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Silicon-induced resistance in rice (*Oryza sativa* L.) against the brown spot pathogen *Cochliobolus miyabeanus*

Jonas Van Bockhaven

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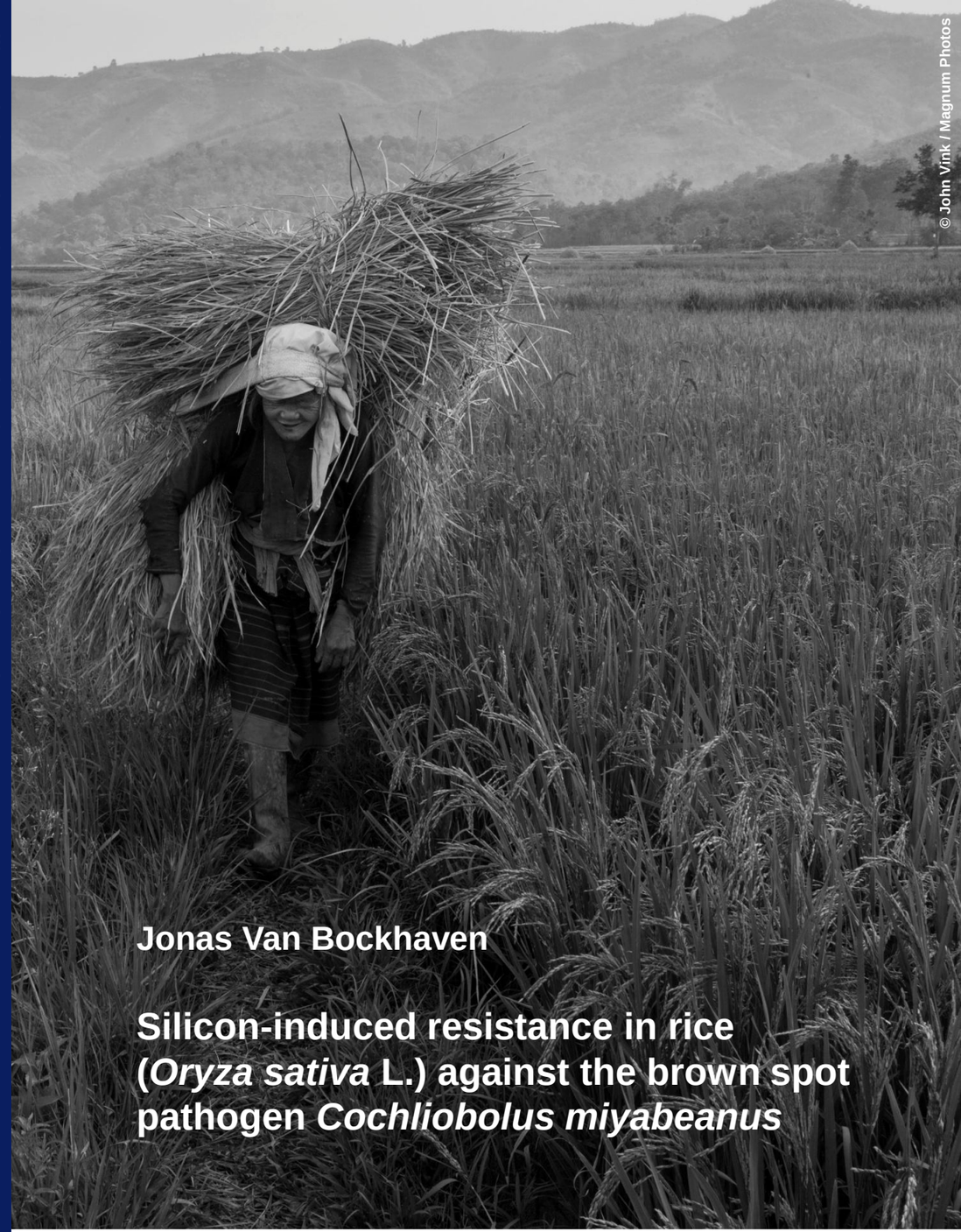

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UNIVERSITEIT GENT

FACULTY OF BIOSCIENCE ENGINEERING

Silicon-induced resistance in rice (*Oryza sativa* L.) against the brown spot pathogen *Cochliobolus miyabeanus*

Jonas VAN BOCKHAVEN

Thesis submitted in fulfillment of the
requirements for the degree of Doctor (PhD) in
Applied Biological Sciences: Agricultural Sciences

"We are all in the gutter, but some of us are looking at the stars"

Oscar Wilde

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Cover illustration:
The cover picture is made by John Vink (www.johnvink.com/JohnVinkSite), a Belgian photographer who is a member of the renowned photographic cooperative Magnum Photos. He took the cover picture in Myanmar showing rice fields and an elderly Palaung woman carrying rice stems to her house, some 3 kilometers away. I cropped the picture to make it fit the cover, the original picture is displayed below. There are several reasons why I chose this picture. First of all, I think this image is beautiful and one can not overestimate the importance of aesthetics. Second and more importantly, this picture represents the main motivations for starting my PhD project. Even though my research is quite fundamental, the potential social and environmental implications of it have always been more important to me than impact factors and amount of citations.

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dat ik werk rond 'brown spot disease'.
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List of Abbreviations

2,2-BP	2,2-bipyridyl
ABA	abscisic acid
ABF	abscisic acid-responsive element binding transcription factor
ACC	1-aminocyclopropane-1-carboxylic acid
AMT	ammonium transporter
AOA	aminooxyacetic acid
ATP	adenosine triphosphate
AUX	auxin
Avr	avirulence
BR	brassinosteroid
BTH	benzothiadiazole
Ca	calcium
Cd	cadmium
cDNA	complementary DNA
CERK1	elicitor receptor kinase 1
CK	cytokinins
CLC	chloride chanel protein
CLC1	nitrate transporting chloride chanel protein
cv.	cultivar
DAB	3,3'-diaminobenzidine
DMSO	dimethyl sulfoxide
dpi	days post inoculation
DREB	dehydration-responsive element-binding transcription factor
EDTA	ethylenediaminetetraacetic acid
EFE	ethylene forming enzyme
EFR	EF-Tu receptor
EIN2	ethylene insensitive 2 protein
ERF	ethylene responsive transcription factor
ET	ethylene
ETI	effector-triggered immunity
FLS2	flagellin sensing 2
FAO	food and agriculture organization of the united nations
Fe	iron
FW	fresh weight
FWO	fonds wetenschappelijk onderzoek - Vlaanderen

GDC	glycine dehydrogenase
GA	gibberellin
GAMYB	gibberellin-responsive MYB transcription factor
GDH	glutamate dehydrogenase
GGAT	glutmate:glyoxylate aminotransferase
GO	gene ontology
GOGAT	glutamate synthase
GOX	glycolate oxidase
GS	glutamine synthetase
H_2O_2	hydrogen peroxide
hpi	hours post inoculation
HPR	hydroxypyruvate reductase
HR	hypersensitive response
IAA	indole-3-acetic acid
IRRI	international rice research institure
ISR	induced systemic resistance
IWT	agentschap voor innovatie door wetenschap en technologie
JA	jasmonic acid
K	potassium
Lsi	silicic acid transporter
MADS	MCM1-AGAMOUS-DEFICIENS-SRF-like transcription factor
MAPK	mitogen-activated protein kinase
Mn	manganese
MYB	myeloblastosis proto-oncogene-like transcription factor
N	nitrogen
NahG	salicylate hydroxylase
NPR1	non-expressor of PR genes 1 protein
NRT	nitrate transporter
P	phosphorus
PAL	phenylalanine ammonia lyase
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
PDA	potato dextrose agar
PGK	phosphoglycerate kinase
PGLP	phosphoglycolate phosphatase
PPO	polyphenol oxidase
PRK	phosphoribulokinase
PRR	plant pattern recognition receptor
PTI	PAMP-triggered immunity
QTL	quantitative trait loci
RNAi	RNA interference
ROS	reactive oxygen species
RPI	ribose-5-phosphate isomerase
Rubisco	ribulose-1
Si	silicon
SA	salicylic acid
SAR	systemic acquired resistance
SGAT	serine:glyoxylate aminotransferase
SHMT	serine hydroxymethyltransferase.
STS	silver thiosulfate
TF	transcription factor
TGA	TGACG-motif binding transcription factor
WT	wild type

1

Problem statement and research outline

Since the 1940s, intensification of agricultural production via modern high-yielding varieties, adequate irrigation, use of fertilizers and other complementary inputs, led to a substantial increase in food production, which is referred to as the green revolution. In recent years however, agricultural growth (especially cereal yields) has slowed down (FAO, 2009; Ray et al., 2013). The combination of a growing population and a finite planet puts the global food production under pressure, which led to the so-called 'world food crisis' in 2007-2008. This crisis resulted in worldwide social upheaval due to skyrocketing prices of many staple foods, especially wheat and rice (McMichael, 2009). Rice provides the majority of the caloric intake worldwide and is a staple food for more than half of the global population living mainly in tropical and subtropical Asia and Africa (FAO, 2009; Matsumura et al., 2009). The increasing demand for rice due to the growing population and urbanization together with the fact that many people in Asia and Africa live in poverty and are extremely vulnerable to fluctuating rice prices highlight the need for an adequate rice production in the future (Savary et al., 2011; Seck et al., 2012). Predictions of the changing climate also show that Africa and Asia will suffer more extreme weather conditions in the future. Recent estimations have calculated that the current increases in rice production will not be sufficient to ensure food security in Asia and particularly in Africa in 2050 (Ray et al., 2013).

The solutions for ensuring global food security lie in establishing a sustainable production and consumption and in the reduction of global poverty (De Schutter, 2014). Sustainable rice production can be ensured by continued and improved research efforts to close yield gaps by improving rice production methods and preventing post-harvest yield losses and yield declines in the field due to abiotic stress and pathogens (Seck et al., 2012). Pests and diseases form a continuous threat to the global food production (Flood, 2010; Schut et al., 2014). A striking example is the great Bengal rice famine of 1942-43 caused by the necrotrophic rice leaf fungus *Cochliobolus miyabeanus*, which led to the death of approximately 2 million people (Lenné, 2000). In rice fields, yield losses can average up to 20-30%, indicating the potential contribution of crop protection in increasing global rice production (Mew et al., 2004; Savary et al., 2012; Seck et al., 2012). Up to date, research on crop protection has focused on the development, transfer and adoption of unilateral technologies such as new resistant varieties or agrochemicals (Kropff et al., 2001; Schut et al., 2014). Although these protection strategies have proven to be effective in the field, they are also associated with some typical disadvantages. Excessive pesticide usage leads to environmental and human safety issues, whereas the lack of alternated agrochemical usage promotes pesticide resistance in different plant pathogen species. Resistant cultivars, on the other hand, often cannot withstand the high degree of variability

in the plant pathogen population and their level of resistance in the field quickly deteriorates over time. Moreover, the use of resistant cultivars and agrochemicals are usually not affordable for the majority of rice growing farmers in developing countries. A more holistic approach in crop protection research has emerged which promotes the use of sustainable, affordable and broad spectrum protection strategies to safeguard future food production (FAO, 2009; Seck et al., 2012; Schut et al., 2014). The application of silicon (Si) emerges as an interesting protection strategy that is both safe for the environment as for human consumption and offers protection against a wide range of both abiotic and biotic stresses. Moreover, Si application is generally associated with growth promotion in the field (see section 2.5).

The main objective of this dissertation was to elucidate the mechanisms that underlie Si-induced broad spectrum resistance, by using the rice-*C. miyabeanus* interaction as a model pathosystem. More specifically, we sought to:

1. shed further light onto the molecular underpinnings of resistance and susceptibility in the rice-*C. miyabeanus* pathosystem
2. to decipher the impact of Si on the physiology and transcriptome of healthy and pathogen-infected crop plants
3. to gain insight into the tapestry of signal transduction pathways governing Si-induced disease resistance and delineate the type of immune responses modulated by Si
4. to advance our molecular understanding of how Si is able to elicit resistance against pathogens with different lifestyles and infection strategies

The introductory **Chapter 2** gives a concise overview of our current knowledge on rice production, the basic mechanisms and regulation of plant innate immunity and pathogen's virulence strategies. Since this dissertation focusses on *Cochliobolus miyabeanus*, a summary of the taxonomy and epidemiology of this fungus is provided as well as the main molecular aspects of the rice-brown spot pathosystem and methods of disease management. The last part of the introduction is dedicated to the the uptake of silicon (Si) and its ability to protect plants against a broad spectrum of pathogens. Although several initiators of induced resistance have been described, Si application is one of the few disease control strategies that is able to induce broad spectrum disease resistance by boosting the plant's basal defense mechanisms. The prophylactic effect of Si is considered to be the result of both passive and active defenses. Although the phenomenon has been known for decades, the molecular basis of Si-induced disease control remains largely unknown. By

combining knowledge on how Si interacts with cell metabolism in diatoms and plants, this chapter describes Si-induced regulatory mechanisms that might account for broad-spectrum disease resistance in plants. Priming of plant immune responses, alterations in phytohormone homeostasis, regulation of iron homeostasis, Si-driven photorespiration and interaction with defense signaling components are all potential mechanisms involved in regulating Si-triggered resistance responses. Further elucidating how Si exerts its beneficial properties may create new avenues for developing plants that are better able to cope with multiple stresses.

Even though the positive effect of Si on disease resistance has been known for decades, the underlying mechanisms remain unclear. **Chapter 3** starts with the analysis of available transcriptome and proteome analyses on Si-treated plants which demonstrated two different hypotheses.
First, Si-induced broad spectrum disease resistance is generally attributed to Si-mediated priming which is generally associated to the accumulation of inactive signaling components such as transcription factors (TFs). A multiparallel quantitative RT-PCR based transcriptional analysis on the influence of silicon on the expression of all rice TF genes was performed to test whether Si might induce priming due to a differential expression of TFs. This analysis showed that Si has an apparent influence on the expression of TFs, which could explain the earlier flower induction and increased plant growth observed in Si-treated plants. However, no major modulator of Si could be characterized, but the list of differentially expressed TFs might provide an interesting dataset for future research on the potential role of Si on defense priming.
Second, to investigate the influence of Si on the rice metabolism during infection, a microarray analysis was performed on brown spot-infected and/or Si-treated rice leaves. Based on this transcriptome analysis, several potential mechanisms underlying *C. miyabeanus*-induced senescence and Si-induced brown spot resistance were hypothesized. *C. miyabeanus* seems to uncouple photosynthesis from photo harvesting and the subsequent photo-oxidative damage is likely responsible for the induction of senescence. Application of Si on the other hand, impeded pathogen-induced photooxidative damage and alleviated premature senescence by enhancing photorespiration activity. Together these findings support a scenario whereby the plant's central metabolism plays a pivotal role in the rice-*C. miyabeanus* pathosystem, leading to either susceptibility via pathogen-induced senescence or resistance through Si-induced prevention of senescence due to photorespiration. Moreover, in common with earlier microarray studies, our results suggest that Si nullifies the impact of pathogen inoculation on the plant's transcriptome, rather than

creating resistance where is none by directing massive transcriptional reprogramming of defense-related genes.

In rice leaves, ethylene (ET) is known to play a negative role in mediating defense responses against the necrotrophic fungus *Cochliobolus miyabeanus*, the causal agent of brown spot disease. *C. miyabeanus* has been hypothesized to exploit the rice ET signaling pathway in order to suppress plant defense responses. **Chapter 4** elaborates on this hypothesis in an attempt to unravel the role of ET in the rice-*C. miyabeanus* pathosystem. We found that *C. miyabeanus* produces ET via a pathway that is distinct from plant ET biosynthesis, a process that requires 2-oxoglutarate and arginine and is catalyzed by a fungal ethylene forming enzyme (EFE). Several plant-pathogens have been reported to produce ET following the 2-oxoglutarate dependent pathway. Due to the availability of a fast growing assembly of microbial genome and proteome sequences, many EFEs have been identified and annotated based on the homology with characterized EFEs. These findings led to the assumption that microbial 2-oxoglutarate dependent ET biosynthesis might play a significant role for many plant pathogens and even outside the field of plant-pathogen interactions. Fungal ET produced by *C. miyabeanus*, both directly and indirectly via the activation of plant ET biosynthesis, induces ET signaling in rice. The pathogen-mediated onset of ET signaling impairs plant defense responses via the induction of senescence and inhibition of phenylpropanoid-driven defense responses against *C. miyabeanus*. Moreover, we also show that the virulence of *C. miyabeanus* isolates is at least partly correlated with their ET producing abilities. While providing novel insights into the multifaceted role of ET in the plant's defense signaling network, this chapter underscores the importance of microbial ET in modulating plant immunity.

