, McGarrell DM, Marsh T, Garrity GM, Tiedje JM** (2009) The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Research* **37**: D141-D145
- Costacurta A, Vanderleyden J** (1995) Synthesis of phytohormones by plant-associated bacteria. *Critical Reviews in Microbiology* **21**: 1-18
- Craker LE, Abeles FB** (1969) Abscission - Role of abscisic acid. *Plant Physiology* **44**: 1144
- da Rocha UN, van Overbeek L, van Elsas JD** (2009) Exploration of hitherto-uncultured bacteria from the rhizosphere. *Fems Microbiology Ecology* **69**: 313-328

- Danhorn T, Fuqua C** (2007) Biofilm formation by plant-associated bacteria. Annual Review of Microbiology **61**: 401-422
- Daval S, Lebreton L, Gazengel K, Boutin M, Guillermin-Erckelboudt AY, Sarniguet A** (2011) The biocontrol bacterium *Pseudomonas fluorescens* Pf29Arp strain affects the pathogenesis-related gene expression of the take-all fungus *Gaeumannomyces graminis* var. tritici on wheat roots. Molecular Plant Pathology **12**: 839-854
- De Vleeschauwer D, Djavaheiri M, Bakker P, Hofte M** (2008) *Pseudomonas fluorescens* WCS374r-induced systemic resistance in rice against *Magnaporthe oryzae* is based on pseudobactin-mediated priming for a salicylic acid-repressible multifaceted defense response. Plant Physiology **148**: 1996-2012
- De Vleeschauwer D, Hofte M** (2009) Rhizobacteria-induced systemic resistance. In LC VanLoon, ed, Plant Innate Immunity, Vol 51. Academic Press Ltd-Elsevier Science Ltd, London, pp 223-281
- de Weert S, Vermeiren H, Mulders IHM, Kuiper I, Hendrickx N, Bloemberg GV, Vanderleyden J, De Mot R, Lugtenberg BJJ** (2002) Flagella-driven chemotaxis towards exudate components is an important trait for tomato root colonization by *Pseudomonas fluorescens*. Molecular Plant-Microbe Interactions **15**: 1173-1180
- de Werra P, Pechy-Tarr M, Keel C, Maurhofer M** (2009) Role of gluconic acid production in the regulation of biocontrol traits of *Pseudomonas fluorescens* CHA0. Applied and Environmental Microbiology **75**: 4162-4174
- Dean R, Van Kan JAL, Pretorius ZA, Hammond-Kosack KE, Di Pietro A, Spanu PD, Rudd JJ, Dickman M, Kahmann R, Ellis J, Foster GD** (2012) The Top 10 fungal pathogens in molecular plant pathology. Molecular Plant Pathology **13**: 414-430
- deJong JC, McCormack BJ, Smirnov N, Talbot NJ** (1997) Glycerol generates turgor in rice blast. Nature **389**: 244-245
- Del Sorbo G, Schoonbeek HJ, De Waard MA** (2000) Fungal transporters involved in efflux of natural toxic compounds and fungicides. Fungal Genetics and Biology **30**: 1-15
- Denance N, Sanchez-Vallet A, Goffner D, Molina A** (2013) Disease resistance or growth: the role of plant hormones in balancing immune responses and fitness costs. Frontiers in Plant Science **4**

- Desjardins AE, Hohn TM, McCormick SP** (1993) Tricothecene biosynthesis in *Fusarium* species – chemistry, genetics, and significance. *Microbiological Reviews* **57**: 595-604
- Desjardins AE, Proctor RH, Bai GH, McCormick SP, Shaner G, Buechley G, Hohn TM** (1996) Reduced virulence of tricothecene-nonproducing mutants of *Gibberella zeae* in wheat field tests. *Molecular Plant-Microbe Interactions* **9**: 775-781
- Deweger LA, Jann B, Jann K, Lugtenberg B** (1987) Lipopolysaccharides of *Pseudomonas* spp that stimulate plant-growth - composition and use for strain identification. *Journal of Bacteriology* **169**: 1441-1446
- Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM, Zoeller RT, Gore AC** (2009) Endocrine-disrupting chemicals: An endocrine society scientific statement. *Endocrine Reviews* **30**: 293-342
- Dong SM, Yin WX, Kong GH, Yang XY, Qutob D, Chen QH, Kale SD, Sui YY, Zhang ZG, Dou DL, Zheng XB, Gijzen M, Tyler BM, Wang YC** (2011) *Phytophthora sojae* avirulence effector Avr3b is a secreted NADH and ADP-ribose pyrophosphorylase that modulates plant immunity. *Plos Pathogens* **7**
- Dong SM, Yu D, Cui LK, Qutob D, Tedman-Jones J, Kale SD, Tyler BM, Wang YC, Gijzen M** (2011) Sequence variants of the *Phytophthora sojae* RXLR Effector Avr3a/5 are differentially recognized by Rps3a and Rps5 in soybean. *Plos One* **6**
- Donofrio NM, Wilson RA** (2014) Redox and rice blast: newtools for dissecting molecular fungal-plant interactions. *New Phytologist* **201**: 367-369
- Dowling DN, Ogara F** (1994) Metabolites of *Pseudomonas* involved in the biocontrol of plant-disease. *Trends in Biotechnology* **12**: 133-141
- Dridi B, Raoult D, Drancourt M** (2011) Archaea as emerging organisms in complex human microbiomes. *Anaerobe* **17**: 56-63
- Dubuis C, Keel C, Haas D** (2007) Dialogues of root-colonizing biocontrol pseudomonads. *European Journal of Plant Pathology* **119**: 311-328
- Durrant WE, Dong X** (2004) Systemic acquired resistance. *Annual Review of Phytopathology* **42**: 185-209

- Egan MJ, Wang ZY, Jones MA, Smirnov N, Talbot NJ** (2007) Generation of reactive oxygen species by fungal NADPH oxidases is required for rice blast disease. *Proceedings of the National Academy of Sciences of the United States of America* **104**: 11772-11777
- Farooq M, Kobayashi N, Wahid A, Ito O, Basra SMA** (2009) Strategies for producing more rice with less water. *In* DL Sparks, ed, *Advances in Agronomy*, Vol 101, Vol 101. Elsevier Academic Press Inc, San Diego, pp 351-388
- Fernando WGD, Ramarathnam R, Krishnamoorthy AS, Savchuk SC** (2005) Identification and use of potential bacterial organic antifungal volatiles in biocontrol. *Soil Biology & Biochemistry* **37**: 955-964
- Filippi MCC, da Silva GB, Silva-Lobo VL, Cortes M, Moraes AJG, Prabhu AS** (2011) Leaf blast (*Magnaporthe oryzae*) suppression and growth promotion by rhizobacteria on aerobic rice in Brazil. *Biological Control* **58**: 160-166
- Fujita Y, Yoshida T, Yamaguchi-Shinozaki K** (2013) Pivotal role of the AREB/ABF-SnRK2 pathway in ABRE-mediated transcription in response to osmotic stress in plants. *Physiologia Plantarum* **147**: 15-27
- Fukui R, Poinar EI, Bauer PH, Schroth MN, Henderson M, Wang XL, Hancock JG** (1994) Spatial colonization patterns and interaction of bacteria on inoculated sugar-beet seed. *Phytopathology* **84**: 1338-1345
- Fuller R** (1989) Probiotics in man and animals. *Journal of Applied Bacteriology* **66**: 365-378
- Gandon S, Capowiez Y, Dubois Y, Michalakis Y, Olivieri I** (1996) Local adaptation and gene-for-gene coevolution in a metapopulation model. *Proceedings of the Royal Society of London Series B-Biological Sciences* **263**: 1003-1009
- Ganguly M, Roychoudhury A, Sarkar SN, Sengupta DN, Datta SK, Datta K** (2011) Inducibility of three salinity/abscisic acid-regulated promoters in transgenic rice with gusA reporter gene. *Plant Cell Reports* **30**: 1617-1625
- Gibson GR, Roberfroid MB** (1995) Dietary modulation of the human colonic microbiota - introducing the concept of prebiotics. *Journal of Nutrition* **125**: 1401-1412