Chapter 5 investigates the importance and function of hormone signaling in Si-induced brown spot resistance. Exogenous application of a range of known defense hormones along with screening of transgenic and mutant rice lines impaired in different hormone biosynthesis or signaling pathways showed that ethylene (ET) is the main modulator of basal and Si-induced brown spot resistance in rice. Blocking the ET signaling pathway in rice mimics Si-induced brown spot resistance, producing a resistance phenotype hallmarked by small necrotic lesions due to the accumulation of fungitoxic phenolic compounds at the site of infection. Chapter 4 has shown that *C. miyabeanus* produces ET as a virulence factor and the data presented in this chapter suggest that Si confers brown spot resistance by preventing fungal ET biosynthesis. In conclusion, these observations not only reaffirm the significance of fungal ET biosynthesis as an important virulence factor

for *C. miyabeanus*, but also coincide with earlier findings that Si application induces brown spot resistance by disarming fungal virulence factors.

Finally, in **Chapter 6**, we summarize the main findings and discuss the practical implications and future prospects of the research conducted.

2

Introduction

2.1 Rice

2.1.1 Taxonomy, characteristics and production

Rice is an extremely versatile crop that is grown in both dry and wetland conditions at both low and high altitudes. The genus *Oryza* is a member of the grass family (*Poaceae*) and contains species distributed across the tropical, sub-tropical and temperate regions of Asia, Africa, central and south America and Australia (Lu, 1999; Vaughan et al., 2008). Only two species are cultivated, *Oryza glaberrima* (Steudel) and *Oryza sativa* (L.). *O. glaberrima* is indigenous to Africa, while *O. sativa* has its origin in Asia. *O. sativa* comprises two subspecies, *indica* and *japonica*. *Indica* rice is prevalent in tropical regions, whereas *japonica* rice occurs mainly in the subtropical and temperate regions of East Asia.

A typical rice field consists of a specific soil system that is formed after long and extensive muddling, hereby creating an impermeable layer that prevents the water from leaching (Lu, 1999; Vaughan et al., 2008) (Fig 2.1). In Asia, rice is mainly grown in fields that are permanently inundated, mostly by irrigation. Irrigated rice fields are responsible for 75% of the global rice production. When irrigation is not feasible, rainfall can provide sufficient water. These rainfed rice fields occur mainly in Africa and account for 20% of the global rice production. Due to fluctuations in rainfall, rainfed rice fields are more prone to drought stress. Other rice production systems, such as rice grown in very deep paddy fields (deepwater rice) or rice grown in non-irrigated fields (aerobic rice) only account for a small portion of the global rice production. For growing under submerged conditions, the rice roots have developed specific structures called aerenchyma. A root aerenchyma consists of cortical root tissue that is interlaced with internal air spaces, allowing oxygen to diffuse from the leaf stomata to the meristematic tissue in the roots (Webster and Gunnell, 1992).

Rice is a C3 plant which means that the first product of photosynthetic CO_2 assimilation is the 3-carbon compound 3-phosphoglycerate as opposed to C4 plants, where the carboxylation results in the 4-carbon compound oxaloacetate (Siedow and Day, 2000; Foyer et al., 2009). Carbon fixation in rice, barley, Arabidopsis and other C3 plants is restricted by a process called photorespiration. This metabolic bypass occurs under conditions that prevent photosynthesis during the daytime, often due to stress-induced stomatal closure (Foyer et al., 2009; Bauwe et al., 2010). By incorporating O_2 instead of CO_2, photorespiration prevents overreduction of the electron transfer pathway and subsequent photooxidative damage in the chloroplast. Photorespiration is commonly thought of as a wasteful and obsolete process in C3 plants (Foyer et al., 2009; Bauwe et al., 2010;

Peterhansel et al., 2013). However, recent publications suggest a novel role for photorespiration as a plant defense mechanism (Wingler et al., 2000; Kangasjärvi et al., 2012; Sørhagen et al., 2013). C4 plants, like maize, sugarcane and sorghum, on the other hand do not suffer from impeded carbon fixation and are therefore more productive under extreme environmental conditions. From an evolutionary point of view, C3 plants predate C4 plants; however, C3 plants still represent the majority of the earth's plant biomass (Ehleringer and Cerling, 2002).

In 2010, global rice consumption was 439 million tons and the demand is increasing each year (Seck et al., 2012). The current increases in rice yield are not sufficient to meet the predicted demand for rice of 450 million tons in 2020 (Timmer et al., 2011). One of the great challenges that lie ahead is the sustainable increase in rice production and a solution can be found in the reduction of yield losses (see Chapter 1 FAO, 2009; Seck et al., 2012). Therefore one of the main goals for global rice production is ensuring sustainable crop protection that reduces rice yield losses due to abiotic stress and pathogens.

Figure 2.1: Rice plants in an irrigated paddy field A) after planting and B) during grain ripening stage (IRRI)

2.2 Plant immune system

2.2.1 Molecular plant immunity

Plants are frequently challenged by a wide variety of microbial pathogens. Due to an intricate network of plant defense mechanisms that can be either constitutively expressed or induced upon pathogen attack, plant disease is an exception rather than a rule (Thordal-Christensen, 2003; Glazebrook, 2005; Boyd et al., 2013). Defense mechanisms often demand substantial amounts of the plant's energy and it is generally assumed that inducible defense mechanisms have evolved in order to save energy under stress-free

conditions, thus reducing defense-related fitness costs (Walters and Heil, 2007; Denancé et al., 2013). The so-called 'zigzag' model depicts the different stages of plant-pathogen interactions as seen from an evolutionary perspective (Fig 2.2; Jones and Dangl, 2006).

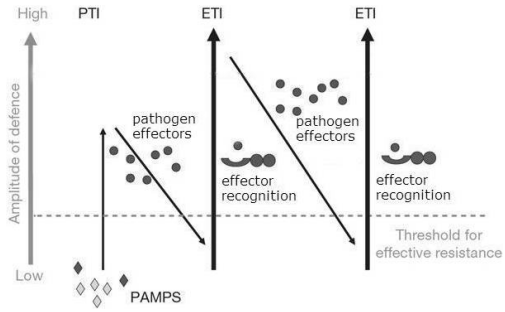

Figure 2.2: Molecular mechanisms of the plant immune system according to the 'zigzag' model, adapted from (Jones and Dangl, 2006). First, plants detect microbial/pathogen-associated molecular patterns (PAMPs) via plant pattern recognition receptors (PRR) which triggers PAMP-triggered immunity (PTI). Successful pathogens secrete effectors that abolish PTI, or otherwise enable pathogen nutrition and dispersal, resulting in susceptibility. Pathogen effectors can be recognized by a specific plant receptor often referred to as resistance (R) proteins activating effector-triggered immunity (ETI). ETI is an amplified version of PTI that is often more effective in fending off pathogen invasion. Natural selection and horizontal gene transfer reshape the pathogen population, favoring isolates that secrete effectors which cannot be recognize by the plant and can suppress ETI.

The first step is the recognition of conserved microbial signatures, also called pathogen-associated molecular patterns (PAMPs). PAMPs include a long list of common microbial molecules, among which the intensively studied bacterial flagellin, bacterial Elongation Factor-Tu (EF-Tu) and fungal chitin (Zhang et al., 2010). Pathogen-derived elicitors are recognized by highly specific plant pattern recognition receptors (PRR) which are often receptor-like kinases, such as FLAGELLIN SENSING 2 (FLS2), EF-Tu RECEPTOR (EFR) and ELICITOR RECEPTOR KINASE 1 (CERK1) (Boyd et al., 2013). Elicitor recognition by PRRs initiates the first line of inducible defense called PAMP-triggered immunity (PTI). Depending on the pathosystem, PTI can be sufficient to fend off pathogen invasion (Katagiri and Tsuda, 2010). In turn, pathogens can deregulate or suppress components of PTI with effectors that are secreted inside the host plant. Effectors can be proteins or host-specific toxins that not only suppress PTI but often also alter the cell's metabolism in order to create an environment in which the pathogen can grow and reproduce (Friesen et al., 2008; Grant et al., 2013; Vleeshouwers and Oliver, 2014). The list of identified effectors has rapidly expanded in recent years due the growing

availability of genome sequences of a plethora of pathogens (Alfano, 2009). A second line of inducible plant defense responses consists of the recognition of pathogen's effectors by plant receptors, encoded by so-called resistance (R) genes. The collection of known R genes is continuously expanding and this knowledge is vital for breeding resistant varieties both via classical breeding and genetic engineering (Vleeshouwers and Oliver, 2014). Although the presence of R proteins results in a clearly resistant phenotype due to effector-triggered immunity (ETI), this type of resistance is not always durable. Effectors are under selection to evade detection by plant R proteins, whilst maintaining their virulence activities. A combination of mutation, horizontal gene transfer and natural selection leads to a shift in pathogen population which favors pathogens with effectors that are not recognized by the plant's R genes and that are able to abolish ETI. In turn, plant R proteins adapt and evolve to acquire and maintain effector recognition (Ma and Yamaji, 2008). The fascinating evolutionary battle between pathogens and plants during which selection enables pathogens to abolish ETI, whereas breeders attempt to obtain resistant cultivars by incorporating other R genes is hallmarked by a continuous pattern of susceptibility and resistance towards the pathogen, hence the term 'zigzag' model. The widespread implementation of monoculture and the genetic narrowing of the grown crops hasten the breakdown of resistant cultivars. Moreover, monoculture with densely planted, homogenous fields of host plants facilitate the spread of diseases (Zhu et al., 2000).

2.2.2 Main mechanisms of induced resistance

The emerging need for a stable food production in the future partly calls for a sustainable and durable crop protection against a broad range of pathogens (FAO, 2009). Multiple articles report broad spectrum disease resistance due to a genetically modified increase in defense signaling (Gómez-Ariza et al., 2007; Wally et al., 2009; Shi et al., 2010), priming (Gómez-Ariza et al., 2007; Ahmad et al., 2010) or systemic resistance induced by previous infections (systemic acquired resistance or SAR) (Colebrook et al., 2012; Fu and Dong, 2013) or beneficial soil microorganisms (De Vleesschauwer and Höfte, 2009). Systemic acquired resistance (SAR) is an induced defense mechanism that is activated by previous infection with an avirulent pathogen. SAR is typically associated with increased levels of salicylic acid, leading to a protected state of the plant (Vlot et al., 2009; Fu and Dong, 2013). Induced systemic resistance (ISR) develops in response to colonization of plant roots by certain non-pathogenic rhizosphere micro-organisms (De Vleesschauwer and Höfte, 2009; Shoresh et al., 2010). Beneficial bacteria like *Pseudomonas fluorescens* and *Serratia* spp. and fungi such as *Trichoderma* spp. have been reported to cause ISR in various host plants (De Vleesschauwer et al., 2008; De Vleesschauwer and Höfte, 2009; De

Vleesschauwer et al., 2009; Shoresh et al., 2010). The perception of these beneficial microorganisms or their influence on the rhizosphere confers resistance to a wide range of plant pathogens. Priming refers to the pre-conditioning of the plant's immune system either by perception of pathogenic or beneficial soil organisms or by natural or synthetic compounds and wounding (Conrath et al., 2006; Conrath, 2011; Pieterse et al., 2012). Although the molecular aspects of priming are still poorly understood, the process is known to be associated with altered levels of inactive signaling components such as mitogen-activated protein (MAP) kinases and transcription factors (Conrath, 2011). Plants in a primed state are believed to be in a higher 'state of alertness' which allows them to adapt to different types of stress leading to a less costly and more broad-spectrum resistance (van Hulten et al., 2006; Van der Ent et al., 2009).

2.2.3 Plant defense signaling: phytohormones

The activation of defense responses during PTI and ETI is governed by a complex network of signaling components that defines the outcome of the plant-pathogen interaction and in which plant hormones play pivotal roles (Pieterse et al., 2009; Grant and Jones, 2009; Robert-Seilaniantz et al., 2011). The most widely accepted model in Arabidopsis states that salicylic acid (SA) confers resistance towards biotrophic pathogens, whereas jasmonic acid (JA) and ethylene (ET) activate defense responses against pathogens with a necrotrophic lifestyle. Biotrophic pathogens derive nutrients from living host tissues, whereas necrotrophs extract nutrients from dead or dying cells (Glazebrook, 2005). Distinct differences between defense responses against necrotrophic and biotrophic pathogens often lead to -offs. The occurrence of trade-offs implies that induction of biotrophic resistance often leads to increased susceptibility towards necrotrophic pathogens and *vice versa* (Spoel et al., 2007; Mengiste, 2012). Hormone crosstalk is one of the main modulators of trade-offs and the antagonism between different hormone pathways explains the distinct roles of SA and JA/ET in mediating respectively biotrophic and necrotrophic resistance (Spoel and Dong, 2008; Denancé et al., 2013). In other plant species the role of the SA-JA/ET model is not that apparent. For instance, in the model organism rice (*Oryza sativa* L.) the role of SA, JA and ET appears pathogen-dependent rather than merely linked to the pathogen's lifestyle (De Vleesschauwer et al., 2013). Aside from the typical defense hormones SA, JA and ET, other hormones including abscisic acid (ABA), gibberellins (GA), auxins, cytokinins and brassinosteroids have emerged as important defense regulators as well. Given the complexity of the plant hormone signaling network, a more holistic approach seems more appropriate. Depending on the pathosystem, a specific blend of signaling components, the so-called signal signature, shapes the outcome

of the plant-pathogen interaction (De Vos et al., 2005). Despite the significant progress in recent years, more research is necessary to improve our current understanding of the role of hormones in mediating plant defense responses.

2.3 Plant pathogenic virulence factors

Pathogens have evolved a range of strategies that interfere with their host's metabolism, among which the secretion of effector proteins, phytotoxins and phytohormones (Arshad and Frankenberger, 1991; Jones and Dangl, 2006; Friesen et al., 2008; Sacristan and Garcia-Arenal, 2008; Dou and Zhou, 2012; Seifi et al., 2013). These virulence factors generally aim at impeding plant defense mechanisms and ensuring sufficient nutrient supply for the pathogen (Wolpert et al., 2002; Sacristan and Garcia-Arenal, 2008; Rico et al., 2011; Vleeshouwers and Oliver, 2014).

One of the most-studied plant pathogens, *Pseudomonas syringae*, deploys a wide variety of virulence factors that show a specific mode of action (Rico et al., 2011; Ichinose et al., 2013). Bacterial effector proteins are generally injected in host cells via the type 3 secretion system (T3SS), in order to suppress defense responses (Grant and Lamb, 2006; Guo et al., 2009). *P. syringae* secretes a plethora of different effectors, the most typical examples are AvrPto, a protein kinase inhibitor and AvrPtoB, an E3 ubiquitin ligase which prevent the perception of PAMPs by either inhibiting or degrading plant PAMP receptor kinases, such as flagellin receptor kinase FLS2 or chitin receptor kinase CERK1 (Block and Alfano, 2011). Effector proteins do not necessarily target defense mechanism, HrpZ(Psph) for instance, binds to the lipid bilayers of plant cells forming a nutrient-conducting pore that enables nutrient release from the plant thus ensuring nutrient supply for *P. syringae* (Lee et al., 2001; Ichinose et al., 2013). *P. syringae* also produces various phytotoxins, among which phaseolotoxin, tabtoxin and coronatine (Bender et al., 1999). Phaseolotoxin and tabtoxin are inhibitors of central metabolic plant enzymes, respectively ornithine carbamoyl transferase and glutamine synthetase, leading to chlorosis and cell death (Bender et al., 1999; Kimura et al., 2001). Coronatine on the other hand not only facilitates the infection process by inhibiting stomatal closure allowing *P. syringae* to invade leaf tissue (Melotto et al., 2006), it also interferes with plant defense mechanisms. The chemical structure of coronatine mimics JA and once inside plant cells, coronatine hyperactivates JA signaling and abolishes effective SA-dependent defenses in the plant (Brooks et al., 2005; Cui et al., 2005; Melotto et al., 2006). *P. syringae* is even known to produce the plant auxin indole acetic acid (IAA) which induces susceptibility in *Arabidopsis thaliana* in a SA-independent manner (Mutka et al., 2013).

2.4 *Cochliobolus miyabeanus*

2.4.1 Taxonomy, characteristics and life cycle

The ascomycete fungus *C. miyabeanus* (anamorph: *Bipolaris oryzae*) is one of the most devastating and prevalent rice pathogens (Ou, 1985; Ahn et al., 2005) (Table 2.1). *C. miyabeanus* is a necrotrophic fungus that quickly induces cell death in the invaded rice leaves. In temperate regions *C. miyabeanus* survives mainly in seeds. In tropical regions, the inoculum is present year-round in seeds and overlapping rice crops (Teng et al., 1994; Castilla and Savary, 2010; Barnwal et al., 2013). The primary source of inoculum mainly are infected seeds, while secondary infections are the result of wind-borne asexual conidiospores (Ou, 1985; Sato et al., 2008; Barnwal et al., 2013). The pale brown, spindle-like conidiospores germinate at both ends and form specific structures called stromae that penetrate epidermal leaf cells (Fig 2.3 and 2.4 B). Once the rice leaf is penetrated, the mycelial hyphae invade the apoplastic mesophyll. Inside the mesophyll, *C. miyabeanus* induces cell death whereafter the fungus can use the cell contents as a source of nutrients. The pathogen-induced leaf cell death results in the typical brown spots surrounded by a chlorotic halo (Fig 2.4 A and B). The massive occurrence of brown spot lesions and chlorosis causes leaf wilting and premature senescence eventually leading to losses in both rice yield and quality. When the conditions are favorable, new conidiospores develop in the brown spot lesions, forming a new source of airborne inoculum. The incubation period of brown spot infection via conidia is short (<24h), disease symptoms develop within 3-4 days and sporulation on infected leaves peaks around 6 days after infection (Ou, 1985; Teng et al., 1994; Barnwal et al., 2013).

Suboptimal conditions caused by water, nutrient and/or temperature stress sensitize rice plants to infection with *C. miyabeanus* (Ou, 1985; Barnwal et al., 2013). Especially abnormalities in the soil nutrient composition seems to increase the brown spot disease development in rice fields. Deficiencies in N, K, Mn, Mg, Fe and Ca are known to increase brown spot incidence in the field (Ou, 1985; Carvalho et al., 2010). This explains the prevalence of this fungus in extensively managed rice fields in developing countries throughout Asia and Africa (Savary et al., 2005; Yaqoob et al., 2011).

Like many other members of the *Cochliobolus* genus, *C. miyabeanus* is known to produce a variety of toxins which have antibiotic and phytotoxic properties (Kim et al., 1999; Krizsán et al., 2010; Condon et al., 2013; Ahn et al., 2005). The majority of toxins produced by *Cochliobolus* species are members of the ophiobolin group, ophiobolin A and B being the best-characterized toxins in *C. miyabeanus* (Au et al., 2000). Both toxins inhibit K^+ uptake and H^+ extrusion by maize roots and leaves, which leads to depolarization

and disorganization of the plasma membrane, likely due to direct inhibition of plant calmodulin by ophiobolin A (Gianani et al., 1979; Cocucci et al., 1983; Leung et al., 1985; Krizsán et al., 2010). The resulting disruption of the plasma membrane (Chattopadhyay and Samaddar, 1976; Tipton et al., 1977) is probably responsible for ophiobolin A-mediated impairment of many metabolic processes such as photosynthesis, respiration and protein and nucleic acid synthesis eventually leading to cell death (Chattopadhyay and Samaddar, 1980; Kim and Hyeon, 1984; Yun et al., 1988; Krizsán et al., 2010). These phytotoxins facilitate the induction of cell death in the host plant (Xiao et al., 1991; Condon et al., 2013).

Table 2.1: Taxonomy of *Cochliobolus miyabeanus*

Taxonomy	
kingdom	*Fungi*
phylum	*Ascomycota*
order	*Pleosporales*
family	*Pleosporaceae*
genus	*Cochliobolus*
teleomorph species	*Cochliobolus miyabeanus*
anamorph species	*Bipolaris oryzae*

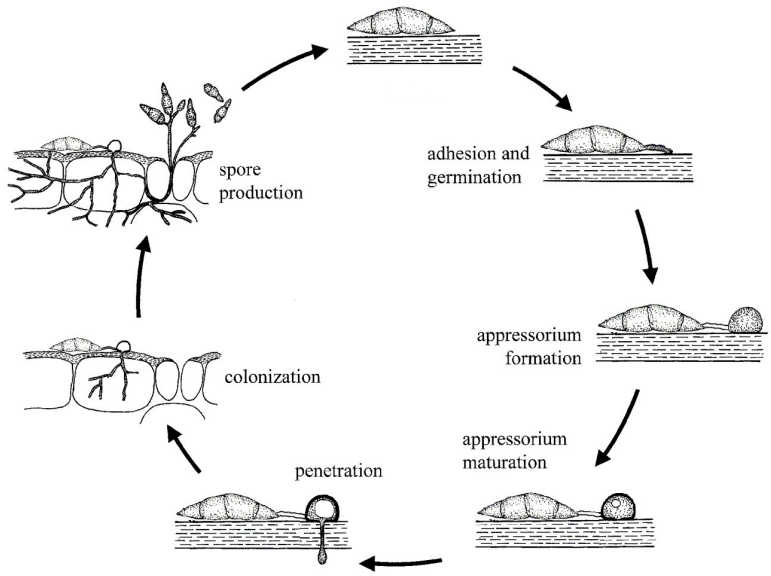

Figure 2.3: Life cycle of *Cochliobolus miyabeanus* (adapted from Thines et al. (2004))

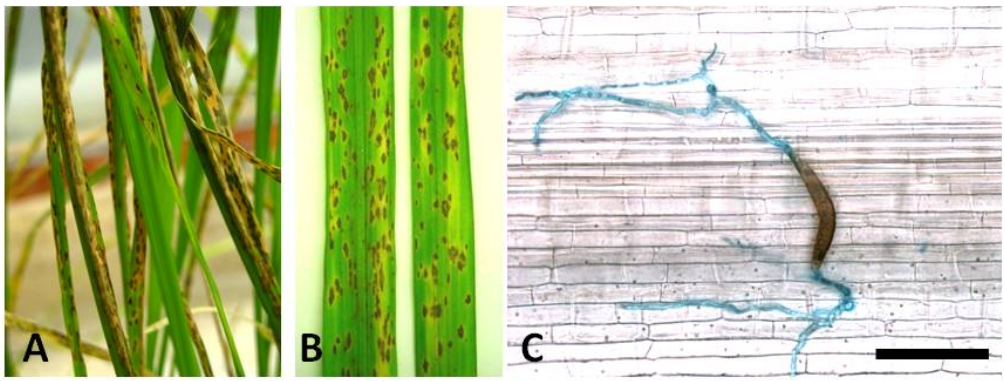

Figure 2.4: Rice leaves infected with *Cochliobolus miyabeanus*. C) *C. miyabeanus* spore on rice sheath epidermis 24h after infection. The germination tubes are stained with trypan blue. Scale bar is 50μm.

2.4.2 Molecular aspects of the rice-brown spot pathosystem

Even though *C. miyabeanus* is a major rice pathogen, the molecular mechanisms that mediate the rice-*C. miyabeanus* interaction have been largely neglected (Ahn et al., 2005). Cytological and microscopic analyses on the effect of inhibitors of the fungal calcium signaling pathway demonstrated the importance of calcium/calmodulin singalling for the induction of prepenetration morphogenesis of *C. miyabeanus* conidia during infection Ahn et al. (2007). Moreover, deletion of the nonribosomal peptide synthetase *CmNPS6* gene results in a substantial reduction of *C. miyabeanus*' virulence and hypersensitivity of the fungus towards plant H_2O_2 (Oide et al., 2006). NPS6 acts as an extracellular siderophore to ensure iron availability for the fungus, which hallmarks the dependency of *C. miyabeanus* on iron during infection.

In rice plants, *C. miyabeanus* generally induces premature senescence in rice plants by downregulating photosynthesis and causing membrane damage and electrolyte leakage in brown spot-infected rice leaves (Dallagnol et al., 2011a). On the other hand, brown spot resistance is commonly asociated with the accumulation of antifungal phenolic components like serotonin, hydroxycinnamic acid and lignin and increased activities of the defense-related enzymes chitinase, peroxidase, polyphenoloxidase (PPO) and phenylalanine ammonia lyase (PAL) Vidhyasekaran et al. (1992); Dallagnol et al. (2011a,b); Ishihara et al. (2011). Chitinases are enzymes that degrade fungal chitin, whereas peroxidase scavenge oxygen radicals that promote HR-like responses and cell death (De Vleesschauwer and Höfte, 2009). PAL and PPO are key enzymes in the phenylpropanoid pathway, an important plant defense pathway that is responsible for the production of phenolic com-

pounds (Dixon et al., 2002).

C. miyabeanus is a known producer of various phytotoxins, which play an essential role in initiating pathogen-induced senescence and cell death (Xiao et al., 1991; Ahn et al., 2005). Several *C. miyabeanus* toxins have been identified such as ophiobolin A, B and I (Kim et al., 1999). Apart from the fact that ophiobolins are toxic to plants, other microbes and even mammals, the mode of action of ophiobolins is still unknown (Au et al., 2000). However, Vidhyasekaran et al. (1992) demonstrated that a crude phytotoxin extract suppressed plant phenol production. The accumulation of fungitoxic phenolic compounds is one of the main defense responses against *C. miyabeanus* (Shabana et al., 2008; Ishihara et al., 2011). These findings demonstrate the importance of toxin secretion as a virulence factor for *C. miyabeanus*.

The activation of plant defense mechanisms against *C. miyabeanus* is modulated by a complex plant signaling network. Ahn et al. (2005) found that the SA-analog BTH and JA do not seem to play a role during brown spot infection. ABA on the other hand, is proven to be an important modulator of brown spot resistance as exogenously administered ABA substantially increases resistance against *C. miyabeanus* (De Vleesschauwer et al., 2010). The driving force behind ABA-induced brown spot resistance is the ABA-responsive MAP kinase, OsMPK6, which inhibits the expression of the ET signaling protein *EIN2*. ET renders rice hyper-susceptible to brown spot infection, whereas reduction of ethylene perception by the application of silver ions or disruption of ET signaling in *EIN2a* antisense plants resulted in a substantial reduction in disease severity (De Vleesschauwer et al., 2010). Moreover, gene expression experiments revealed a strong activation of ET signaling in susceptible but not in resistant rice plants, raising the hypothesis that *C. miyabeanus* exploits ET as a virulence factor and co-opts the rice ET signaling route to suppress other effectual defense pathways. However, how the pathogen is able to tap into the rice ET machinery and how increased ET signaling favors disease development remained unclear.

2.4.3 Disease management

Since *C. miyabeanus* thrives on rice plants grown under suboptimal conditions, the first step in preventing yield losses due to brown spot disease is the maintenance of good agricultural practices. Ensuring sufficient nutrient and water and preventing abiotic stresses as much as possible reduces the risk of brown spot epidemics (Webster and Gunnell, 1992; Barnwal et al., 2013). Up to now no reports on ISR against *C. miyabeanus* are available. On the contrary, De Vleesschauwer and Höfte (2009) showed that even though root colonization by *Serratia plymuthica* IC1270 induced resistance against *M.*

oryzae, it renders rice plants hypersusceptible towards *C. miyabeanus*. Two conventionally used practices offer protection against *C. miyabeanus*. Firstly, the use of resistant cultivars offers the most consistent protection against the pathogen in the field. Although several resistant and partial-resistant cultivars are available, the search for sources of resistance against *C. miyabeanus* has been a difficult endeavor (Barnwal et al., 2013). Three quantitative trait loci (QTL) for disease resistance have been identified (Hossain et al., 2004; Sato et al., 2008), but up to date no real resistance genes have been identified. On the other hand, fungicides like iprodione, propiconazole, azoxystrobin, trifloxystrobin, and carbendazim are also effective in controlling brown spot in the field (Castilla and Savary, 2010). Even though resistant cultivars and agrochemicals are highly effective in reducing brown spot disease severity, there are some major drawbacks (see Chapter 1). The use of resistant cultivars leads to natural selection of pathogen isolates that can break through the plant's defense mechanisms, which reduces the level of resistance of these cultivars (see section 2.2.2). Furthermore, excessive and non-regulated pesticide usage poses risks on environmental and human health. Moreover, *C. miyabeanus* mainly occurs in suboptimal rice production systems where farmers often cannot afford resistant seeds and/or agrochemicals. Other protection strategies exist that are more economical, durable and/or sustainable (Barnwal et al., 2013). First, since seeds are often infected with *C. miyabeanus*, seed treatments with hot water (53-55 °C, Mew and Gonzales (2002))or a small amount of fungicide can reduce the introduction of *C. miyabeanus* to the field (Teng et al., 1994). Second, several reports also show the potential of biocontrol agents in protecting plants from *C. miyabeanus* either via direct antagonism or via the induction of ISR (Singh et al., 2005; see Chapter 2.2.2). Finally, another known method to manage brown spot disease in a sustainable and durable manner is the application of Si which will be discussed in chapter 3.

2.5 Si: broad-spectrum inducer of resistance against biotic and abiotic stress

2.5.1 Background

The second most abundant element in the Earth's crust, silicon (Si) can comprise up to 70% of the soil mass in the form of silicate minerals and water-soluble orthosilicic acid [$Si(OH)_4$] (Epstein, 1994; Savant et al., 1997; Ma and Yamaji, 2006). The concentration of orthosilicic acid in the soil solution averages over 0.1 to 0.6 mM and is affected by its dissolution from soil minerals and its adsorption or resorption by the soil

(McKeague and Cline, 1963; Epstein, 1994; Savant et al., 1997). Extreme conditions including high temperatures and rainfall increase the release of orthosilicic acid, explaining why most weathered soils in the tropics are Si-deficient (Savant et al., 1997; Richmond and Sussman, 2003). Orthosilicic acid [$Si(OH)_4$] is taken up by plant roots and constantly polymerized into insoluble silica [SiO_2.nH2O] in cell walls, intercellular spaces and in a subcuticular layer in the leaves (Ma et al., 2011; Sangster and Hodson, 2001). Si is known to increase the tolerance against both abiotic and biotic stresses in many plant species and it is the only nutrient which is not detrimental when collected in excess (Epstein, 1994; Fauteux et al., 2005; Ma and Yamaji, 2006). According to the universally excepted criteria for the essentiality of a nutrient published by Arnon and Stout (1939), Si is not essential for plants. An important criterion herewith is the intrinsic occurrence of Si in the structure or metabolism of the plants, which to date has not been confirmed. Yet, a more recent definition of essential nutrients by Epstein and Bloom (2005) defines Si as essential because Si-deficient plants exhibit abnormalities in growth, development and reproduction. Amidst the ongoing debate on the essentiality of Si, most authors refer to Si as a 'semi-essential' nutrient Epstein (1999); Ma and Yamaji (2006); Liang et al. (2007).