- Giraldo MC, Dagdas YF, Gupta YK, Mentlak TA, Yi M, Martinez-Rocha AL, Saitoh H, Terauchi R, Talbot NJ, Valent B** (2013) Two distinct secretion systems facilitate tissue invasion by the rice blast fungus *Magnaporthe oryzae*. *Nature Communications* **4**
- Gnanamanickam S, Mew T** (1992) Biological control of blast disease of rice (*Oryza sativa* L.) with antagonistic bacteria and its mediation by a *Pseudomonas* antibiotic. *Japanese Journal of Phytopathology* **58**: 380-385
- Grover M, Ali SZ, Sandhya V, Rasul A, Venkateswarlu B** (2011) Role of microorganisms in adaptation of agriculture crops to abiotic stresses. *World Journal of Microbiology & Biotechnology* **27**: 1231-1240
- Gschwendtner S, Esperschuetz J, Buegger F, Reichmann M, Muller M, Munch JC, Schlöter M** (2011) Effects of genetically modified starch metabolism in potato plants on photosynthate fluxes into the rhizosphere and on microbial degraders of root exudates. *Fems Microbiology Ecology* **76**: 564-575
- Haas D, Keel C** (2003) Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance for biological control of plant disease. *Annual Review of Phytopathology* **41**: 117-153
- Haas D, Keel C, Reimmann C** (2002) Signal transduction in plant-beneficial rhizobacteria with biocontrol properties. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **81**: 385-395
- Hamada M, Hashimoto T, Takahashi S, Yoneyama M, Miyake T, Takeuchi Y, Okami Y, Umezawa H** (1967) Antifungal activity of kasugamycin. *J. Antibiot. B.* **20**: 424-426
- Hammer PE, Hill DS, Lam ST, VanPee KH, Ligon JM** (1997) Four genes from *Pseudomonas fluorescens* that encode the biosynthesis of pyrrolnitrin. *Applied and Environmental Microbiology* **63**: 2147-2154
- Han CU, Lee CH, Jang KS, Choi GJ, Lim HK, Kim JC, Ahn SN, Choi JE, Cha JS, Kim HT, Cho KY, Lee SW** (2004) Identification of rice genes induced in a rice blast-resistant mutant. *Molecules and Cells* **17**: 462-468
- Han JG, Sun L, Dong XZ, Cai ZQ, Sun XL, Yang HL, Wang YS, Song W** (2005) Characterization of a novel plant growth-promoting bacteria strain *Delftia tsuruhatensis* HR4 both as a diazotroph and a potential biocontrol agent against various plant pathogens. *Systematic and Applied Microbiology* **28**: 66-76

- Hartung W** (2010) The evolution of abscisic acid (ABA) and ABA function in lower plants, fungi and lichen. *Functional Plant Biology* **37**: 806-812
- Hauser F, Waadt R, Schroeder JI** (2011) Evolution of abscisic acid synthesis and signaling mechanisms. *Current Biology* **21**: R346-R355
- Havrankova H, Ovesna J** (2012) Genes of trichothecene biosynthesis in the *Fusarium* genus. *Chemicke Listy* **106**: 818-825
- Heil M, Bostock RM** (2002) Induced systemic resistance (ISR) against pathogens in the context of induced plant defences. *Annals of Botany* **89**: 503-512
- Hess RA, Nakai M** (2000) Histopathology of the male reproductive system induced by the fungicide benomyl. *Histology and Histopathology* **15**: 207-224
- Higgins CF** (1992) ABC transporters – from microorganisms to man. *Annual Review of Cell Biology* **8**: 67-113
- Hirai N, Yoshida R, Todoroki Y, Ohigashi H** (2000) Biosynthesis of abscisic acid by the non-mevalonate pathway in plants, and by the mevalonate pathway in fungi. *Bioscience Biotechnology and Biochemistry* **64**: 1448-1458
- Hirano SS, Upper CD** (2000) Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae* - a pathogen, ice nucleus, and epiphyte. *Microbiology and Molecular Biology Reviews* **64**: 624-+
- Hoitink HAJ, Boehm MJ** (1999) Biocontrol within the context of soil microbial communities: A substrate-dependent phenomenon. *Annual Review of Phytopathology* **37**: 427-446
- Howard RJ, Valent B** (1996) Breaking and entering: Host penetration by the fungal rice blast pathogen *Magnaporthe grisea*. *Annual Review of Microbiology* **50**: 491-512
- Howell CR, Beier RC, Stipanovic RD** (1988) Production of ammonia by *Enterobacter-cloacae* and its possible role in the biological control of *Pythium* preemergence damping-off by the bacterium. *Phytopathology* **78**: 1075-1078
- Howell CR, Stipanovic RD** (1979) Control of *Rhizoctonia solani* on cotton seedlings with *Pseudomonas fluorescens* and with an antibiotic produced by the bacterium. *Phytopathology* **69**: 480-482

- Howell CR, Stipanovic RD** (1980) Suppression of *Pythium ultimum*-induced damping-off of cotton seedlings by *Pseudomonas fluorescens* and its antibiotic, pyoluteorin. *Phytopathology* **70**: 712-715
- Hugouvieux V, Barber CE, Daniels MJ** (1998) Entry of *Xanthomonas campestris* pv. *campestris* into hydathodes of *Arabidopsis thaliana* leaves: A system for studying early infection events in bacterial pathogenesis. *Molecular Plant-Microbe Interactions* **11**: 537-543
- Iavicoli A, Boutet E, Buchala A, Mettraux JP** (2003) Induced systemic resistance in *Arabidopsis thaliana* in response to root inoculation with *Pseudomonas fluorescens* CHA0. *Molecular Plant-Microbe Interactions* **16**: 851-858
- Imamura T, Yasuda M, Kusano H, Nakashita H, Ohno Y, Kamakura T, Taguchi S, Shimada H** (2010) Acquired resistance to the rice blast in transgenic rice accumulating the antimicrobial peptide thanatin. *Transgenic Research* **19**: 415-424
- Iwata M** (2001) Probenazole- a plant defense activator. *Pesticide outlook*: 28-31
- Jaeger CH, Lindow SE, Miller S, Clark E, Firestone MK** (1999) Mapping of sugar and amino acid availability in soil around roots with bacterial sensors of sucrose and tryptophan. *Applied and Environmental Microbiology* **65**: 2685-2690
- Jansen C, von Wettstein D, Schafer W, Kogel KH, Felk A, Maier FJ** (2005) Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted *Fusarium graminearum*. *Proceedings of the National Academy of Sciences of the United States of America* **102**: 16892-16897
- Jha S, Chattoo BB** (2010) Expression of a plant defensin in rice confers resistance to fungal phytopathogens. *Transgenic Research* **19**: 373-384
- Jiang CJ, Shimono M, Sugano S, Kojima M, Yazawa K, Yoshida R, Inoue H, Hayashi N, Sakakibara H, Takatsuji H** (2010) Absciscic acid interacts antagonistically with salicylic acid signaling pathway in rice-*Magnaporthe grisea* interaction. *Molecular Plant-Microbe Interactions* **23**: 791-798
- Jiang JH, Yun YZ, Fu J, Shim WB, Ma ZH** (2011) Involvement of a putative response regulator FgRrg-1 in osmotic stress response, fungicide resistance and virulence in *Fusarium graminearum*. *Molecular Plant Pathology* **12**: 425-436

- Jiang Y, Peng D, Bai LP, Ma H, Chen LJ, Zhao MH, Xu ZJ, Guo ZF** (2013) Molecular switch for cold acclimation - anatomy of the cold-inducible promoter in plants. *Biochemistry-Moscow* **78**: 342-354
- Jiao Z, Wu N, Hale L, Wu W, Wu D, Guo Y** (2013) Characterisation of *Pseudomonas chlororaphis* subsp *aurantiaca* strain Pa40 with the ability to control wheat sharp eyespot disease. *Annals of Applied Biology* **163**: 444-453
- Jonkers W, Dong YH, Broz K, Kistler HC** (2012) The Wor1-like protein Fgpl regulates pathogenicity, toxin synthesis and reproduction in the phytopathogenic fungus *Fusarium graminearum*. *Plos Pathogens* **8**
- Jung HI, Kuk YI, Kim HY, Back K, Lee DJ, Lee S, Burgos NR** (2010) Resistance levels and fitness of protoporphyrinogen oxidase (PROTOX) inhibitor-resistant transgenic rice in paddy fields. *Field Crops Research* **115**: 125-131
- Kai M, Haustein M, Molina F, Petri A, Scholz B, Piechulla B** (2009) Bacterial volatiles and their action potential. *Applied Microbiology and Biotechnology* **81**: 1001-1012
- Kanjanamaneesathian M, Kusonwiriawong C, Pengnoo A, Nilratana L** (1998) Screening of potential bacterial antagonists for control of sheath blight in rice and development of suitable bacterial formulations for effective application. *Australasian Plant Pathology* **27**: 198-206
- Kankanala P, Czymmek K, Valent B** (2007) Roles for rice membrane dynamics and plasmodesmata during biotrophic invasion by the blast fungus. *Plant Cell* **19**: 706-724
- Kaplan JL, Shi HN, Walker WA** (2011) The role of microbes in developmental immunologic programming. *Pediatric Research* **69**: 465-472
- Karthikeyan V, Gnanamanickam SS** (2008) Biological control of *Setaria* blast (*Magnaporthe grisea*) with bacterial strains. *Crop Protection* **27**: 263-267
- Kato H** (2001) Rice blast disease. *Pesticide Outlook* **12**: 23-25
- Kato-Noguchi H** (2009) Stress-induced allelopathic activity and momilactone B in rice. *Plant Growth Regulation* **59**: 153-158
- Keel C, Weller DM, Natsch A, Defago G, Cook RJ, Thomashow LS** (1996) Conservation of the 2,4-diacetylphloroglucinol biosynthesis locus among fluorescent *Pseudomonas* strains from diverse geographic locations. *Applied and Environmental Microbiology* **62**: 552-563

- Kepka M, Benson CL, Gonugunta VK, Nelson KM, Christmann A, Grill E, Abrams SR** (2011) Action of natural abscisic acid precursors and catabolites on abscisic acid receptor complexes. *Plant Physiology* **157**: 2108-2119
- Kim H, Hwang H, Hong JW, Lee YN, Ahn IP, Yoon IS, Yoo SD, Lee S, Lee SC, Kim BG** (2012) A rice orthologue of the ABA receptor, OsPYL/RCAR5, is a positive regulator of the ABA signal transduction pathway in seed germination and early seedling growth. *Journal of Experimental Botany* **63**: 1013-1024
- Kim H, Lee K, Hwang H, Bhatnagar N, Kim DY, Yoon IS, Byun MO, Kim ST, Jung KH, Kim BG** (2014) Overexpression of PYL5 in rice enhances drought tolerance, inhibits growth, and modulates gene expression. *Journal of Experimental Botany* **65**: 453-464
- Kim SY** (2007) Recent advances in ABA signaling. *Journal of Plant Biology* **50**: 117-121
- Kim TH** (2012) Plant stress surveillance monitored by ABA and disease signaling interactions. *Molecules and Cells* **33**: 1-7
- Kim TH** (2014) Mechanism of ABA signal transduction: Agricultural highlights for improving drought tolerance. *Journal of Plant Biology* **57**: 1-8
- Knief C, Delmotte N, Vorholt JA** (2011) Bacterial adaptation to life in association with plants - A proteomic perspective from culture to in situ conditions. *Proteomics* **11**: 3086-3105
- Koga H, Dohi K, Mori M** (2004) Absciscic acid and low temperatures suppress the whole plant-specific resistance reaction of rice plants to the infection of *Magnaporthe grisea*. *Physiological and Molecular Plant Pathology* **65**: 3-9
- Kohler T, Curty LK, Barja F, van Delden C, Pechere JC** (2000) Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. *Journal of Bacteriology* **182**: 5990-5996
- Kong HDH** (2011) Skin microbiome: genomics-based insights into the diversity and role of skin microbes. *Trends in Molecular Medicine* **17**: 320-328
- Koornneef M, Bentsink L, Hilhorst H** (2002) Seed dormancy and germination. *Current Opinion in Plant Biology* **5**: 33-36

- Kravchenko LV, Shapozhnikov AI, Makarova NM, Azarova TS, L'Vova KA, Kostyuk, II, Lyapunova OA, Tikhonovich IA** (2011) Exometabolites of bread wheat and tomato affecting the plant-microbe interactions in the rhizosphere. *Russian Journal of Plant Physiology* **58**: 936-940
- Krishnamurthy K, Gnanamanickam SS** (1998) Biological control of rice blast by *Pseudomonas fluorescens* strain Pf7-14: Evaluation of a marker gene and formulations. *Biological Control* **13**: 158-165
- Krishnamurthy K, Gnanamanickam SS** (1998) Induction of systemic resistance and salicylic acid accumulation in *Oryza sativa*, L. in the biological suppression of rice blast caused by treatments with *Pseudomonas* spp. *World Journal of Microbiology & Biotechnology* **14**: 935-937
- Kulik A, Wawer I, Krzywinska E, Bucholc M, Dobrowolska G** (2011) SnRK2 protein kinases-key regulators of plant response to abiotic stresses. *Omics-a Journal of Integrative Biology* **15**: 859-872
- Kulik T, Lojko M, Jestoi M, Perkowski J** (2012) Sublethal concentrations of azoles induce tri transcript levels and trichothecene production in *Fusarium graminearum*. *Fems Microbiology Letters* **335**: 58-67
- Kumar AS, Lakshmanan V, Caplan JL, Powell D, Czymmek KJ, Levia DF, Bais HP** (2012) Rhizobacteria *Bacillus subtilis* restricts foliar pathogen entry through stomata. *Plant Journal* **72**: 694-706
- Lakshmanan V, Kitto SL, Caplan JL, Hsueh YH, Kearns DB, Wu YS, Bais HP** (2012) Microbe-associated molecular patterns-triggered root responses mediate beneficial rhizobacterial recruitment in *Arabidopsis*. *Plant Physiology* **160**: 1642-1661
- Lamb C, Dixon RA** (1997) The oxidative burst in plant disease resistance. *Annual Review of Plant Physiology and Plant Molecular Biology* **48**: 251-275
- Lan A, Huang J, Zhao W, Peng Y, Chen Z, Kang D** (2013) A salicylic acid-induced rice (*Oryza sativa* L.) transcription factor OsWRKY77 is involved in disease resistance of *Arabidopsis thaliana*. *Plant Biology* **15**: 452-461
- Laville J, Blumer C, Von Schroetter C, Gaia V, Defago G, Keel C, Haas D** (1998) Characterization of the hcnABC gene cluster encoding hydrogen cyanide synthase and anaerobic regulation by ANR in the strictly aerobic biocontrol agent *Pseudomonas fluorescens* CHAO. *Journal of Bacteriology* **180**: 3187-3196

- Lee KH, Piao HL, Kim HY, Choi SM, Jiang F, Hartung W, Hwang I, Kwak JM, Lee IJ** (2006) Activation of glucosidase via stress-induced polymerization rapidly increases active pools of abscisic acid. *Cell* **126**: 1109-1120
- Lee YJ, Yamamoto K, Hamamoto H, Nakaune R, Hibi T** (2005) A novel ABC transporter gene ABC2 involved in multidrug susceptibility but not pathogenicity in rice blast fungus, *Magnaporthe grisea*. *Pesticide Biochemistry and Physiology* **81**: 13-23
- Leelasuphakul W, Sivanunsakul P, Phongpaichit S** (2006) Purification, characterization and synergistic activity of beta-1,3-glucanase and antibiotic extract from an antagonistic *Bacillus subtilis* NSRS 89-24 against rice blast and sheath blight. *Enzyme and Microbial Technology* **38**: 990-997
- Leveau JHJ, Lindow SE** (2001) Appetite of an epiphyte: Quantitative monitoring of bacterial sugar consumption in the phyllosphere. *Proceedings of the National Academy of Sciences of the United States of America* **98**: 3446-3453
- Li H, Zhou SY, Zhao WS, Su SC, Peng YL** (2009) A novel wall-associated receptor-like protein kinase gene, OsWAK1, plays important roles in rice blast disease resistance. *Plant Molecular Biology* **69**: 337-346
- Li P, Pei Y, Sang XC, Ling YH, Yang ZL, He GH** (2009) Transgenic indica rice expressing a bitter melon (*Momordica charantia*) class I chitinase gene (McCHIT1) confers enhanced resistance to *Magnaporthe grisea* and *Rhizoctonia solani*. *European Journal of Plant Pathology* **125**: 533-543
- Li QL, Jiang YH, Ning P, Zheng L, Huang JB, Li GQ, Jiang DH, Hsiang T** (2011) Suppression of *Magnaporthe oryzae* by culture filtrates of *Streptomyces globisporus* JK-1. *Biological Control* **58**: 139-148
- Li SH, Oakley CE, Chen GF, Han XY, Oakley BR, Xiang X** (2005) Cytoplasmic dynein's mitotic spindle pole localization requires a functional anaphase-promoting complex, gamma-tubulin, and NUDF/LIS1 in *Aspergillus nidulans*. *Molecular Biology of the Cell* **16**: 3591-3605
- Li W, Wang BH, Wu J, Lu GD, Hu YJ, Zhang X, Zhang ZG, Zhao Q, Feng QY, Zhang HY, Wang ZY, Wang GL, Han B, Wang ZH, Zhou B** (2009) The *Magnaporthe oryzae* avirulence gene AvrPiz-t encodes a predicted secreted protein that triggers the immunity in rice mediated by the blast resistance gene Piz-t. *Molecular Plant-Microbe Interactions* **22**: 411-420
- Lim CW, Baek W, Lim S, Lee SC** (2012) ABA signal transduction from ABA receptors to ion channels. *Genes & Genomics* **34**: 345-353