2.5.2 Si uptake in plants

Si is readily absorbed by plant roots in the form of noncharged monosilicic acid [$Si(OH)_4$] (Ma et al., 2006). Various plant species, especially monocots, are known to actively absorb Si (Liang et al., 2005a). Since rice is a well-known Si accumulator and an important scientific model organism, the Si uptake mechanism has been most intensively studied in this plant species. In rice roots two Si transporters with a different mode of action are responsible for the transport of silicic acid past the casparian strips in exo- and endodermis cells (Figure 2.5). The influx transporter Lsi1 is located on the plasma membrane at the distal side of exo- and endodermis cells. Silicic acid is transported out of the exo- and endodermis cells through the Lsi2 transporters at the proximal side of these cells (Ma et al., 2006, 2007, 2011). The uptake of silicic acid by Lsi1 is a passive process, while the transport via the Lsi2 transporters is actively driven by an ATP-consuming H^+-pump. Once taken up by Lsi1 in the exodermis and released by Lsi2, silicic acid diffuses through the apoplast of the aerenchyma. Lsi1 transporters in endodermis cells take up the silicic acid from the aerenchyma and Lsi2 transporters load it into cortical cells. An unknown transporter is responsible for the xylem loading of silicic acid. Leaf cells take up silicic acid from the xylem by means of a Lsi1-like transporter, Lsi6. Inside the leaf cells, a natural polymerisation process takes place, transforming water soluble silicic acid into

insoluble silica (SiO_2.nH2O,) either inside the cell, as phytoliths and colloidal cytoplasmic silica or outside the cell, as a silica layer or silica bodies located just beneath the cuticle (Yamanaka et al., 2009; Ma et al., 2011).

In maize, barley, pumpkin and wheat, orthologues of rice Lsi1 and Lsi2 have been shown to be involved in Si absorption (Chiba et al., 2009; Mitani et al., 2009b,a, 2011; Montpetit et al., 2012). Although the Si transporters in different plant species are homologous to OsLsi1 and OsLsi2 in rice, the uptake of Si differs considerably between rice and other plant species due to differences in root architecture (Mitani et al., 2009b). In contrast with rice, the roots of most other plants lack both exodermal casparian strips and aerenchyma (Ma et al., 2011). The Lsi1 transporter in barley, maize and pumpkin occur at the distal side of all root cells between the epidermis and hypodermis, while Lsi2 is localized in endodermal cells without polarity. In the latter plant species Si appears to be taken up from the soil solution by Lsi1 outside the endodermis, while in rice Lsi1 transports Si exclusively at the exodermis (Mitani et al., 2009a,b; Bauer et al., 2011; Ma et al., 2011).

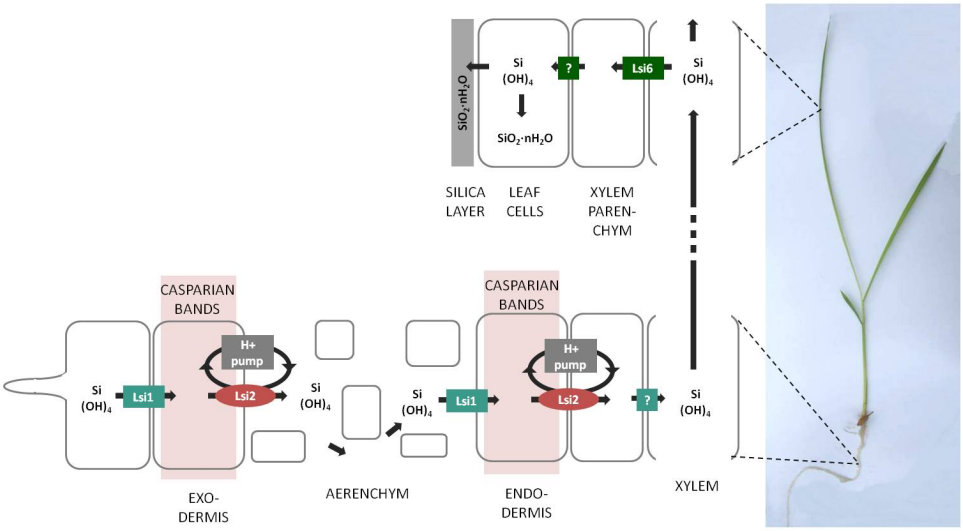

Figure 2.5: General uptake of Si in rice plants from root to shoot. From root epidermis cells silicic acid is transported through exodermis cells by the passive Lsi1 and the active Lsi2 Si transporters. In the aerenchyma silicic acid moves apoplastically until it reaches the endodermis where the Lsi1 and Lsi2 transporters load silicic acid in the cortical cells. An undefined transporter loads the silicic acid in the xylem. Via the xylem silicic acid arrives in the shoots, where the Lsi6 transporter unloads the silicic acid into the xylem parenchyma cells. An undefined protein transports the silicic acid in the leaf cells where it is polymerised either as silica in the cell or as a subcuticular silica layer outside the cell. Adapted from Ma and Yamaji (2006); Ma et al. (2011).

2.5.3 The importance of resistance trade-offs in rice production systems

Rice is the most important food crop of the developing world and the staple food of more than half of the world's population. Diseases caused by microbial pathogens have always had a significant impact on rice production. Historically, severe epidemics have led to serious food shortages, claiming the lives of millions (Ou, 1985). Nowadays, diseases are still among the major constraints on high rice productivity. Fungal diseases such as rice blast (caused by *Magnaporthe oryzae*), sheath blight (*Rhizoctonia solani*), brown spot (*Cochliobolus miyabeanus* (sexual stage), also called *Bipolaris oryzae* (asexual stage)), and bacterial blight (*Xanthomonas oryzae* pv. *oryzae*) are the most serious constraints on high productivity (Webster and Gunnell, 1992). *R. solani* and *B. oryzae* are necrotrophic pathogens, *X. oryzae* pv. *oryzae* is considered to be a biotrophic pathogen while *M. oryzae* is hemi-biotrophic. Studies by Savary et al. (2000) demonstrate that among the many diseases occurring in rice fields, brown spot along with sheath blight, account for the highest yield losses across all production systems (5 and 6%, respectively). In comparison, estimated yield losses for rice blast and bacterial blight are 0.3-5% and less than 1%, respectively. These figures indicate that the sustained efforts of resistance breeding against blast and bacterial blight have paid off (Leung et al., 2003). Diseases such as sheath blight and brown spot have become more prominent due to either a lack of effective resistance in the germplasm or a lack of breeding effort. According to Leung et al. (2003) the challenge ahead is to develop broad-spectrum resistance and production systems that suppress multiple biotic stresses. Among these strategies, approaches based on the plant's own defensive repertoire seem very promising for sustainable rice production (Song and Goodman, 2001).

Table 2.2 summarizes our current knowledge on the role of plant hormones and other selected plant activators on disease resistance in rice against its four major pathogens. Salicylic acid or compounds that act up- or downstream of SA in the SA-signaling pathway such as the plant activator probenazole or the SA analogues benzothiadiazole (BTH) and triadinil are effective against the rice blast fungus *M. oryzae* and the bacterial blight pathogen *X. oryzae* pv. *oryzae* but do not induce resistance to *C. miyabeanus* or *R. solani* (Iwai et al., 2006; De Vleesschauwer et al., 2010; Takatsuji et al., 2010; Shen et al., 2011). Jasmonate plays a positive role in the resistance to *M. oryzae*, *X. oryzae* pv. *oryzae* and *R. solani* (Taheri and Tarighi, 2010), but is ineffective against *C. miyabeanus*. Application of ABA enhances susceptibility to rice blast (Koga et al., 2004; Jiang et al., 2010) and bacterial blight (Xu et al., unpublished), has no effect on *R. solani*, but induces

resistance to the brown spot fungus *C. miyabeanus* (De Vleesschauwer et al., 2010). ET is involved in rice blast resistance and it was shown that ET biosynthesis, but not ET itself is necessary for resistance to rice blast in young rice plants (Iwai et al., 2006). A recent study by Shen et al. (2011) suggests, however, that ET has a negative role on resistance to bacterial blight. De Vleesschauwer et al. (2010) have likewise demonstrated that ET is involved in susceptibility to brown spot and that ABA-induced suppression of the ET response is involved in induced resistance to brown spot. The plant hormone auxin also plays contrasting roles in the interaction of rice with blast and bacterial blight on one hand, and brown spot on the other hand. Exogenous application of the auxin indole-3-acetic acid (IAA) increased susceptibility to bacterial blight (Ding et al., 2008) and blast (Fu et al., 2011), while it increased resistance to brown spot (Fonteyne, 2011). A rice line overexpressing *OsGH3.1*, a gene encoding an IAA amido synthetase that inactivates IAA by conjugating it to amino acids, was more resistant to *M. oryzae* (Domingo et al., 2009), but Fonteyne (2011) revealed that this line is very susceptible to *C. miyabeanus*. These data clearly show that rice requires distinct signal transduction pathways to defend itself to its major pathogens and that trade-offs are especially apparent between pathogens with a contrasting life style such as *M. oryzae* and *C. miyabeanus*. Although both *R. solani* and *C. miyabeanus* are necrotrophic fungal pathogens, accumulating evidence suggests that also against the latter pathogens distinct resistance mechanisms are operative. Exogenous ABA, although highly effective against *C. miyabeanus*, failed to reduce sheath blight severity, whereas application of riboflavin, a water-soluble B vitamin thought to function via activation of JA-dependent defenses (Taheri and Tarighi, 2010), induces resistance to *R. solani* while increasing susceptibility to *C. miyabeanus* (De Vleesschauwer et al., unpublished).

A similar picture emerges from our studies on rhizobacteria-mediated resistance in rice. The root-colonizing *Pseudomonas aeruginosa* strain 7NSK2 was found to activate ISR against *M. oryzae* and the blue phenazine pigment pyocyanin appeared to be an essential determinant of 7NSK2-mediated ISR. However, pyocyanin acts as a two-faced ISR elicitor, positively modulating protection against *M. oryzae*, but repressing *R. solani* resistance. Transient generation of low-level micro-oxidative bursts by redox-active pyocyanin *in planta* most likely accounts for the dual role of this compound in 7NSK2-ISR as exogenous application of H_20_2-quenching sodium ascorbate alleviated the contrasting effects of pyocyanin on *R. solani* and *M. oryzae* pathogenicity (De Vleesschauwer et al., 2006). Later it was shown that topical application of pyocyanin also triggers susceptibility to *C. miyabeanus* (De Vleesschauwer et al., 2009). Similar findings were obtained in response to root treatment with *Serratia plymuthica* strain IC1270. Although highly effective

against *M. oryzae*, arresting the pathogen in its biotrophic phase by boosting infection-induced H_20_2 accumulation in the epidermis, IC1270 colonization resulted in enhanced tissue colonization by both *R. solani* and *C. miyabeanus* (De Vleesschauwer et al., 2009). The effect of reactive oxygen species-fueled hypersensitive response (HR) like cell death thus clearly varies according to the mode of infection of the invading pathogen. In this context, it can be hypothesized that the widespread circulation of high-yielding, semi-dwarf varieties carrying multiple blast resistance genes might be an important factor driving the overall increase in sheath blight incidence that is typically observed in intensified rice production systems (Mew et al., 2004). In this respect we have observed that pre-inoculation with an avirulent HR-triggering *M. oryzae* isolate favours subsequent infection with *R. solani* (De Vleesschauwer et al, unpublished).

Table 2.2 reveals that there is not one a single hormone, plant activator or resistance elicitor that is active against all four major rice pathogens. Moreover, in many cases, resistance to one pathogen is coupled to enhanced susceptibility against others, clearly demonstrating the occurrence of resistance trade-offs. The notable exception, however, is Si, which triggers broad-spectrum resistance against all four pathogens.

Table 2.2: Effect of plant activators on the defence response of rice against its major fungal and bacterial pathogens.

	Magnaporthe oryzae	*Xanthomonas oryzae* pv. oryzae	*Cochliobolus miyabeanus*	*Rhizoctonia solani*	References
Plant hormones or analogues					
Ethylene	+	-	-	NT	De Vleesschauwer et al. (2010); Iwai et al. (2006); Shen et al. (2011)
Salicylic acid, benzothiadiazole, tiadinil	+	+	**0**	**0**	Ahn et al. (2005); Babu et al. (2003); Takatsuji et al. (2010)
Jasmonic acid	+	+	**0**	+	Ahn et al. (2005); Mei et al. (2006); Schweizer et al. (1998); Taheri and Tarighi (2010); Tao et al. (2009)
Abscisic acid	-	-	+	**0**	Jiang et al. (2010); De Vleesschauwer et al. (2010)
Auxin	-	-	+	NT	Domingo et al. (2009); Fonteyne (2011); Fu et al. (2011)
Plant activators					
Probenazole	+	+	**0**	**0**	Takatsuji et al. (2010); Watanabe (1977)
Riboflavin	+	NT	-	+	Aver'yanov et al. (2000); Taheri and Tarighi (2010)
Bacterial elicitors					
Pseudobactin	+	NT	NT	**0**	De Vleesschauwer et al. (2008)
Pyocyanin	+	NT	-	-	De Vleesschauwer et al. (2006, 2009)
Silicon	+	+	+	+	Chang et al. (2002); Dallagnol et al. (2011b); Rodrigues et al. (2003, 2004)

2.5.4 Si-induced broad spectrum resistance in rice

Si application is known to induce broad spectrum stress tolerance in many plant species without the occurrence of resistance trade-offs and/or growth and yield penalties (Epstein, 1999; Fauteux et al., 2005; Ma and Yamaji, 2006; Currie and Perry, 2007; Epstein, 2009; Tripathi et al., 2014). As such, addition of Si is one of the only plant protection strategies that enable plants to maximize efficiency in responding to the exact set of environmental conditions encountered, at the same time as conserving resources for growth and development. These traits make Si nutrition one of the most promising approaches for sustainable, environmentally sound and broad-spectrum disease control in various agricultural contexts.

Originally, the prophylactic role of Si treatment was attributed to the deposition of silica in the leaves, which was believed to act as a physical barrier (Jones and Handreck, 1967). Though important, accumulating evidence indicates that this passive role of Si is not solely determinant for the Si-elicited stress protection. Indeed, analyses of different plant species showed that Si nutrition can boost the expression of a large spectrum of inducible defense responses dependent on the type of stress.

Many articles report the positive influence of Si on rice plants during drought, radiation, salt, cold and heavy metal stress (Chen et al., 2011; Fang et al., 2011; Fu et al., 2012; Ming et al., 2012; Chalmardi et al., 2013; Shi et al., 2013; Tripathi et al., 2013; Kim et al., 2014; Tripathi et al., 2014). In most of these reports, Si is believed to confer stress tolerance by preventing stress-induced premature senescence and cell death due to impairment of the photosynthesis machinery. Si also protects rice plants against herbivore attacks (Reynolds et al., 2009) presumably due to priming of JA-mediated defense responses (Ye et al., 2013).

Furthermore, Si increases the level of resistance against many plant pathogens. For instance, in cucumber roots, Si treatment ensures an enhanced activity of chitinases, peroxidases, polyphenol oxidases and flavonoid phytoalexins after infection with *Pythium* spp. (Chérif et al., 1994), while in leaves an increased concentration of antifungal components protects the plant against the powdery mildew pathogen *Podosphaera xanthii* and the necrotrophic fungus *Colletotrichum lagenarium* (Fawe et al., 1998; Liang et al., 2005b). In cucumber leaves, a strongly cationic protein reinforces the cell wall at the site of attempted pathogen ingress by enhancing silica deposition, thus preventing infection by *C. lagenarium* (Kauss et al., 2003). Similarly, recent research on the beneficial effect of Si in roses clearly shows that the heightened resistance against *Podosphaera pannosa* is the result of an increased formation of papillae and deposition of callose and H_2O_2, along with an upregulation of the phenylpropanoid pathway producing antimicrobial

phenolic compounds and flavonoids (Shetty et al., 2011, 2012). Si-treatment also protects *Arabidopsis* from powdery mildew (*Erysiphe cichoracearum*), due to the accumulation of fungitoxic phenolic compounds and silica depositions at the site of infection (Ghanmi et al., 2004; Fauteux et al., 2005). In wheat, Si-induced resistance against *Blumeria graminis* f. sp. tritici is associated with increased papillae formation and accumulation of callose, fungitoxic phenolic compounds and methylated forms of trans-aconitate (Bélanger et al., 2003; Rémus-Borel et al., 2005, 2009).

However, the mode of action of Si against different pathogens is best-described for rice. Against the necrotrophic sheath blight fungus *Rhizoctonia solani*, Si application increases the activity of the defense-related enzymes polyphenoloxidase (PPO) and phenylalanine ammonialyase (PAL) (Zhang et al., 2013). The resulting accumulation of phenolic compounds is a well-described defense response (Benhamou and Nicole, 1999; Cheynier et al., 2013) which explains the positive influence of Si on rice resistance against *R. solani* (Zhang et al., 2013). Furthermore, application of Si significantly increases resistance against the necrotrophic fungus, *Cochliobolus miyabeanus* (Dallagnol et al., 2009). Si-induced brown spot resistance is hallmarked by increased photosynthesis, chitinase activity and the accumulation of fungitoxic phenolic compounds (Dallagnol et al., 2011a, 2013). The prophylactic effect of Si is best characterized for rice leaves infected with rice blast, caused by the hemibiotrophic fungus, *Magnaporthe oryzae*. The defense response in resistant cultivars is commonly associated with the induction of rapid cell death around the site of infection preventing fungal invasion of rice leaves, also referred to as a hypersensitive response (HR). However, Si application seems to prevent both HR-mediated cell death in both susceptible and resistant cultivars as well as *M. oryzae*-induced senescence and subsequent cell death (Rodrigues et al., 2005). Si-induced rice blast resistance is characterized by an increase in photosynthetic abilities, augmented activity of PPO and PAL and the accumulation of fungitoxic phytoalexins and phenolic compounds at the site of infection (Rodrigues et al., 2003, 2004; Gao et al., 2011). Elaborate microscopic analysis showed that in Si-treated rice leaves infected with *M. oryzae*, fungal hyphae were embedded in an amorphous matrix containing silica and aromatic phenolic components which restricted fungal growth (Kim et al., 2002; Rodrigues et al., 2003; Cai et al., 2008). In an attempt to unravel the mechanisms of Si-induced resistance against *M. oryzae*, a transcriptional analysis on the effect of Si and rice blast infection on rice leaves was performed (Brunings et al., 2009). In this study, Si application alone led to the up- and downregulation of 105 and 116 genes respectively, among which metal transporters, transcription factors, peroxidases and defense-related proteins. Interestingly, Si also dictated transcriptional reprogramming of extensive gene sets in blast-infected rice leaves,

triggering the up- and downregulation of 272 and 26 genes respectively, including ethylene signaling genes, chitinases, a thaumatin/pathogenesis-related protein, a class III peroxidase and a number of transcription factors and protein kinases.

Although the significance and causal roles of many of these responses remain to be resolved, the wide variety of immune responses influenced by Si amendment clearly demonstrates its potential to act as a biological inducer of plant innate defense responses. Moreover, the observation that all prophylactic effects are lost within a short period of time after Si feeding is interrupted, clearly suggests that the role of Si as a modulator of basal defense responses is dominant over its function as a mechanical barrier (Samuels et al., 1991; Fawe et al., 1998; Fauteux et al., 2005, 2006; Chain et al., 2009; Zargar et al., 2010; Ghareeb et al., 2011). Despite the fact that many of the previous mentioned articles provide a first insight into the myriad cellular processes targeted by Si and identifying numerous Si-responsive transcripts in both healthy and infected rice plants, these studies have only scratched the surface and much remains to be discovered about the precise mechanisms underpinning Si-induced disease resistance.

2.6 Mechanisms of Si action

Although the beneficial effects of Si on disease resistance in plants have been known for years, few reports in the literature have focused on understanding the mechanistic basis and regulation of this response. Here we aim to propose several potential mechanisms that can explain the prophylactic role of Si by approaching this enigma from two different sides, i.e. from a diatom and plant point of view.

Even though the essentiality of Si in plant biology is still heavily debated (see above), in a few primitive life forms, such as diatoms, Si is required for growth and development (Martin-jézéquel et al., 2000; Kinrade et al., 2002). Diatoms are encased with a silica-containing cell wall, called a frustule and the polymerisation of Si to a viable frustule is an energy-consuming process that depends on photorespiration (Martin-jézéquel et al., 2000). However, diatoms also depend heavily on Si for many non-cell wall related processes, including protein phosphorylation, DNA replication and DNA-protein interactions (Sullivan and Volcani, 1973; Okita and Volcani, 1978; Reeves and Volcani, 1984). Like plants, diatoms contain several Si transporters, often arranged in gene families, but these transporters are different in both their structures and functions from their plant counterparts (Hildebrand et al., 1998; Ma et al., 2004). Most tellingly, ectopic expression of a Si transporter gene from diatoms in transgenic tobacco had no significant impact on Si uptake, indicating fundamental differences in Si absorption between plants and diatoms

(Ma et al., 2004). These differences notwithstanding, insights into the significance and regulation of Si-mediated processes in diatoms may potentially shed new light on the poorly understood role of Si in plant stress responses. In the subsequent parts of this review, we therefore aim to uncover Si-mediated regulatory mechanisms in diatoms that also may apply to higher plants and evaluate whether these processes can contribute to broad spectrum disease resistance (Raven, 2003; Thamatrakoln et al., 2006; Currie and Perry, 2007; Pondaven et al., 2007). Moreover, building upon recent progress in identifying and characterizing the genes and molecular pathways that are involved in regulating Si-induced plant defense, we propose five hypothetical mechanisms that may explain how Si elicits broad spectrum disease resistance.

2.6.1 Si-induced priming for enhanced defense

Over the past decade, a number of transcriptomic and proteomic studies have been performed to explain the protective role of Si in various pathosystems (Watanabe et al., 2004; Fauteux et al., 2006; Chain et al., 2009; Zargar et al., 2010; Ghareeb et al., 2011; Nwugo and Huerta, 2011). One of the most salient results of these studies is that Si has very little impact on the metabolism of non-stressed plants. In rice, for instance, Si treatment was found to alter the abundance of as few as 4 proteins in the absence of stress, as compared to 57 in plants responding to both Si and cadmium stress (Nwugo and Huerta, 2011). Similar findings were obtained in several microarray studies on the effect of Si in rice, wheat, *Arabidopsis* and tomato (Watanabe et al., 2004; Fauteux et al., 2006; Chain et al., 2009; Ghareeb et al., 2011). Together with the ability of Si-treated plants to adapt to multiple types of stresses without the occurrence of resistance trade-offs, these data are compatible with the view that Si application does not directly induce immunity but rather primes plants for enhanced defense in response to pathogen attack.

One notable exception, however, is a study by Brunings et al. (2009) in which Si was shown to significantly alter the basal expression level of more than 220 rice genes. This result strikingly contrasts with previous work by Watanabe et al. (2004) who, using a similar hydroponic rice growing system, found approximately ten times less genes to be differentially expressed. Although differences in rice cultivars, microarray platforms and statistical settings used in both studies cannot be excluded, none of these factors justifies a ten-fold difference in the number of Si-responsive genes. Another confounding factor, however, involves the plant growth conditions. The literature is replete with reports that Si promotes plant growth and development especially when the plant is under some form of stress (Epstein, 1999; Fauteux et al., 2005, 2006). In line with this, one could speculate that Si-treated plants display very little differential gene expression when grown

under optimal conditions, whereas short and/or moderate stress episodes that may go unnoticed at the phenotypic level potentially amplify the influence of Si on the plant's basal transcriptome.

Because priming initiates a state of readiness that does not confer resistance *per se*, but allows for accelerated induced resistance once an attack occurs, one presumed benefit of priming is that it entails less fitness costs than direct activation of defense (van Hulten et al., 2006). Moreover, priming is thought to confer flexibility to adapt the defense response to a specific challenge, leading to a less costly and broad-spectrum resistance (Van der Ent et al., 2008; Conrath, 2011). Although the molecular aspects of priming are still poorly understood, the induction of priming is increasingly associated with a subtle increase in the level of inactive signaling components such as MAP kinases and transcription factors (Conrath, 2011). After perception of a second, pathogen-derived signal, the enhanced signaling capacity in primed plants would facilitate a faster and stronger immune response. Consistent with available articles on Si, it is not inconceivable that the broad spectrum disease resistance in Si-treated rice is at least in part the result of priming.

2.6.2 Si-hormone interactions

An additional mechanism by which Si may impact pathological outcomes is by influencing endogenous hormone balances. Corroborating this concept, mounting evidence suggests that Si is intimately associated with plant hormone signaling. In soybean, for instance, Si treatment reportedly induces synthesis of gibberellic acid, while Si-treated rice accumulates slightly higher levels of gibberellin and jasmonic acid and lower levels of ethylene (Lee et al., 2010; Hwang et al., 2007).

However, consistent with its putative role as a biological priming agent (see above), major effects of Si on plant hormone responses are only seen upon pathogen attack. In one of the first microarray studies on Si-treated plants, Fauteux et al. (2006) demonstrated the stimulating effect of Si on the biosynthesis of the stress hormones salicylic acid, jasmonic acid and ethylene in leaves challenged with the powdery mildew pathogen *Erysiphe cichoracearum*. Similarly, microarray analysis of rice infected with *Magnaporthe oryzae* showed that Si triggers activation of the ethylene signaling pathway, the role of which in resistance to blast is well established (Iwai et al., 2006; De Vleesschauwer et al., 2008; Brunings et al., 2009). Furthermore, in Si treated tomato plants infected with *Ralstonia solanacearum* both jasmonic acid and ethylene signaling pathways were found to be induced, leading to increased resistance (Zhang et al., 2004; Chen et al., 2009;

Kawamura et al., 2009; Ghareeb et al., 2011).
Although the underlying molecular mechanisms remain poorly understood, these data clearly demonstrate the potential of Si to interfere at multiple levels with hormone biosynthesis and response pathways. Moreover, these findings suggest that Si does not impose continuous changes in phytohormone homeostasis, but rather primes hormone biosynthesis and signaling processes, creating a flexible signaling network that allows the plant to finely tune its defense response to the invaders encountered. Verifying whether the versatile role of Si may indeed be attributed to high-dimensional interactions with the plant's hormone signaling network is an important challenge ahead.

2.6.3 Targeted alterations in iron homeostasis: an alternative mechanism for Si-induced disease resistance?

Iron (Fe) is a ubiquitous redox-active element and an essential micronutrient for plants and associated microorganisms. Despite its paramount importance for plant growth and reproduction, iron has only recently been identified as a central factor regulating plant pathogen defenses. Consistent with disease-related alterations in iron homeostasis in animals (Rouault, 2006; Liu and Mehdy, 2007; Brissot et al., 2011; Abed-Ashtiani et al., 2012) proposed a model whereby pathogen attack elicits the targeted redistribution of Fe to the apoplast, leading to Fe depletion in the cytosol of attacked cells and resultant activation of redox-dependent defense gene expression. Interestingly, plant Fe titers have also been shown to be a central factor in the induction of systemic resistance by beneficial rhizobacteria (De Vleesschauwer et al., 2008; Van der Ent et al., 2008). Many rhizobacteria competitively acquire ferric iron by producing large amounts of low-molecular-weight compounds or siderophores, called pyoverdins or pseudobactins. Given the scarcity of bio-available iron and the high affinity of pseudobactins for this ferric iron, pseudobactin-producing rhizobacteria are thought to interfere with the iron acquisition of other soil organisms, including the host plant. Accordingly, we recently showed a strict correlation between the resistance-inducing potential of bacterial pseudobactins and their ability to deprive young rice seedlings from iron (De Vleesschauwer and Höfte, 2009). Considering that the total iron content of Si-treated plants is reduced by on average 20% (Islam and Saha, 1969; Ma and Takahashi, 1990), it is tempting to speculate that Si may likewise induce disease resistance by perturbing iron homeostasis.
In favour of this assumption, transcriptome analysis of Si-treated rice leaves revealed transcriptional reprogramming of several genes implicated in regulating intracellular iron homeostasis (Brunings et al., 2009). Moreover, the expression patterns of these genes

mirrors those observed in iron-deficient rice leaves, further supporting our hypothesis (Gross et al., 2003; Kobayashi et al., 2005; dos Santos and de Oliveira, 2007; Walker and Connolly, 2008). It is important to note, however, that Si amendment does not impose severe levels of iron stress as evidenced by its growth-promoting abilities. Rather, Si application may trigger dynamic yet subtle changes in plant Fe status, thereby preconditioning naive tissues to respond faster and stronger upon subsequent pathogen attack.
Several mechanisms can explain Si-induced alterations in iron homeostasis. First, Si-application is well known to protect plants from iron toxicity by enhancing the oxidizing power of root tissues, which leads to increased oxidation of iron into iron oxides (Savant et al., 1997; Fleck et al., 2011). Being water-insoluble, these oxides cannot be absorbed by the roots and thereby lower the total amount of bio-available iron in the rhizosphere, potentially resulting in intracellular iron depletion (Okuda and Takahashi, 1964). Secondly, Si and iron are able to interact and many iron molecules can be co-precipitated in silica. There are, for instance, reports of Fe^{2+} binding directly to silica and also Fe^{3+}-chelating siderophores can bind Si (Perry and Keeling-Tucker, 1998; Saeki, 2004; Liang et al., 2007; Schmiederer et al., 2011). Finally and as indicated by aforementioned microarray data (Brunings et al., 2009), Si may impinge on the plant's Fe status by interfering either directly or indirectly with specific components of the iron uptake and signaling machinery.

2.6.4 Linking Si-driven photorespiration to plant immunity

The polymerisation of Si in diatoms is essential for the formation of cell walls and is therefore determining for the growth and viability of diatoms. The driving force behind Si polymerisation is respiration rather than photosynthesis. Especially photorespiration is essential in providing ATP, serine and glycine, all of which are necessary for the polymerisation of silicic acid in diatoms (Martin-Jézéquel, 1998; Martin-jézéquel et al., 2000). Photorespiration is generally considered a wasteful process that occurs in C3-plants under specific conditions. Mounting evidence, however, suggests that photorespiration might also be an important mechanism for many C3-plants to cope with abiotic and biotic stress by maintaining electron flow to prevent photoinhibition. Many processes are involved in photorespiration-mediated stress defense, including the production of oxygen radicals, increased assimilation of ammonium and replenishment of mitochondrial respiration. The outcome of these processes are extremely diverse, ranging from rapid cell death to increased longevity of the plants (Wingler et al., 2000; Foyer et al., 2009; Guan and Gu, 2009; Kangasjärvi et al., 2012). Whereas induced cell death is often effective against biotrophic pathogens, an increase in cell viability generally leads to an

increased resistance against necrotrophic pathogens (Glazebrook, 2005). Accordingly, recent advances have brought several exciting new molecular links to support a central role of photorespiration in biotic and abiotic stress-response signaling. For instance, transgenic rice lines with increased chloroplastic glutamine synthetase activities were recently shown to be more resistant towards salt stress, an effect which the authors attributed to the increased photorespiration capacity associated with the transgenic phenotype (Hoshida et al., 2000; Cai et al., 2009). Similarly, expression of the photorespiratory enzymes, formate-tetrahydrofolate ligase and hydroxypyruvate kinase was found to be increased under cadmium stress in *Arabidopsis* cells and pea plants (Romero-Puertas et al., 2007; Sarry et al., 2006). In a different example, overexpression of three key photorespiratory genes encoding glycolate oxidase, serine:glyoxylate aminotransferase and glutamate:glyoxylate aminotransferase increased resistance in melon and *Arabidopsis* against the (hemi)biotrophs *Pseudoperonospora cubensis* and *Pseudomonas syringae*, respectively (Kenigsbuch and Cohen, 1992; Taler et al., 2004; Rojas et al., 2012). In accordance with this, loss-of-function mutations in another important photorespiratory enzyme, serine hydroxylmethyltransferase, resulted in broad spectrum susceptibility of *Arabidopsis* against the biotroph *P. syringae* pv. *tomato* DC3000 and the necrotrophic fungi *Alternaria brassicicola* and *Botrytis cinerea* (Moreno et al., 2005).