- Linares JF, Gustafsson I, Baquero F, Martinez JL** (2006) Antibiotics as intermicrobial signaling agents instead of weapons. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 19484-19489
- Lindow SE, Arny DC, Upper CD** (1982) Bacterial ice nucleation - A factor in frost injury to plants. *Plant Physiology* **70**: 1084-1089
- Ling N, Raza W, Ma JH, Huang QW, Shen QR** (2011) Identification and role of organic acids in watermelon root exudates for recruiting *Paenibacillus polymyxa* SQR-21 in the rhizosphere. *European Journal of Soil Biology* **47**: 374-379
- Liotenberg S, North H, Marion-Poll A** (1999) Molecular biology and regulation of abscisic acid biosynthesis in plants. *Plant Physiology and Biochemistry* **37**: 341-350
- Liu HJ, Colavitti R, Rovira, II, Finkel T** (2005) Redox-dependent transcriptional regulation. *Circulation Research* **97**: 967-974
- Liu XG, Bimerew M, Ma YX, Mueller H, Ovadis M, Eberl L, Berg G, Chernin L** (2007) Quorum-sensing signaling is required for production of the antibiotic pyrrolnitrin in a rhizospheric biocontrol strain of *Serratia plymuthica*. *Fems Microbiology Letters* **270**: 299-305
- Loake G, Grant M** (2007) Salicylic acid in plant defence-the players and protagonists. *Current Opinion in Plant Biology* **10**: 466-472
- Lucas JA, Solano BR, Montes F, Ojeda J, Megias M, Manero FJG** (2009) Use of two PGPR strains in the integrated management of blast disease in rice (*Oryza sativa*) in Southern Spain. *Field Crops Research* **114**: 404-410
- Lucero ME, Unc A, Cooke P, Dowd S, Sun SL** (2011) Endophyte microbiome diversity in micropropagated *Atriplex canescens* and *Atriplex torreyi* var griffithsii. *Plos One* **6**: 12
- Lugtenberg B, Kamilova F** (2009) Plant-Growth-Promoting Rhizobacteria. *Annual Review of Microbiology* **63**: 541-556
- Lugtenberg BJJ, Dekkers L, Bloembergen GV** (2001) Molecular determinants of rhizosphere colonization by *Pseudomonas*. *Annual Review of Phytopathology* **39**: 461-+

- Machado CR, deOliveira RLC, Boiteux S, Praekelt UM, Meacock PA, Lenck CFM** (1996) Thi1, a thiamine biosynthetic gene in *Arabidopsis thaliana*, complements bacterial defects in DNA repair. *Plant Molecular Biology* **31**: 585-593
- Malamy J, SanchezCasas P, Hennig J, Guo AL, Klessig DF** (1996) Dissection of the salicylic acid signaling pathway in tobacco. *Molecular Plant-Microbe Interactions* **9**: 474-482
- Marcel S, Sawers R, Oakeley E, Angliker H, Paszkowski U** (2010) Tissue-adapted invasion strategies of the rice blast fungus *Magnaporthe oryzae*. *Plant Cell* **22**: 3177-3187
- Mares D, Romagnoli C, Andreotti E, Forlani G, Guccione S, Vicentini CB** (2006) Emerging antifungal azoles and effects on *Magnaporthe grisea*. *Mycological Research* **110**: 686-696
- Marshall R, Kombrink A, Motteram J, Loza-Reyes E, Lucas J, Hammond-Kosack KE, Thomma B, Rudd JJ** (2011) Analysis of two in planta expressed LysM effector homologs from the fungus *Mycosphaerella graminicola* reveals novel functional properties and varying contributions to virulence on wheat. *Plant Physiology* **156**: 756-769
- Martinez-Romero E** (2009) Coevolution in rhizobium-legume symbiosis? *DNA and Cell Biology* **28**: 361-370
- Mathioni SM, Belo A, Rizzo CJ, Dean RA, Donofrio NM** (2011) Transcriptome profiling of the rice blast fungus during invasive plant infection and in vitro stresses. *Bmc Genomics* **12**
- Meguro A, Sato Y** (2014) Salicylic acid antagonizes abscisic acid inhibition of shoot growth and cell cycle progression in rice. *Scientific Reports* **4**
- Mei CS, Qi M, Sheng GY, Yang YN** (2006) Inducible overexpression of a rice allene oxide synthase gene increases the endogenous jasmonic acid level, PR gene expression, and host resistance to fungal infection. *Molecular Plant-Microbe Interactions* **19**: 1127-1137
- Mendes R, Kruijt M, de Bruijn I, Dekkers E, van der Voort M, Schneider JHM, Piceno YM, DeSantis TZ, Andersen GL, Bakker P, Raaijmakers JM** (2011) Deciphering the rhizosphere microbiome for disease-suppressive bacteria. *Science* **332**: 1097-1100

- Meng SW, Brown DE, Ebbole DJ, Torto-Alalibo T, Oh YY, Deng JX, Mitchell TK, Dean RA** (2009) Gene Ontology annotation of the rice blast fungus, *Magnaporthe oryzae*. *Bmc Microbiology* **9**
- Mentlak TA, Kombrink A, Shinya T, Ryder LS, Otomo I, Saitoh H, Terauchi R, Nishizawa Y, Shibuya N, Thomma B, Talbot NJ** (2012) Effector-mediated suppression of chitin-triggered immunity by *Magnaporthe oryzae* is necessary for rice blast disease. *Plant Cell* **24**: 322-335
- Merhej J, Richard-Forget F, Barreau C** (2011) Regulation of trichothecene biosynthesis in *Fusarium*: recent advances and new insights. *Applied Microbiology and Biotechnology* **91**: 519-528
- Miller MB, Bassler BL** (2001) Quorum sensing in bacteria. *Annual Review of Microbiology* **55**: 165-199
- Morris CE, Monier JM** (2003) The ecological significance of biofilm formation by plant-associated bacteria. *Annual Review of Phytopathology* **41**: 429-453
- Mosquera-Espinosa AT, Bayman P, Prado GA, Gomez-Carabali A, Otero JT** (2013) The double life of *Ceratobasidium*: orchid mycorrhizal fungi and their potential for biocontrol of *Rhizoctonia solani* sheath blight of rice. *Mycologia* **105**: 141-150
- Mousivand M, Jouzani GS, Monazah M, Kowsari M** (2012) Characterization and antagonistic potential of some native biofilm-forming and surfactant-producing *Bacillus subtilis* strains against six pathotypes of *Rhizoctonia solani*. *Journal of Plant Pathology* **94**: 171-180
- Nakano T, Suzuki K, Fujimura T, Shinshi H** (2006) Genome-wide analysis of the ERF gene family in *Arabidopsis* and rice. *Plant Physiology* **140**: 411-432
- Nambara E, Marion-Poll A** (2005) Absciscic acid biosynthesis and catabolism. *Annual Review of Plant Biology* **56**: 165-185
- Nandakumar R, Babu S, Viswanathan R, Raguchander T, Samiyappan R** (2001) Induction of systemic resistance in rice against sheath blight disease by *Pseudomonas fluorescens*. *Soil Biology & Biochemistry* **33**: 603-612
- Nandi S, Suzuki YA, Huang JM, Yalda D, Pham P, Wu LY, Bartley G, Huang N, Lonnerdal B** (2002) Expression of human lactoferrin in transgenic rice grains for the application in infant formula. *Plant Science* **163**: 713-722