Evidence connecting photorespiration to Si-afforded stress tolerance comes from Nwugo and Huerta (2011), who reported that the beneficial effect of Si in protecting rice from cadmium stress is associated with enhanced accumulation of the photorespiratory enzymes phosphoglycolate phosphatase and glycine dehydrogenase. Furthermore, intensive screening of the photosynthetic capacities in Si-treated rice plants revealed significant increases in photorespiratory ability due to Si treatment (Van Bockhaven et al., unpublished). Additional evidence supporting a role of photorespiration in Si's mechanism(s) of action is currently missing; however, given the fact that photorespiration is important for disease resistance against various pathogens (see above), it is not unlikely that Si-driven photorespiration may be an important mechanism leading to broad-spectrum disease resistance.

2.6.5 Interaction of Si with plant molecules

Although complex formation of orthosilicic acid by certain sugars and hydroxyl-amino acids has been demonstrated *in vitro* (Jugdaohsingh, 2007), there is no definitive evidence yet that Si binds to proteins or has direct biochemical functions at physiological pH. Nevertheless, consistent with the emerging role of Si as a biologically active element capable of inducing plant defense responses, Si is increasingly being associated with

modulation of primary signal transduction. Fauteux et al. (2005) hypothesized that Si's mode of action in signaling events could results from interactions with phosphorus and/or cationic metals such as Mn and Fe, which act as cofactors for many enzymes. Alternatively, though less parsimoniously, it was suggested that Si may impinge on protein activity and/or conformation by binding hydroxyl groups on amino acid residues, thereby interfering with the phosphorylation status of these signaling proteins (Fauteux et al., 2005). As for other molecular interactions, complex formation and/or interaction of cellular components and silicic acid may alter the location, activity, transport and/or selectivity of the complexed molecules. In this scenario, Si could influence the plant's defense responses at the post-translational stage, providing yet another mechanistic framework for how Si improves disease resistance without inducing major changes in the transcriptome and proteome of non-stressed plants. Additional research is essential in exploring this train of thought, but more advanced proteomics analyses on Si-treated plants might shed more light on the potential roles of Si as a post-translational modifier of plant defense signaling.

2.6.6 Interaction of Si with xenobiotics

Echoing the interaction between Si and plant molecules, Si might possibly interact with xenobiotics, such as excessive concentrations of metal ions, pathogen effectors and phytotoxins in a similar manner. Even though, there is no evidence for this hypothesis, inhibiting the negative effect of xenobiotics due to adsorption by Si might explain why pathogens often fail to cause damage to Si-treated plants (see Section 2.5.4).

For instance, Si application is known to prevent the toxic effects of excessive uptake of metal ions by adsorption of the ions on silica outside the cell (Wu et al., 2013). X-ray analysis of Si-treated pumpkin under Mn stress (Rogalla and Römheld, 2002) and rice plants under Cd and Zn stress (Shi et al., 2005; Gu et al., 2011; Song et al., 2011) showed a significant increase in deposition of a subcuticular silica layer (Shi et al., 2005). Moreover, the silica layer in rice plants under Cd and Zn stress contained higher amounts of these metals (Zhang et al., 2008). These findings indicate that the co-precipitation of Si and excessive metal ions might be responsible for the increase in tolerance towards metal toxicity in Si-treated plants. Since the export of xenobiotics towards the apoplast is a common defense strategy in plants (Horst et al., 2010; Seth, 2012), it is possible that metal ions, effectors and phytotoxins are exported to the plant's apoplast, adsorbed and embedded in the subcuticular silica layer which probably prevents their negative influence on plant metabolism.

An alternative mechanism is based on the assumption that Si itself might be 'harmful'

for plants. Si uptake results in the continuous deposition of a subcuticular layer, which might be necessary to ensure normal functioning of plants (Ma and Yamaji, 2006). The importance of exporting Si outside plant cells is demonstrated by Montpetit et al. (2012). Constitutively expressing the rice and wheat Si influx transporters *OsLsi1* and *TaLsi1* in Arabidopsis which is characterized by a low Si uptake demonstrated that Arabidopsis can not cope with a massive intake of Si in the cytosol of leaf cells. Due to Arabidopis' inability to export large quantities of silicic acid outside leaf cells, a silica layer is deposited inside the cell which inhibits all transport in- and outside the cell, leading to cell death and necrosis (Montpetit et al., 2012). The fact that the efflux of Si from plant leaves is essential, led to several hypotheses on the physical effect of Si in plant cells. First, it is possible that both plant molecules and/or xenobiotics that are adsorbed to Si inside the cytosol might be exported outside the cell along with the continuous efflux of Si towards the subcuticular silica layer where they are embedded and immobilized. Second, the constant efflux of Si is hypothesized to activate other transport systems in order to maintain the membrane pH gradient, cytosolic osmotic potential, ion concentration etc. thus transporting other components, such as xenobiotics, outside the cell ass well, where they might possibly be adsorbed by Si outside the cell, thus inhibiting their negative effects on the plant metabolism.

2.7 Conclusion

Although many treatments are reported to induce resistance against plant pathogens, there are very little strategies that induce broad spectrum disease resistance without trade-offs. Si is one of the only exceptions, rendering plants more resistant towards a wide range of abiotic and biotic stresses. The prophylactic role of Si is the result of both passive and active effects. Many studies on Si-induced broad spectrum resistance report that the active effect is prevalent. However, the molecular underpinnings of Si-mediated broad spectrum disease resistance are still poorly understood. By combining knowledge on Si metabolism in diatoms and higher plants, we propose five potential mechanisms that may explain how Si activates plant innate immune responses. First, evidence is accumulating that Si induces resistance against a wide range of stress factors by modifying the intensity and/or timing of basal defense responses. The differential expression of several transcription factors in Si-treated rice plants strengthens the hypothesis that Si primes the plant's own defensive repertoire, resulting in rapid deployment of natural defense mechanisms upon pathogen attack. In a similar vein, Si application may induce disease resistance by affecting plant hormone homeostasis, the role of which in shaping the outcome of plant-pathogen interactions is well established (Robert-Seilaniantz et al.,

2011). Third, the diverse beneficial role of photorespiration in plants under biotic stress, the dependency of Si polymerisation on photorespiration in diatoms and the accumulation of photorespiratory enzymes in Si-treated plants following stress treatment, argues that photorespiration may be an important factor in Si-induced disease resistance. Another putative mechanism involves the role of Si in maintaining and adjusting cellular iron homeostasis. Si treatment seems to be accompanied by subtle yet dynamic changes in iron homeostasis, a phenomenon which shows strong commonalities to the mechanism of action of several resistance-inducing rhizobacteria. Finally, Si may interact either directly or indirectly with various signal transduction components, resulting in enhanced signaling capacity in Si-treated plants and fortified defense responses. Hypothetical in nature, each of these mechanisms requires extensive experimental validation, but may serve as a primer for future research aimed at delineating the molecular basis and regulation of Si-afforded disease control.

Given the huge potential and value of Si nutrition in stress management, the application of a range of biotechnological strategies based on the modulation of Si content and its signaling effects could provide a unique tool for the genetic improvement of crop productivity in a sustainable manner. Classic genetic approaches and genome-wide transcriptional analyses are now beginning to unveil large numbers of Si targets, shedding light on the complexity and diverse activity of Si in plants. An important challenge in the coming years, however, will be unraveling the exact mechanisms of Si-induced pathogen defenses in a systems biology-based manner. This is especially important as studying how Si is able to induce plant broad-spectrum disease resistance without inducing resistance trade-offs and appreciable fitness penalties will require profound knowledge of both the transcriptional and post-transcriptional fate of the target response. Special efforts should also be paid to uncovering the crosstalk mechanisms between Si and other plant growth regulators. At the same time, controlled field experiments will be critical in understanding the physiological behavior of Si-induced plants under various stress conditions. By combining all of abovementioned approaches, we may finally make sense of Si-induced disease control.

3

Transcriptome analysis of Si-induced brown spot resistance in rice

Authors

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Abstract

In face of the ever-increasing loss of arable land, shrinking fresh water resources and global climate change, feeding the world's burgeoning population in coming decades will be a daunting task. One of the solutions to safeguard global food security is to minimize yield losses caused by biotic and abiotic stresses. Over the past decades, application of silicon (Si) has emerged as a promising disease control strategy that is able to protect plants against a broad range of microbial pathogens, among which the necrotrophic rice brown spot fungus *Cochliobolus miyabeanus*. However, despite this huge potential, relatively little is known about the precise mechanisms by which Si induces broad-spectrum disease resistance. In an attempt to unveil the molecular underpinnings of Si-induced disease resistance, we studied the transcriptome of control and Si-treated rice plants subjected to *C. miyabeanus* infection using Agilent 44K oligo DNA arrays. The data obtained were analysed using a variety of bioinformatic approaches suitable for rice functional genomics. Brown spot development is hallmarked by the formation of necrotic disease lesions surrounded by extensive chlorosis. Interestingly, analysis of brown-spot infected plants suggested that *C. miyabeanus* actively represses photosynthetic processes in order to trigger premature senescence and, hence, inflict disease. In Si-treated plants, however, this pathogen-induced suppression of photosynthesis was strongly impaired, suggesting that Si alleviates biotic stress imposed by the pathogen. Further analysis of the effect of Si in brown spot infected leaves demonstrated significantly increased levels of photorespiration in these plants. Even though photorespiration is often considered a wasteful process in C3 plants like rice, recent studies indicate that this metabolic bypass also indirectly enhances resistance during abiotic stress and pathogen attack by protecting the plant's photosynthetic machinery. Taking these facts into account, our findings favor a scenario whereby Si enhances brown spot resistance at least in part by boosting photorespiration-associated metabolism, thereby counteracting *C. miyabeanus*-induced senescence and cell death. Moreover, our results shed light onto the mechanistic basis of Si-afforded disease control and support the view that in addition to activating plant immune responses, Si may also reduce disease severity by interfering with pathogen virulence strategies.

3.1 Introduction

Silicon (Si), a commonly available element in many soils is absorbed by plant roots in the form of noncharged silicic acid [$Si(OH)_4$] (Ma and Yamaji, 2006). A specific transporter system transports Si through the xylem into leaf cells (see Chapter 2.5). Over time, water-soluble silicic acid is deposited into an insoluble, subcuticular silica layer in the leaves, which renders Si-treated leaves more rigid. The application of Si has manifold effects of plants leading to an increased growth and yield and conferring resistance against a variety of biotic and abiotic stresses (Fauteux et al., 2005; Ma and Yamaji, 2006; Epstein, 2009; Guntzer et al., 2011)). In order to disentangle the molecular underpinnings of Si-induced broad spectrum disease resistance, a series of transcriptional and proteome analyses in different plant species including rice, Arabidopsis, wheat and tomato have been performed (Fauteux et al., 2005; Brunings et al., 2009; Chain et al., 2009; Fleck et al., 2011; Ghareeb et al., 2011; Nwugo and Huerta, 2011).

Two different views emerge from theses studies, namely the hypothesis that Si-induced priming is responsible for broad spectrum resistance on one hand and the hypothesis that Si induces stress tolerance at least in part through its effects on the plant's metabolism on the other hand.

Si-mediated priming is often suggested to be responsible for enabling faster and/or stronger activation of defense responses when necessary, which offers a more efficient disease protection at lower energy costs (Fauteux et al., 2005; Ghareeb et al., 2011; Ye et al., 2013; see section 2.6.1). The current understanding of the molecular mechanisms that govern priming is still in TFs) (Pastor et al., 2013). During pathogen infection post-translational activation of the signaling components subsequently leads to rapid recruitment of defense responses resulting in increased disease resistance (Conrath et al., 2006; Conrath, 2011). For instance, in Arabidopsis plants treated with the chemical priming agent benzothiadiazole (BTH) or in the constitutive priming mutant *edr1*, priming of defense responses is associated with accumulation of inactive MPK3 and MPK6 (Beckers et al., 2009). Subsequent infection with the pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) leads to a stronger accumulation of active MPK3 and MPK6 in the primed plants resulting in increased levels of resistance, whereas in *mpk3* and *mpk6* mutants BTH-mediated resistance against *Pst* is attenuated. Moreover, Arabidopsis root colonization with *Pseudomonas fluorescens* WCS417r is known to prime defense responses against *Pst* and the downy mildew pathogen *Hyaloperonospora arabidopsidis* (Pieterse et al., 1998; Pozo et al., 2008). The WCS417r-mediated priming process depends on the expression of both ET-inducible *AP2/EREBP* TFs and jasmonic acid (JA)-responsive *MYC2* TF (Pozo et al.,

2008; Van der Ent et al., 2009). In primed Arabidopsis plants elevated *MYC2* expression is responsible for priming the expression of JA-responsive genes during infection with *Pst*, ultimately leading to increased resistance. Moreover, in Arabidopsis mutants impaired in the *MYC2* gene, WCS417r is unable to induce resistance against *Pst* and *H. arabidopsidis* (Pozo et al., 2008). Furthermore, the increased resistance against *Pst* and *H. arabidopsidis* in Arabidopsis plant treated with the chemical priming agent β-aminobutyric acid (BABA) appears to depend on the upregulation of several salicylic acid (SA)-responsive *WRKY* TFs (Van der Ent et al., 2009).

Another recurrent finding in the different transcriptional and proteome analyses that aimed to unravel Si-induced resistance (Fauteux et al., 2005; Brunings et al., 2009; Chain et al., 2009; Fleck et al., 2011; Ghareeb et al., 2011; Nwugo and Huerta, 2011) is that the broad spectrum mode of action of Si is often accompanied by alterations of primary metabolism generally due to increased levels of photosynthesis during abiotic stress (Nwugo and Huerta, 2008; Shen et al., 2010; Chen et al., 2011; Farooq et al., 2013) and during pathogen infection (Resende et al., 2012; Dallagnol et al., 2013; Perez et al., 2014). In plants, primary metabolic pathway consists of photosynthesis, gluconeogenesis and respiration that provide carbohydrates and energy, whereas nitrogen metabolism accounts for the incorporation of inorganic and organic nitrogen in amino acids (Rontein et al., 2002). Plant-pathogen interactions are generally determined by the pathogen's need for carbohydrates and nutrients and the plant's need for energy to fuel energy-demanding plant defense mechanisms (Berger et al., 2007; Bolton, 2009). Since the plant's primary metabolism is at the crossroad of these contrasting interests, central metabolic processes often shape the outcome of pathogen infection, either leading to 'evasion' that promotes cell death or to 'endurance' which maintains cell viability during infection (Mur et al., 2013; Seifi et al., 2013). The evasion strategy often is effective against pathogens with a biotrophic lifestyle, whereas endurance generally confers resistance towards necrotrophic pathogens (Glazebrook, 2005; Seifi et al., 2013). In Arabidopsis, for instance, infection with an avirulent isolate of the (hemi)-biotrophic bacterium *Pseudomonas syringae* leads to a faster decrease in photosynthesis compared to infection with a virulent isolate (Bonfig et al., 2006). The resulting induction of rapid programmed cell death around the site of infection, often referred to as a HR, leads to resistance (Mur et al., 2008). On the other hand, rapid induction of cell death around the site of infection has the opposite effect against the necrotrophic pathogens *Botrytis cinerea* and *Sclerotinia sclerotium*. Moreover, these pathogens even induce a hypersensitive response to facilitate the infection process (Govrin and Levine, 2000).

The induction of senescence and cell death is often exploited as a virulence strategy by

several other necrotrophic pathogens (Glazebrook, 2005), which also appears to be the case for the necrotrophic rice leaf fungus *C. miyabeanus* (see Chapter 4). This toxin-producing fungus also called *Bipolaris oryzae* (Breda de Haan) causes brown spot disease in rice (Xiao et al., 1991; Dela Paz et al., 2006). The link between senescence and brown spot severity explains why brown spot disease is prevalent in rainfed rice fields which are prone to premature senescence (Ou, 1985; Leung et al., 2003; Zadoks, 2003). Even though semi-resistant rice cultivars are available, brown spot disease is mainly managed by using fungicides (Castell-Miller and Samac, 2012). In this light, application of Si emerges as a sustainable protection strategy against *C. miyabeanus* (Dallagnol et al., 2009, 2011a). Dallagnol et al. (2013) showed that Si impedes pathogen-induced inhibition of the photosynthesis apparatus resulting in increased photosynthetic activity of Si-treated rice leaves after brown spot infection.