- Naureen Z, Price AH, Hafeez FY, Roberts MR** (2009) Identification of rice blast disease-suppressing bacterial strains from the rhizosphere of rice grown in Pakistan. *Crop Protection* **28**: 1052-1060
- Neidig N, Paul RJ, Scheu S, Jousset A** (2011) Secondary metabolites of *Pseudomonas fluorescens* CHA0 drive complex non-trophic interactions with bacterivorous nematodes. *Microbial Ecology* **61**: 853-859
- Nielsen TH, Christophersen C, Anthoni U, Sorensen J** (1999) Viscosinamide, a new cyclic depsipeptide with surfactant and antifungal properties produced by *Pseudomonas fluorescens* DR54. *Journal of Applied Microbiology* **87**: 80-90
- Nielsen TH, Thrane C, Christophersen C, Anthoni U, Sorensen J** (2000) Structure, production characteristics and fungal antagonism of tensin - a new antifungal cyclic lipopeptide from *Pseudomonas fluorescens* strain 96.578. *Journal of Applied Microbiology* **89**: 992-1001
- Nikolaev ON, Averyanov AA** (1991) Involvement of superoxide radical in the mechanism of fungicidal action of phthalide and probenazol. *Soviet Plant Physiology* **38**: 375-381
- Nomoto S, Mori K** (1997) Synthetic microbial chemistry .30. Synthesis of acetophthalidin, a fungal metabolite which inhibits the progression of the mammalian cell cycle. *Liebigs Annalen-Recueil*: 721-723
- Nowak-Thompson B, Chaney N, Wing JS, Gould SJ, Loper JE** (1999) Characterization of the pyoluteorin biosynthetic gene cluster of *Pseudomonas fluorescens* Pf-5. *Journal of Bacteriology* **181**: 2166-2174
- Ofek M, Hadar Y, Minz D** (2011) Colonization of cucumber seeds by bacteria during germination. *Environmental Microbiology* **13**: 2794-2807
- Oide S, Bejai S, Staal J, Guan N, Kaliff M, Dixelius C** (2013) A novel role of PR2 in abscisic acid (ABA) mediated, pathogen-induced callose deposition in *Arabidopsis thaliana*. *New Phytologist* **200**: 1187-1199
- Osharov N, May GS** (2001) The molecular mechanisms of conidial germination. *Fems Microbiology Letters* **199**: 153-160
- Pareja L, Colazzo M, Perez-Parada A, Besil N, Heinzen H, Bocking B, Cesio V, Fernandez-Alba AR** (2012) Occurrence and distribution study of residues from pesticides applied under controlled conditions in the field during rice processing. *Journal of Agricultural and Food Chemistry* **60**: 4440-4448

- Patten CL, Glick BR** (1996) Bacterial biosynthesis on indole-3-acetic acid. *Canadian Journal of Microbiology* **42**: 207-220
- Pedraza RO, Motok J, Salazar SM, Ragout AL, Mentel MI, Tortora ML, Guerrero-Molina MF, Winik BC, Diaz-Ricci JC** (2010) Growth-promotion of strawberry plants inoculated with *Azospirillum brasilense*. *World Journal of Microbiology & Biotechnology* **26**: 265-272
- Peng XX, Hu YJ, Tang XK, Zhou PL, Deng XB, Wang HH, Guo ZJ** (2012) Constitutive expression of rice *WRKY30* gene increases the endogenous jasmonic acid accumulation, PR gene expression and resistance to fungal pathogens in rice. *Planta* **236**: 1485-1498
- Pessi G, Haas D** (2000) Transcriptional control of the hydrogen cyanide biosynthetic genes *hcnABC* by the anaerobic regulator ANR and the quorum-sensing regulators LasR and RhIR in *Pseudomonas aeruginosa*. *Journal of Bacteriology* **182**: 6940-6949
- Pieterse CMJ, Van Pelt JA, Van Wees SCM, Ton J, Leon-Kloosterziel KM, Keurentjes JJB, Verhagen BWM, Knoester M, Van der Sluis I, Bakker P, Van Loon LC** (2001) Rhizobacteria-mediated induced systemic resistance: Triggering, signalling and expression. *European Journal of Plant Pathology* **107**: 51-61
- Pieterse CMJ, vanWees SCM, Hoffland E, vanPelt JA, vanLoon LC** (1996) Systemic resistance in *Arabidopsis* induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. *Plant Cell* **8**: 1225-1237
- Pimbucha R, Kaiser HM** (2009) Measuring the effectiveness of US rice export promotion programs. *Agribusiness* **25**: 215-230
- Podila GK, Sreedasyam A, Muratet MA** (2009) Populus rhizosphere and the ectomycorrhizal interactome. *Critical Reviews in Plant Sciences* **28**: 359-367
- Popov VO, Lamzin VS** (1994) NAD(+)-dependent formate dehydrogenase. *Biochemical Journal* **301**: 625-643
- Porcel R, Zamarreno AM, Garcia-Mina JM, Aroca R** (2014) Involvement of plant endogenous ABA in *Bacillus megaterium* PGPR activity in tomato plants. *Bmc Plant Biology* **14**

- Porras-Alfaro A, Bayman P** (2011) Hidden fungi, emergent properties: Endophytes and microbiomes. *In* NK VanAlfen, G Bruening, JE Leach, eds, Annual Review of Phytopathology, Vol 49, Vol 49. Annual Reviews, Palo Alto, pp 291-315
- Pozo MJ, Van Der Ent S, Van Loon LC, Pieterse CMJ** (2008) Transcription factor MYC2 is involved in priming for enhanced defense during rhizobacteria-induced systemic resistance in *Arabidopsis thaliana*. *New Phytologist* **180**: 511-523
- Pratt LA, Kolter R** (1998) Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. *Molecular Microbiology* **30**: 285-293
- Priest DM, Ambrose SJ, Vaistij FE, Elias L, Higgins GS, Ross ARS, Abrams SR, Bowles DJ** (2006) Use of the glucosyltransferase UGT71B6 to disturb abscisic acid homeostasis in *Arabidopsis thaliana*. *Plant Journal* **46**: 492-502
- Pushpalatha HG, Sudisha J, Geetha NP, Amruthesh KN, Shetty HS** (2011) Thiamine seed treatment enhances LOX expression, promotes growth and induces downy mildew disease resistance in pearl millet. *Biologia Plantarum* **55**: 522-527
- Qin XQ, Zeevaart JAD** (1999) The 9-cis-epoxycarotenoid cleavage reaction is the key regulatory step of abscisic acid biosynthesis in water-stressed bean. *Proceedings of the National Academy of Sciences of the United States of America* **96**: 15354-15361
- Quilis J, Penas G, Messeguer J, Brugidou C, Segundo BS** (2008) The *Arabidopsis* AtNPR1 inversely modulates defense responses against fungal, bacterial, or viral pathogens while conferring hypersensitivity to abiotic stresses in transgenic rice. *Molecular Plant-Microbe Interactions* **21**: 1215-1231
- Raaijmakers JM, Weller DM, Thomashow LS** (1997) Frequency of antibiotic-producing *Pseudomonas* spp. in natural environments. *Applied and Environmental Microbiology* **63**: 881-887
- Radutoiu S, Madsen LH, Madsen EB, Felle HH, Umehara Y, Gronlund M, Sato S, Nakamura Y, Tabata S, Sandal N, Stougaard J** (2003) Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. *Nature* **425**: 585-592
- Rainey PB** (1999) Adaptation of *Pseudomonas fluorescens* to the plant rhizosphere. *Environmental Microbiology* **1**: 243-257