In an attempt to shed more light on the rice - *C. miyabeanus* interaction and the beneficial effect of Si on brown spot resistance in rice, a microarray experiment was performed during early stages of infection (12hpi). Our data support a central role of nitrogen- and photosynthesis-related metabolic processes in shaping the outcome of rice-brown spot interactions. Moreover, we propose that Si protects rice from brown spot attack by preventing the pathogen from hijacking the plant's primary metabolism for its own benefit.

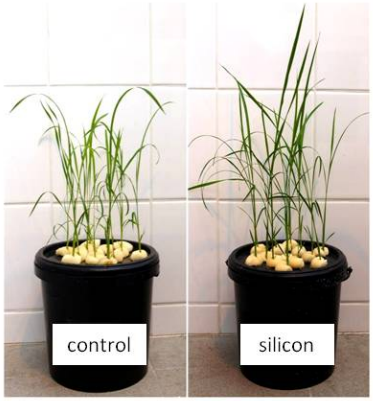
3.2 Results

3.2.1 Effect of Si on rice growth and yield

The positive effect of Si on rice plants was studied in a hydroponic nutrient solution containing 0 or 2mM Si under the form of silicic acid, ensuring a continuous supply of Si to the rice roots. The application of Si increased plant height, biomass and yield due to a combination of increased tillering and ear filling (Fig 3.1 A and B). Si also shortened the time to flower initiation with on average 10 days (Fig 3.1 C). Furthermore, leaf gas exchange measurement on untreated and Si-treated rice plants (Fig 3.1 D) showed that even though the level of respiration (Rd) was similar for both treatments, Si application increased both net photosynthesis as well as the level of photorespiration. Si-treated plants demonstrated higher levels of photochemical quenching (qP) which is a non-linear measure for the proportion of open photosystem II (PSII) centers. Effective PSII quantum yield (ΦPSII) was also increased by Si, indicating that more photons were absorbed to drive the photochemical reactions. Si seemed to increase water-use efficiency since net photosynthesis was improved without altering the transpiration rate. The positive effect

of Si on photosynthesis was probably due to increased levels of chlorophyll (Fig 3.1 E) enabling Si-treated plants to use more light energy than untreated plants. Si-treated plants lost more energy to photorespiration, which could be explained by the deposition of a subcuticular silica layer which is thought to be driven by photorespiration (see Chapter 2.5). Despite the energy-losses due to enhanced photorespiration, net photosynthesis was still significantly higher in Si-treated plants compared to untreated plants.

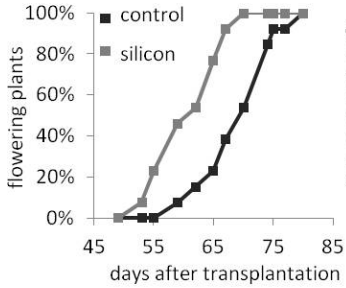
A



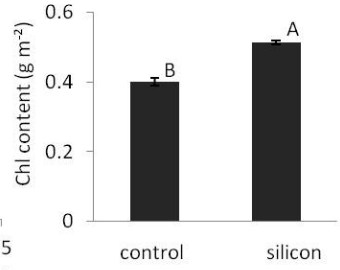
B

characteristics	control mean	control SE	silicon mean	silicon SE
dry plant weight (g)	3.86	0.31 $_b$	9.71	0.91 $_a$
# tillers / plant	1.23	0.14 $_b$	2.46	0.28 $_a$
ear weight (g)	0.91	0.09 $_b$	1.82	0.14 $_a$
ear filling (%)	0.67	0.04 $_b$	0.83	0.13 $_a$

C



D



E

Leaf gas exchange measurements	control mean	control SE	silicon mean	silicon SE
net photosynthesis (An; µmol CO_2 m^{-2} s^{-1})	9.61	1.14 $_b$	12.88	1.42 $_a$
respiration (Rd, µmol CO_2 m^{-2} s^{-1})	3.45	0.23 $_a$	3.61	0.16 $_a$
photorespiration (Ri; µmol CO_2 m^{-2} s^{-1})	3.44	0.18 $_b$	3.99	0.23 $_a$
photosystem II efficiency (ϕPSII)	0.14	0.01 $_b$	0.17	0.00 $_a$
photochemical quenching (qP)	0.30	0.02 $_b$	0.36	0.01 $_a$
transpiration (Tr; mmol H_2O)	2.36	0.22 $_b$	2.88	0.13 $_a$

Figure 3.1: A) Untreated (control) and Si-treated (Si) 3-weeks old rice plants. B) Yield characteristics of untreated (control) and Si-treated (Si) rice plants (n=26, parametric, α=0.05). C) Flowering time of untreated (control) and Si-treated (Si) rice plants in four biological repeats (n=48). D) Chlorophyll contents of untreated (control) and Si-treated (silicon) rice leaves in four biological repeats (n=36, parametric, α=0.05). E) Leaf gas exchange and chlorophyll a fluorescence measurements on untreated (control) and Si-treated (Si) rice plants in two biological repeats (n=24, parametric, α=0.05).

3.2.2 Si-induced brown spot resistance

Consistent with previous results (Dallagnol et al., 2009; Rezende et al., 2009; Silva et al., 2012; Dallagnol et al., 2013), the application of Si resulted in a reduction of brown spot disease severity (Fig 3.2 A, B and C). Si-treated rice leaves clearly showed smaller lesions and substantially less chlorosis and necrosis upon brown spot infection when Si was applied continuously. The prophylactive effect of Si on disease resistance is due to the combined effect of passive protection offered by the hard, subcuticular silica layer and active Si-mediated recruitment of defense responses. In an attempt to investigate whether the active or passive effect of Si on brown spot resistance is prevalent and the time span during which Si is effective, the influence of different Si treatments on brown spot resistance was investigated (Fig 3.2 D). All Si treatments are compared to control plants that were grown in the absence of Si (Si-/Si-). The first treatment existed of Si-treated plants that had been deprived of Si three days before inoculation (Si+/Si-). Another treatment consisted of plants that only had been treated with Si for three days before inoculation (Si-/Si+). The final group comprised plants that had continuously been treated with Si (Si+/Si+). This experiment showed that short term Si deprivation significantly tackled the level of brown spot resistance (Si+/Si-). The deposition of silica in rice plants leads to harder leaves due to the accumulation of a subcuticular silica layer and to the silification of the trichomes on the leaf surface. The level of silica deposition in the leaves was assessed based on these phenotypical characteristics. After three days of Si deprivation, the active effect of Si on disease resistance appeared to be already abolished, while the subcuticular silica layer still appeared intact and might still have offered some degree of protection against *C. miyabeanus* compared to control plants (Si-/Si-). Short term Si application (Si-/Si+), on the other hand, induced the same level of resistance as continuously Si-treated rice plants (Si+/Si+). The seemingly absence of a silica layer suggested that the active induction of brown spot resistance is predominant in the outcome of Si-induced brown spot resistance.

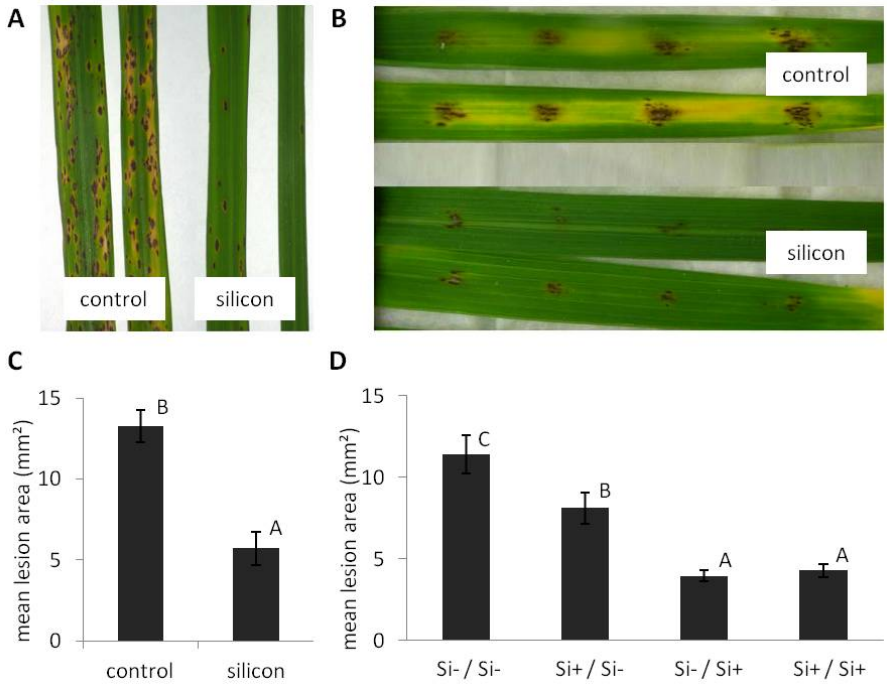


Figure 3.2: Si-induced brown spot resistance in rice three days after A) spray-infection or B) infection with 4 droplets (15μl) of spore solution (1 x 10^4 conidia.ml^{-1}). C) Mean lesion area on untreated (control) and Si-treated leaves (silicon) 3 days after spray-inoculation (1 x 10^4 conida.ml^{-1}, non-parametric, α=0.05). D) Mean lesion area three days after spray-inoculation (1 x 10^4 conidia.ml^{-1}) on leaves from untreated plants (Si- / Si-), Si-treated plants that have been deprived from Si for three days prior inoculation (Si+ / Si-), plants that have been treated with Si for three days prior inoculation (Si- / Si+) and plants that were treated with Si continuously (Si+ / Si+) in three biological repeats (Bonferoni, n=36, α=0.05). The experiment has been repeated at least three times.

3.2.3 High-throughput expression profiling of rice transcription factors

In a first attempt to elucidate whether Si-induced priming for enhanced defense responses might involve accumulation of inactive TFs, we assessed the influence of Si-treatment on the expression of all rice transcription factors using a quantitative RT PCR platform (Table 3.1).

Table 3.1: List of transcription factors that are significantly up- and downregulated by silicon application in uninfected rice plants (three biological repeats, n=12, paired t-test, ($\alpha = 0.05$).

TF type	Identifier	silicon vs. control (log2)	p-value
bHLH	LOC_Os01g09900	2.15	0.008
MADS	LOC_Os01g67890	5.67	0.050
putative TF	LOC_Os04g51130	1.82	0.003
C3HC4	LOC_Os05g33830	3.37	0.041
RING-H2	LOC_Os06g16060	2.99	0.010
NAM	LOC_Os11g31360	2.66	0.016
MYB	LOC_Os12g07610	5.25	0.035
MYB	LOC_Os01g50110	-3.31	0.004
C2H2	LOC_Os01g62190	-3.10	0.004
C3HC4	LOC_Os01g62640	-2.28	0.006
TGA	LOC_Os02g16680	-1.73	0.008
F-box containing TF	LOC_Os03g28130	-2.00	0.005
AP2/EREBP	LOC_Os04g34970	-1.89	0.005
RAV	LOC_Os05g47650	-3.18	0.007
ERF	LOC_Os05g49700	-2.42	0.009
bZIP	LOC_Os06g41100	-1.70	0.001
MYB	LOC_Os06g45890	-3.26	0.006
bHLH	LOC_Os08g37730	-1.96	0.003
putative TF	LOC_Os09g31050	-1.72	0.004
NAC	LOC_Os09g33490	-1.81	0.003
AP2	LOC_Os11g03540	-2.18	0.004
MADS	LOC_Os12g21850	-1.71	0.005

The differentially expressed TFs might account for the positive influence of Si on plant growth (see Chapter 3.2.1) and more importantly on broad spectrum disease resistance. However, none of the differentially expressed TFs has been reported to play a significant role in rice defense responses. To identify potential key regulators that might mediate Si-induced broad spectrum resistance, binding sites enrichment analysis was performed on the promoters (1kb) of the differentially expressed TFs (Table 3.2). This analysis revealed an overrepresentation of binding sites for MYB, MADS and GAMYB TFs and TGA, ERF, ABF and DREB TFs in the promoters of TFs that are respectively up- and downregulated by Si-application (Table 3.2).

Table 3.2: *Cis*-elements that are overrepresented in the promoters of the up- (+) and downregulated (-) transcription factors from Table 3.1 using TRANSFAC (Matys et al., 2006).

	Cis-element	p-value	Sequence	Function	Reference
+	GAMYB	3.1E-04	YAACSGMC	GA-inducecd flower initiation	Kaneko et al. (2004)
+	MYB	5.4E-04	YAACNNNY	gametogenesis	Yang et al. (2001)
+	MADS	2.7E-03	AAAAATGG	flower initiation	Riechmann et al. (1996)
-	TGA	1.6E-03	ACGT	abiotic stress response	Kesarwani et al. (2007)
-	DREB	9.5E-04	CCGAC	abiotic stress response	Srivasta et al. (2010)
-	ABF	4.3E-03	CACGTG	SA-induced defense response	Roychoudhury et al. (2008)
-	ERF	6.9E-05	CGCCGCC	ABA-signalling	Hao et al. (2002)

3.2.4 Microarray experiment

To shed further light on the molecular base underpinning Si-induced growth promotion and resistance against *C. miyabeanus*, a microarray experiment was performed in three biological repeats (2x2 factorial design: control and Si-treated, mock-treated and infected). This study revealed that of 43494 gene probes on the Agilent slides, 2906 were differentially expressed (FDR <0.01, -2 >$\log_2$ fold change >2) in the four comparisons (GEO accession GSE55330). The volcano plots show the distribution of the significance (-log p-value) over the expression ($\log_2$ fold change) of a given gene, represented as a dot (Fig 3.3). A schematic overview shows the amount of genes that are up- and downregulated (Fig 3.4 A). The application of Si largely had the same effect in both uninfected (si,m vs. co,m) and infected leaves (si,I vs. co,I) leading to the differential expression of a large dataset of genes, mostly downregulated. Brown spot infection resulted in a less pronounced transcriptional profile, both in control (co,I vs. co,m) and Si-treated leaves (si,I vs. si,m). A Venn diagram was constructed (Oliveros, 2007) to visualize specificity of the genes and the number of commonly regulated genes (Fig 3.4 B). Hierarchical clustering analysis (Fig 3.4 C) showed two clusters, on containing the comparisons Si, mock vs. control, mock (si,m vs. co,m) and Si, infected vs. control, infected (si,I vs. co,I), whereas the other cluster comprised the comparisons control, infected vs. control, mock (co,I vs. co,m) and Si, infected vs. Si, mock (si,I vs. si,m).

Surprisingly, the outcome of the high-throughput expression profiling of rice transcription factors (section 3.2.3) does not coincide with the results of the microarray experiment (si,m vs. co,m), which do not show a significant differential expression of rice *TFs*. The influence of Si on the expression of *TFs* (Table 3.1) is not very pronounced and these more subtle differences in expression are not detected in the microarray experiment, probably due to the lower levels of sensitivity compared to the quantitative RT-PCR platform used in section 3.2.3.