- Raio A, Puopolo G, Cimmino A, Danti R, Della Rocca G, Evidente A** (2011) Biocontrol of cypress canker by the phenazine producer *Pseudomonas chlororaphis* subsp aureofaciens strain M71. *Biological Control* **58**: 133-138
- Ramarathnam R, Fernando WGD, de Kievit T** (2011) The role of antibiosis and induced systemic resistance, mediated by strains of *Pseudomonas chlororaphis*, *Bacillus cereus* and *B. amyloliquefaciens*, in controlling blackleg disease of canola. *Biocontrol* **56**: 225-235
- Ramey BE, Koutsoudis M, von Bodman SB, Fuqua C** (2004) Biofilm formation in plant-microbe associations. *Current Opinion in Microbiology* **7**: 602-609
- Rashid MH, Kornberg A** (2000) Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences of the United States of America* **97**: 4885-4890
- Rasmussen TB, Givskov M** (2006) Quorum sensing inhibitors: a bargain of effects. *Microbiology-Sgm* **152**: 895-904
- Reid RJD, Sunjevaric I, Kedacche M, Rothstein R** (2002) Efficient PCR-based gene disruption in *Saccharomyces* strains using intergenic primers. *Yeast* **19**: 319-328
- Robert-Seilaniantz A, Grant M, Jones JDG** (2011) Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. *In* NK VanAlfen, G Bruening, JE Leach, eds, *Annual Review of Phytopathology*, Vol 49, Vol 49, pp 317-343
- Roinestad KS, Montville TJ, Rosen JD** (1994) Mechanism for sodium-bicarbonate inhibition of trochothecene biosynthesis in *Fusarium tricinctum*. *Journal of Agricultural and Food Chemistry* **42**: 2025-2028
- Rosenberg E, Sharon G, Atad I, Zilber-Rosenberg I** (2010) The evolution of animals and plants via symbiosis with microorganisms. *Environmental Microbiology Reports* **2**: 500-506
- Roychoudhury A, Paul S, Basu S** (2013) Cross-talk between abscisic acid-dependent and abscisic acid-independent pathways during abiotic stress. *Plant Cell Reports* **32**: 985-1006

- Roychoudhury A, Roy C, Sengupta DN** (2007) Transgenic tobacco plants overexpressing the heterologous *lea* gene *Rab16A* from rice during high salt and water deficit display enhanced tolerance to salinity stress. *Plant Cell Reports* **26**: 1839-1859
- Rudramurthy SM, Chakrabarti A, Geertsens E, Mouton JW, Meis JF** (2011) In vitro activity of isavuconazole against 208 *Aspergillus flavus* isolates in comparison with 7 other antifungal agents: assessment according to the methodology of the European Committee on Antimicrobial Susceptibility Testing. *Diagnostic Microbiology and Infectious Disease* **71**: 370-377
- Rudrappa T, Biedrzycki M, Kunjeti S, Donofrio N, Czymmek K, Pare P, Bais H** (2010) The rhizobacterial elicitor acetoin induced systemic resistance in *Arabidopsis thaliana*. *Communicative & Integrative Biology* **3**: 1-9
- Rudrappa T, Biedrzycki ML, Bais HP** (2008) Causes and consequences of plant-associated biofilms. *Fems Microbiology Ecology* **64**: 153-166
- Rudrappa T, Czymmek KJ, Pare PW, Bais HP** (2008) Root-Secreted malic acid recruits beneficial soil bacteria. *Plant Physiology* **148**: 1547-1556
- Rudrappa T, Splaine RE, Biedrzycki ML, Bais HP** (2008) Cyanogenic pseudomonads influence multitrophic interactions in the rhizosphere. *Plos One* **3**
- Ruiz-Roldan C, Puerto-Galan L, Roa J, Castro A, Di Pietro A, Roncero MIG, Hera C** (2008) The *Fusarium oxysporum* *sti35* gene functions in thiamine biosynthesis and oxidative stress response. *Fungal Genetics and Biology* **45**: 6-16
- Ryder LS, Dagdas YF, Mentlak TA, Kershaw MJ, Thornton CR, Schuster M, Chen JS, Wang ZH, Talbot NJ** (2013) NADPH oxidases regulate septin-mediated cytoskeletal remodeling during plant infection by the rice blast fungus. *Proceedings of the National Academy of Sciences of the United States of America* **110**: 3179-3184
- Ryu CM, Farag MA, Hu CH, Reddy MS, Kloepper JW, Pare PW** (2004) Bacterial volatiles induce systemic resistance in *Arabidopsis*. *Plant Physiology* **134**: 1017-1026
- Ryu CM, Farag MA, Hu CH, Reddy MS, Wei HX, Pare PW, Kloepper JW** (2003) Bacterial volatiles promote growth in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America* **100**: 4927-4932

- Saika H, Okamoto M, Miyoshi K, Kushihiro T, Shinoda S, Jikumaru Y, Fujimoto M, Arikawa T, Takahashi H, Ando M, Arimura S, Miyao A, Hirochika H, Kamiya Y, Tsutsumi N, Nambara E, Nakazono M** (2007) Ethylene promotes submergence-induced expression of OsABA8ox1, a gene that encodes ABA 8'-hydroxylase in rice. *Plant and Cell Physiology* **48**: 287-298
- Sakurai H, Naito H, Fujita S** (1976) Sensitivity distribution of phytopathogenic bacteria and fungi to antibiotics. *Journal of Antibiotics* **29**: 1230-1236
- Samalova M, Meyer AJ, Gurr SJ, Fricker MD** (2014) Robust anti-oxidant defences in the rice blast fungus *Magnaporthe oryzae* confer tolerance to the host oxidative burst. *New Phytologist* **201**: 556-573
- Sanogo I, Amadou MM** (2010) Rice market integration and food security in Nepal: The role of cross-border trade with India. *Food Policy* **35**: 312-322
- Santiago J, Dupeux F, Betz K, Antoni R, Gonzalez-Guzman M, Rodriguez L, Marquez JA, Rodriguez PL** (2012) Structural insights into PYR/PYL/RCAR ABA receptors and PP2Cs. *Plant Science* **182**: 3-11
- Schardl CL, Leuchtmann A, Chung KR, Penny D, Siegel MR** (1997) Coevolution by common descent of fungal symbionts (*Epichloe* spp) and grass hosts. *Molecular Biology and Evolution* **14**: 133-143
- Schmidt K, Pflugmacher M, Klages S, Maser A, Mock A, Stahl DJ** (2008) Accumulation of the hormone abscisic acid (ABA) at the infection site of the fungus *Cercospora beticola* supports the role of ABA as a repressor of plant defence in sugar beet. *Molecular Plant Pathology* **9**: 661-673
- Schroth MN, Hancock JG** (1982) Disease-suppressive soil and root-colonizing bacteria. *Science* **216**: 1376-1381
- Schulze-Lefert P, Panstruga R** (2011) A molecular evolutionary concept connecting nonhost resistance, pathogen host range, and pathogen speciation. *Trends in Plant Science* **16**: 117-125
- Schwartz SH, Qin XQ, Zeevaart JAD** (2003) Elucidation of the indirect pathway of abscisic acid biosynthesis by mutants, genes, and enzymes. *Plant Physiology* **131**: 1591-1601
- Schwartz SH, Tan BC, Gage DA, Zeevaart JAD, McCarty DR** (1997) Specific oxidative cleavage of carotenoids by VP14 of maize. *Science* **276**: 1872-1874

- Sesma A, Osbourn AE** (2004) The rice leaf blast pathogen undergoes developmental processes typical of root-infecting fungi. *Nature* **431**: 582-586
- Shan HY, Zhao MM, Chen DX, Cheng JL, Li J, Feng ZZ, Ma ZY, An DR** (2013) Biocontrol of rice blast by the phenaminomethylacetic acid producer of *Bacillus methylotrophicus* strain BC79. *Crop Protection* **44**: 29-37
- Shi B, Ni L, Liu YP, Zhang AY, Tan MP, Jiang MY** (2014) OsDMI3-mediated activation of OsMPK1 regulates the activities of antioxidant enzymes in abscisic acid signalling in rice. *Plant Cell and Environment* **37**: 341-352
- Shi SJ, Richardson AE, O'Callaghan M, DeAngelis KM, Jones EE, Stewart A, Firestone MK, Condrón LM** (2011) Effects of selected root exudate components on soil bacterial communities. *Fems Microbiology Ecology* **77**: 600-610
- Shimoi S, Inoue K, Kitagawa H, Yamasaki M, Tsushima S, Park P, Ikeda K** (2010) Biological control for rice blast disease by employing detachment action with gelatinolytic bacteria. *Biological Control* **55**: 85-91
- Shinjo A, Araki Y, Hirano K, Arie T, Ugaki M, Teraoka T** (2011) Transgenic rice plants that over-express the mannose-binding rice lectin have enhanced resistance to rice blast. *Journal of General Plant Pathology* **77**: 85-92
- Siewers V, Kokkelink L, Smedsgaard J, Tudzynski P** (2006) Identification of an abscisic acid gene cluster in the grey mold *Botrytis cinerea*. *Applied and Environmental Microbiology* **72**: 4619-4626
- Silby MW, Winstanley C, Godfrey SAC, Levy SB, Jackson RW** (2011) *Pseudomonas* genomes: diverse and adaptable. *Fems Microbiology Reviews* **35**: 652-680
- Simons M, Permentier HP, deWeger LA, Wijffelman CA, Lugtenberg BJJ** (1997) Amino acid synthesis is necessary for tomato root colonization by *Pseudomonas fluorescens* strain WCS365. *Molecular Plant-Microbe Interactions* **10**: 102-106
- Singh P, Rathinasamy K, Mohan R, Panda D** (2008) Microtubule assembly dynamics: An attractive target for anticancer drugs. *Iubmb Life* **60**: 368-375
- Skamnioti P, Gurr SJ** (2009) Against the grain: safeguarding rice from rice blast disease. *Trends in Biotechnology* **27**: 141-150

- Smalla K, Wieland G, Buchner A, Zock A, Parzy J, Kaiser S, Roskot N, Heuer H, Berg G** (2001) Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: Plant-dependent enrichment and seasonal shifts revealed. *Applied and Environmental Microbiology* **67**: 4742-4751
- Someya N, Nakajima M, Watanabe K, Hibi T, Akutsu K** (2005) Potential of *Serratia marcescens* strain B2 for biological control of rice sheath blight. *Biocontrol Science and Technology* **15**: 105-109
- Spence C, Alff E, Johnson C, Ramos C, Donofrio N, Sundaresan V, Bais H.** (2014) Natural rice rhizospheric microbes suppress rice blast infections. *BMC Plant Biology* **14**:130. DOI: 10.1186/1471-2229-14-130
- Spence CA, Raman V, Donofrio NM, Bais HP** (2014) Global gene expression in rice blast pathogen *Magnaporthe oryzae* treated with a natural rice soil isolate. *Planta* **239**: 171-185
- Stutz EW, Defago G, Kern H** (1986) Naturally-occurring fluorescent pseudomonads involved in suppression of black root rot of tobacco. *Phytopathology* **76**: 181-185
- Sundin GW, Jacobs JL** (1999) Ultraviolet radiation (UVR) sensitivity analysis and UVR survival strategies of a bacterial community from the phyllosphere of field-grown peanut (*Arachis hypogaea* L.). *Microbial Ecology* **38**: 27-38
- Suzuki F, Arai M, Yamaguchi J** (2007) Genetic analysis of *Pyricularia grisea* population by rep-PCR during development of resistance to scytalone dehydratase inhibitors of melanin biosynthesis. *Plant Disease* **91**: 176-184
- Sweigard JA, Chumley FG, Valent B** (1992) Disruption of a *Magnaporthe-grisea* cutinase gene. *Molecular & General Genetics* **232**: 183-190
- Szostkiewicz I, Richter K, Kepka M, Demmel S, Ma Y, Korte A, Assaad FF, Christmann A, Grill E** (2010) Closely related receptor complexes differ in their ABA selectivity and sensitivity. *Plant Journal* **61**: 25-35
- Takagaki M, Kaku K, Watanabe S, Kawai K, Shimizu T, Sawada H, Kumakura K, Nagayama K** (2004) Mechanism of resistance to carpropamid in *Magnaporthe grisea*. *Pest Management Science* **60**: 921-926
- Talbot NJ** (2003) On the trail of a cereal killer: Exploring the biology of *Magnaporthe grisea*. *Annual Review of Microbiology* **57**: 177-202

- Thomashow LS, Weller DM** (1988) Role of a phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis* var tritici. *Journal of Bacteriology* **170**: 3499-3508
- Tilg H, Kaser A** (2011) Gut microbiome, obesity, and metabolic dysfunction. *Journal of Clinical Investigation* **121**: 2126-2132
- Tisdale S, Nelson W** (1975) Soil fertility and fertilizers. Macmillan Publishing, New York, U.S.A.
- Ton J, Flors V, Mauch-Mani B** (2009) The multifaceted role of ABA in disease resistance. *Trends in Plant Science* **14**: 310-317
- Toppari J, Larsen JC, Christiansen P, Giwercman A, Grandjean P, Guillette LJ, Jegou B, Jensen TK, Jouannet P, Keiding N, Leffers H, McLachlan JA, Meyer O, Muller J, Rajpert-DeMeyts E, Scheike T, Sharpe R, Sumpter J, Skakkebaek NE** (1996) Male reproductive health and environmental xenoestrogens. *Environmental Health Perspectives* **104**: 741-803
- Tredway LP, Stevenson KL, Burpee LL** (2003) Components of resistance to *Magnaporthe grisea* in 'Coyote' and 'Coronado' tall fescue. *Plant Disease* **87**: 906-912
- Tukey HB** (1970) Leaching of substances from plants. *Annual Review of Plant Physiology* **21**: 305-&
- Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI** (2007) The human microbiome project. *Nature* **449**: 804-810
- Van der Ent S, Van Wees SCM, Pieterse CMJ** (2009) Jasmonate signaling in plant interactions with resistance-inducing beneficial microbes. *Phytochemistry* **70**: 1581-1588
- van Loon LC** (2007) Plant responses to plant growth-promoting rhizobacteria. *European Journal of Plant Pathology* **119**: 243-254
- van Loon LC, Bakker P, Pieterse CMJ** (1998) Systemic resistance induced by rhizosphere bacteria. *Annual Review of Phytopathology* **36**: 453-483
- Van Wees SCM, Van der Ent S, Pieterse CMJ** (2008) Plant immune responses triggered by beneficial microbes. *Current Opinion in Plant Biology* **11**: 443-448

- Vanpeer R, Niemann GJ, Schippers B** (1991) Induced resistance and phytoalexin accumulation in biological control of *Fusarium* wilt of carnation by *Pseudomonas* sp strain WCS417R. *Phytopathology* **81**: 728-734
- Vidhyasekaran P, Muthamilan M** (1999) Evaluation of a powder formulation of *Pseudomonas fluorescens* Pfl for control of rice sheath blight. *Biocontrol Science and Technology* **9**: 67-74
- Vidhyasekaran P, Rabindran R, Muthamilan M, Nayar K, Rajappan K, Subramanian N, Vasumathi K** (1997) Development of a powder formulation of *Pseudomonas fluorescens* for control of rice blast. *Plant Pathology* **46**: 291-297
- Vitousek PM, Aber JD, Howarth RW, Likens GE, Matson PA, Schindler DW, Schlesinger WH, Tilman D** (1997) Human alteration of the global nitrogen cycle: Sources and consequences. *Ecological Applications* **7**: 737-750
- Vitullo D, Di Pietro A, Romano A, Lanzotti V, Lima G** (2012) Role of new bacterial surfactins in the antifungal interaction between *Bacillus amyloliquefaciens* and *Fusarium oxysporum*. *Plant Pathology* **61**: 689-699
- Voisard C, Keel C, Haas D, Defago G** (1989) Cyanide production by *Pseudomonas fluorescens* helps suppress black root rot of tobacco under gnotobiotic conditions. *Embo Journal* **8**: 351-358
- Wahlund TM, Madigan MT** (1995) Genetic transfer by conjugation in the thermophilic green sulfur bacterium *Chlorobium tepidum*. *Journal of Bacteriology* **177**: 2583-2588
- Walker TS, Bais HP, Grotewold E, Vivanco JM** (2003) Root exudation and rhizosphere biology. *Plant Physiology* **132**: 44-51
- Wang GN, Ding XH, Yuan M, Qiu DY, Li XH, Xu CG, Wang SP** (2006) Dual function of rice OsDR8 gene in disease resistance and thiamine accumulation. *Plant Molecular Biology* **60**: 437-449
- Wang Q, Garrity GM, Tiedje JM, Cole JR** (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology* **73**: 5261-5267
- Waters CM, Bassler BL** (2005) Quorum sensing: Cell-to-cell communication in bacteria. *Annual Review of Cell and Developmental Biology* **21**: 319-346

- Whitehead NA, Barnard AML, Slater H, Simpson NJL, Salmond GPC** (2001) Quorum-sensing in gram-negative bacteria. *Fems Microbiology Reviews* **25**: 365-404
- Wickham H** (2009) ggplot2: Elegant Graphics for Data Analysis. *Ggplot2: Elegant Graphics for Data Analysis*: 1-212
- Wilson RA, Talbot NJ** (2009) Under pressure: investigating the biology of plant infection by *Magnaporthe oryzae*. *Nature Reviews Microbiology* **7**: 185-195
- Wiwattanapatapee R, Pengnoo A, Kanjanamaneesathian M, Matchavanich W, Nilratana L, Jantharangsri A** (2004) Floating pellets containing bacterial antagonist for control sheath blight of rice: formulations, viability and bacterial release studies. *Journal of Controlled Release* **95**: 455-462
- Xie XN, Yoneyama K** (2010) The Strigolactone Story. *Annual Review of Phytopathology*, Vol 48 **48**: 93-117
- Xu J, Audenaert K, Hofte M, De Vleeschauwer D** (2013) Absciscic acid promotes susceptibility to the rice leaf blight pathogen *Xanthomonas oryzae* pv *oryzae* by suppressing salicylic acid-mediated defenses. *Plos One* **8**: 10
- Xu ZJ, Nakajima M, Suzuki Y, Yamaguchi I** (2002) Cloning and characterization of the abscisic acid-specific glucosyltransferase gene from adzuki bean seedlings. *Plant Physiology* **129**: 1285-1295
- Yamada N, Motoyama T, Nakasako M, Kagabu S, Kudo T, Yamaguchi I** (2004) Enzymatic characterization of scytalone dehydratase Val75Met variant found in melanin biosynthesis dehydratase inhibitor (MBI-D) resistant strains of the rice blast fungus. *Bioscience Biotechnology and Biochemistry* **68**: 615-621
- Yang DL, Yang YN, He ZH** (2013) Roles of plant hormones and their interplay in rice immunity. *Molecular Plant* **6**: 675-685
- Yang F, Cao YJ** (2012) Biosynthesis of phloroglucinol compounds in microorganisms-review. *Applied Microbiology and Biotechnology* **93**: 487-495
- Yang XD, Anderson NJ, Dong XH, Shen J** (2008) Surface sediment diatom assemblages and epilimnetic total phosphorus in large, shallow lakes of the Yangtze floodplain: their relationships and implications for assessing long-term eutrophication. *Freshwater Biology* **53**: 1273-1290

- Yazawa K, Jiang CJ, Kojima M, Sakakibara H, Takatsuji H** (2012) Reduction of abscisic acid levels or inhibition of abscisic acid signaling in rice during the early phase of *Magnaporthe oryzae* infection decreases its susceptibility to the fungus. *Physiological and Molecular Plant Pathology* **78**: 1-7
- Ye NH, Jia LG, Zhang JH** (2012) ABA signal in rice under stress conditions. *Rice* **5**
- Ye XD, Al-Babili S, Kloti A, Zhang J, Lucca P, Beyer P, Potrykus I** (2000) Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. *Science* **287**: 303-305
- Yoon MY, Kim YS, Ryu SY, Choi GJ, Choi YH, Jang KS, Cha B, Han SS, Kim JC** (2011) In vitro and in vivo antifungal activities of decursin and decursinol angelate isolated from *Angelica gigas* against *Magnaporthe oryzae*, the causal agent of rice blast. *Pesticide Biochemistry and Physiology* **101**: 118-124
- Yukioka H, Inagaki S, Tanaka R, Katoh K, Miki N, Mizutani A, Masuko M** (1998) Transcriptional activation of the alternative oxidase gene of the fungus *Magnaporthe grisea* by a respiratory-inhibiting fungicide and hydrogen peroxide. *Biochimica Et Biophysica Acta-Gene Structure and Expression* **1442**: 161-169
- Yukioka H, Tanaka R, Inagaki S, Katoh K, Miki N, Mizutani A, Masuko M, Kunoh H** (1997) Mutants of the phytopathogenic fungus *Magnaporthe grisea* deficient in alternative, cyanide-resistant, respiration. *Fungal Genetics and Biology* **22**: 221-228
- Zeigler RS** (1998) Recombination in *Magnaporthe grisea*. *Annual Review of Phytopathology* **36**: 249-275
- Zemanick ET, Sagel SD, Harris JK** (2011) The airway microbiome in cystic fibrosis and implications for treatment. *Current Opinion in Pediatrics* **23**: 319-324
- Zhou JM, Chai J** (2008) Plant pathogenic bacterial type III effectors subdue host responses. *Current Opinion in Microbiology* **11**: 179-185
- Zlosnik JEA, Williams HD** (2004) Methods for assaying cyanide in bacterial culture supernatant. *Letters in Applied Microbiology* **38**: 360-365

APPENDIX A

Copyright permission for Chapter 1



WILEY

Book: Molecular Microbial Ecology of the Rhizosphere, Two Volume Set
Author: Frans J. de Bruijn
Publisher: John Wiley and Sons
Date: Jun 1, 2013
Copyright © 2013, John Wiley and Sons

Account Info Help

Logged in as:
Carla Spence
Account #: 3000765999

LOGOUT

Order Completed

Thank you very much for your order.

This is a License Agreement between Carla A. Spence ("You") and John Wiley and Sons ("John Wiley and Sons"). The license consists of your order details, the terms and conditions provided by John Wiley and Sons, and the [payment terms and conditions](#).

License number	Reference confirmation email for license number
License date	Apr 07, 2014
Licensed content publisher	John Wiley and Sons
Licensed content publication	Wiley Books
Licensed content title	Molecular Microbial Ecology of the Rhizosphere, Two Volume Set
Book title	
Licensed copyright line	Copyright © 2013, John Wiley and Sons
Licensed content author	Frans J. de Bruijn
Licensed content date	Jun 1, 2013
Type of use	Dissertation/Thesis
Requestor type	University/Academic
Format	Print and electronic
Portion	Text extract
Number of Pages	5
Will you be translating?	No
Title of your thesis / dissertation	Elucidation of molecular and biochemical determinants in a natural rice rhizospheric isolate to attenuate rice blast pathogen Magnaporthe oryzae
Expected completion date	Jun 2014
Expected size (number of pages)	175
Billing Type	Invoice
Billing address	27 Cedar Farms Dr Newark, DE 19702 United States
Total	0.00 USD

CLOSE WINDOW

Copyright © 2014 [Copyright Clearance Center, Inc.](#) All Rights Reserved. [Privacy statement](#).
Comments? We would like to hear from you. E-mail us at customerservice@copyright.com

APPENDIX B

Copyright permission for Chapter 2

Chapter 2 is modified from a journal article, “Natural rice rhizospheric microbes suppress rice blast infections” published in BMC Plant Biology, an open access journal. As the author of this article, I hold the copyright and agree to it being modified for use in this dissertation.

APPENDIX C

Copyright Permission for Chapter 3

Planta

Order detail ID:	64655608	Permission Status:	✔ Granted
Order License Id:	3363731114881	Permission type:	Republish or display content
Article Title:	Global gene expression in rice blast pathogen <i>Magnaporthe oryzae</i> treated with a natural rice soil isolate	Type of use:	use in a thesis/dissertation
Author(s):	Raman, Vidhyavathi ; et al	Portion	Full text
DOI:	10.1007/S00425-013-1974-1	Number of copies	10
Date:	Jan 01, 2014	Author of this Springer article	Yes and you are the sole author of the new work
ISSN:	1432-2048	Title of your thesis / dissertation	Elucidation of molecular and biochemical determinants in a natural rice rhizospheric isolate to attenuate rice blast pathogen <i>Magnaporthe oryzae</i>
Publication Type:	e-Journal	Expected completion date	Jun 2014
Volume:	239	Estimated size(pages)	175
Issue:	1		
Start page:	171		
Publisher:	SPRINGER-VERLAG BERLIN/HEIDELBERG		