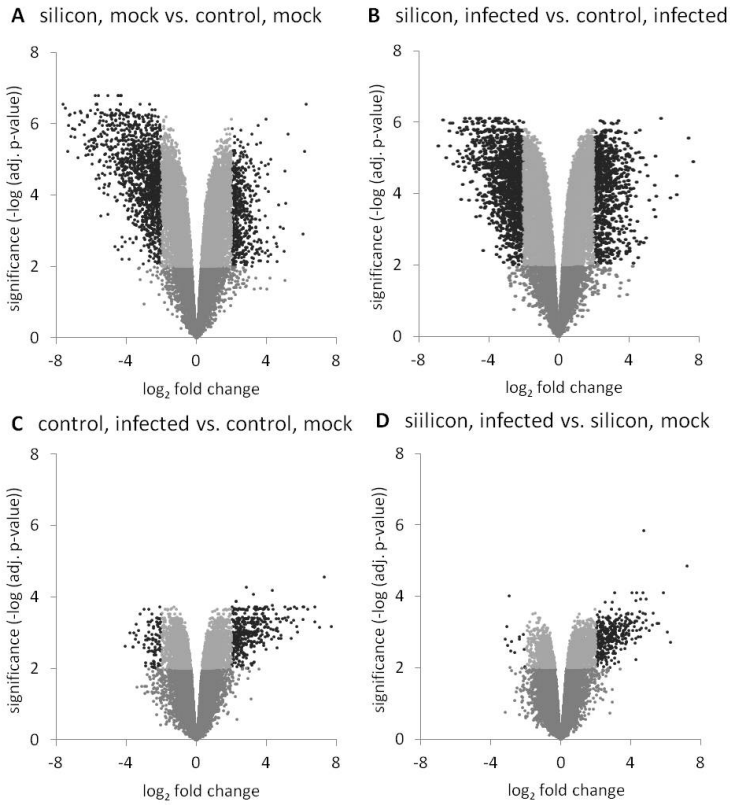


Figure 3.3: Volcano plots of the microarray data in different comparisons. The difference of expression levels of each array spot is represented by a dot. Statistical analysis (LIMMA) only retained the genes with p-value <0.01 or -log $_{10}$(p-value) >2 and a -2 <log$_2$ fold induction <2 (GEO accession GSE55330).

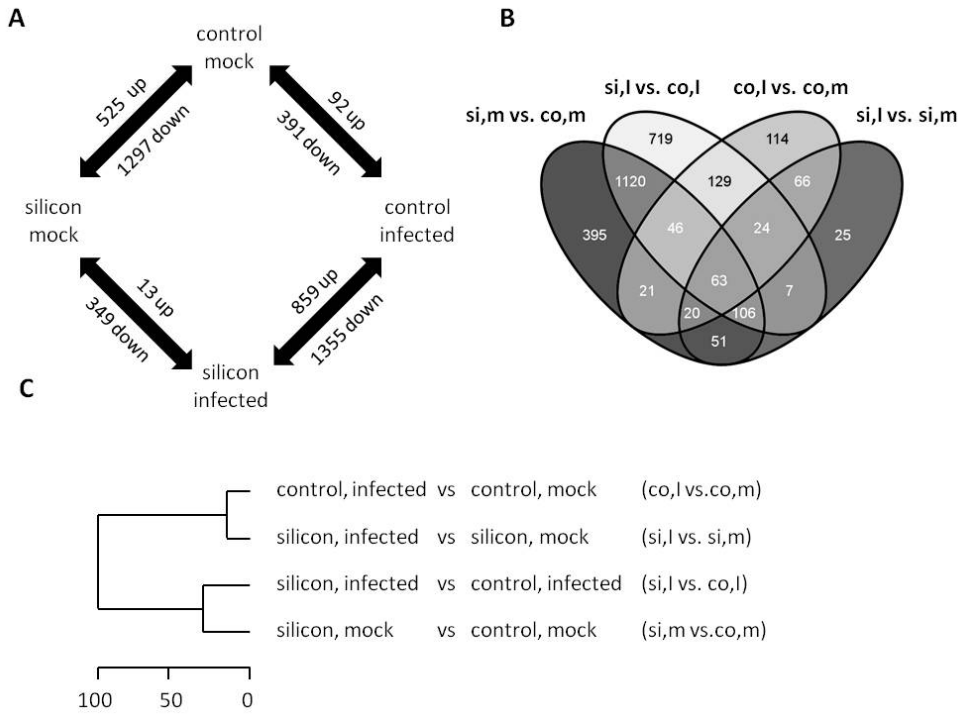


Figure 3.4: A) Schematic overview of the number of differentially expressed genes for each comparison of the microarray experiment (2x2 factorial design) in three biological replicates after statistical analysis (LIMMA, FDR <0.01, -2 >$\log_2$ fold change >2, GEO accession GSE55330). B) Venn diagram (Oliveros, 2007) showing genes differentially expressed for each comparison and all possible combinations. C) Hierarchical clustering (Tamura et al., 2011) of the microarray data identified similar expression patterns in Si-treated vs. control rice leaves and in brown spot-infected vs. uninfected leaves.

Gene ontology enrichment analysis of microarray data

Gene ontology (GO) enrichment analysis using MapMan software (Usadel et al., 2009) showed that mainly primary metabolic processes, including photosynthesis, glycolysis, amino acid metabolism and modulation of the nitrogen cycle (Fig 3.5) and defense response pathways among which defense hormone pathways, the modulation of cell wall and membrane components and the phenylpropanoid pathway (Fig 3.6) were influenced by brown spot infection and Si application. Of special interest are the ET pathway, because it is known to negatively influence brown spot resistance (De Vleesschauwer et al., 2010; see Chapter 4) and the phenylpropanoid pathway which plays an important role during infection with *C. miyabeanus* (Shabana et al. (2008); see Chapter 4).

Influence of Si on uninfected leaves (si,m vs. co,m)

Application of Si led to an increased expression of genes that mediate chlorophyll biosynthesis, light-dependent reactions and nitrate reduction. Genes involved in glycolysis, cell wall biosynthesis and degradation and nitrogen metabolism and transport were differentially expressed. Moreover, Si application downregulated amino acid metabolism-related and defense-associated genes involved in lipid desaturation, phenylpropanoid pathway, biotic stress response and in the metabolism of the defense hormones ethylene (ET), jasmonic (JA) and salicylic acid (SA).

Influence of *C. miyabeanus* (co,I vs. co,m)

One of the major influences of *C. miyabeanus* on primary metabolic processes was the downregulation of genes involved in photosynthetis like chlorophyll biosynthesis, light-dependent reactions, Calvin cycle and photorespiration. In addition, brown spot infection also slightly upregulated amino acid biosynthesis, while enzymes involved in nitrate reduction were downregulated.

Influence of Si on infected leaves (si,I vs. co,I)

Comparing the effect of Si in infected vs. uninfected leaves yielded very similar results (si,m vs. co,m) (si,I vs. co,I). The only notable difference was the higher number of DEGs associated with light-dependent reactions, the Calvin cycle, photorespiration and nitrate reduction in infected versus mock inoculated plants.Very little defense-related pathways were induced by Si in infected leaves.

Influence of *C. miyabeanus* on Si-treated leaves (si,I vs. si,m)

Interestingly, brown spot infection did not significantly affect the transcriptome of Si-treated leaves, except for a slight increase in the number of genes implicated in defense-related processes such as the phenylpropanoid pathway, biotic stress responses and cell wall biosynthesis.

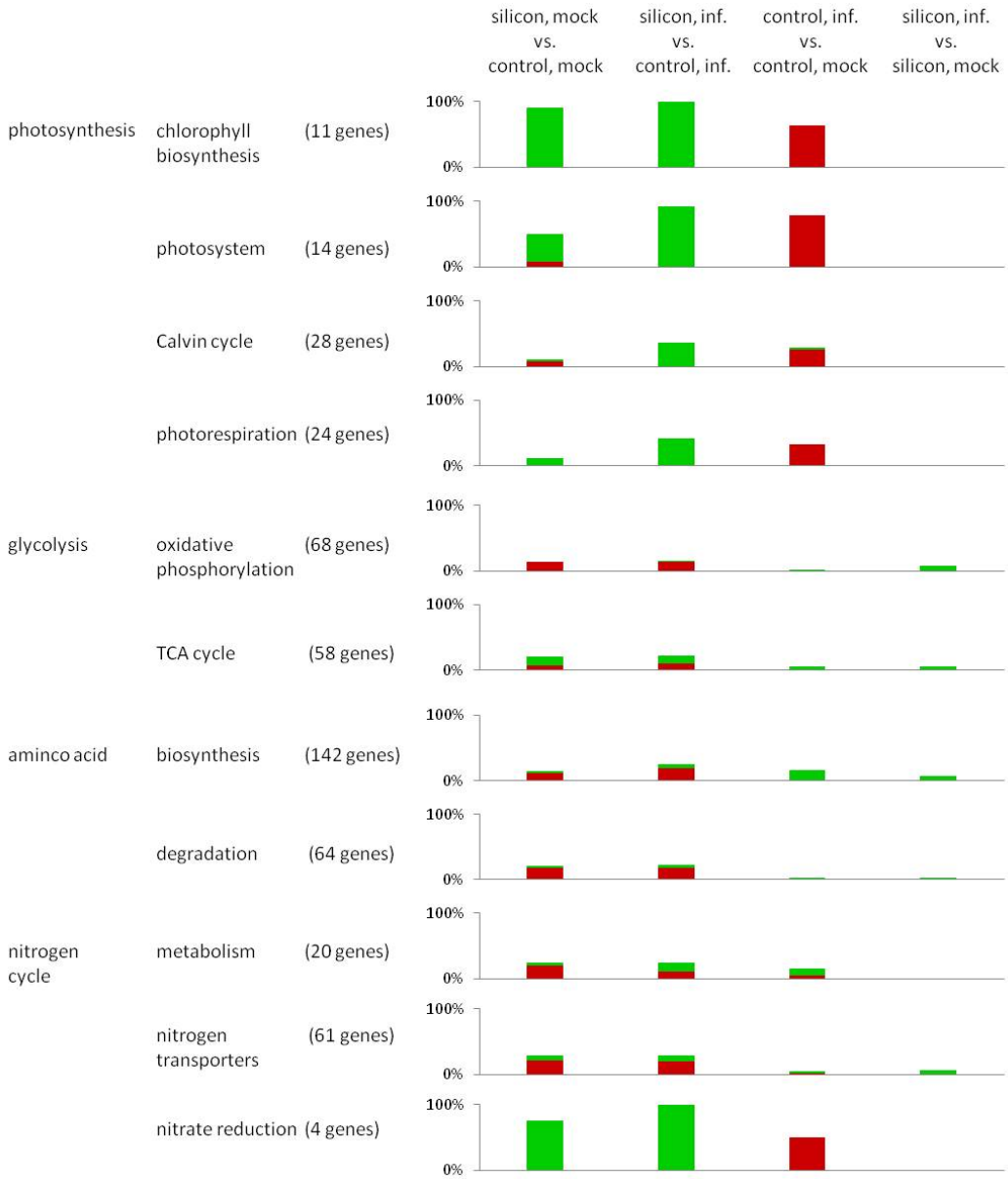


Figure 3.5: Enrichment analyses of gene ontologies of primary metabolism on microarray data (LIMMA, FDR <0.01, GEO accession GSE55330) in four comparisons. For each gene ontology the amount of genes that are represented in the microarray experiment are shown. The colored bars indicate the relative portion of the gene ontology genes that show a substantial difference in expression for the four different comparisons (green bars = upregulation, log2 fold change >2 and red bars = downregulation $\log_2$ fold change <-2). The gene ontologies are derived from the MapMan database (Usadel et al., 2009).

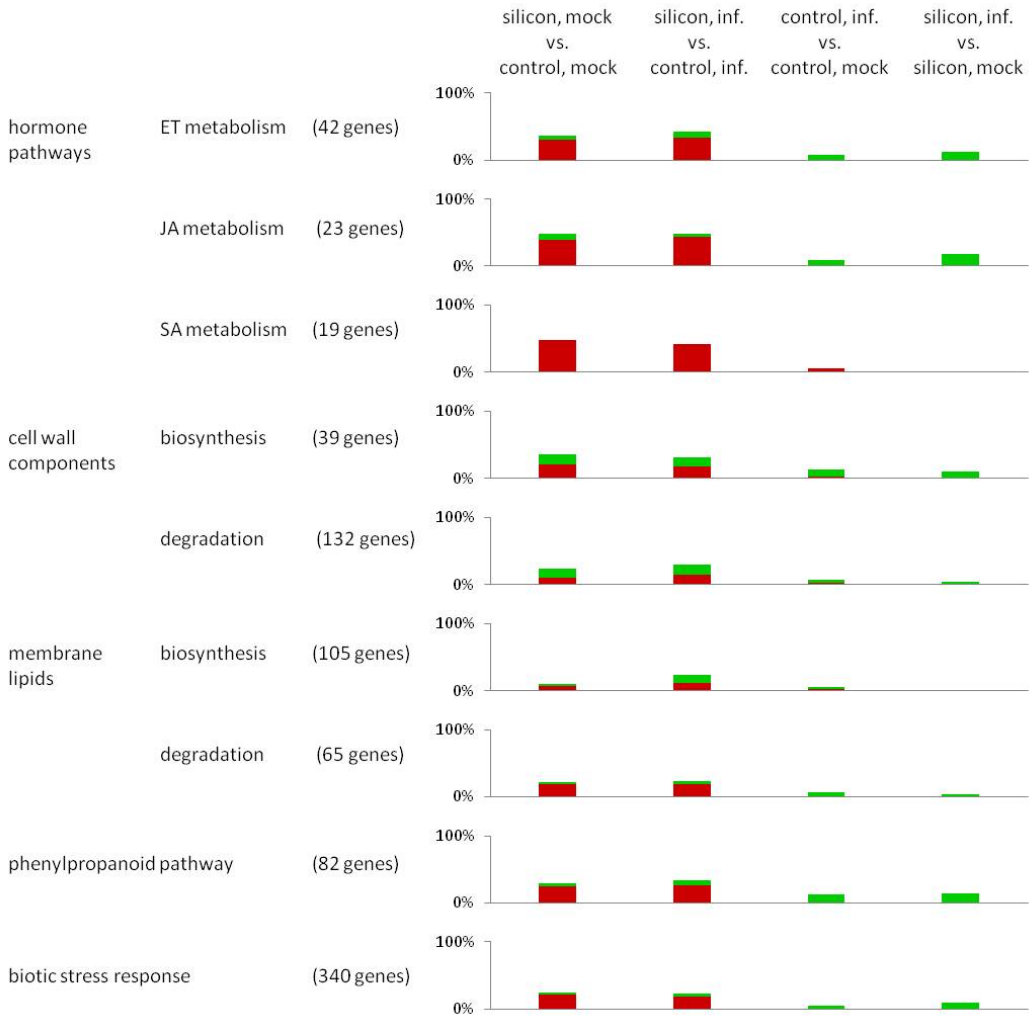


Figure 3.6: Enrichment analyses of gene ontologies of plant defense responses on microarray data (LIMMA, FDR <0.01, GEO accession GSE55330) in four comparisons. For each gene ontology the amount of genes that are represented in the microarray experiment are shown. The colored bars indicate the relative portion of the gene ontology genes that show a substantial difference in expression for the four different comparisons (green bars = upregulation, log2 fold change >2 and red bars = downregulation $\log_2$ fold change <-2). The gene ontologies are derived from the MapMan database (Usadel et al., 2009).

Detailed analysis of the influence of *C. miyabeanus* and Si on the microarray expression of central metabolic genes

Brown spot infected leaves are hallmarked by the typical necrotic lesions surrounded by a chlorotic halo, whereas Si-treated leaves demonstrated smaller lesions and less chlorosis (Fig 3.3 B). Linking these phenotypes to the results of the GO enrichment analysis (Fig 3.5 and 3.6) led to the hypothesis that central metabolism plays an essential role in shaping the outcome of rice-*C. miyabeanus* interaction. To investigate the role of Si and/or *C. miyabeanus* on the central metabolism, microarray data of a subset of primary metabolism genes was studied in more detail.

Influence of Si (si,m vs. co,m) on central metabolism

Si application increased the expression of photosynthetic genes that mediate light harvesting and the Calvin cycle (Fig 3.7 A), which coincides with the observed increase in photosynthesis in Si-treated plants (Fig 3.1 E). The enhanced photosynthetic abilities of Si-treated plants coincided with a decrease in the expression of genes that are responsible for mitochondrial ATP synthesis. Since Si-treated plants are more efficient in using photochemical energy (Fig 3.1 E), chloroplastic ATP synthesis might be enough to account for the majority of the plant's ATP need resulting in a decreased mitochondrial ATP synthesis. Furthermore, Si led to an upregulated expression of *nitrate* and *nitrite reductases* and the nitrate transporters *NRT1.1C*, *NRT1.4* and *CLC1*. Since AtNRT1.1, the homolog of OsNRT1.1C, has been reported to act as a nitrate receptor/transporter (Gojon et al., 2011) and AtCLCa, the homolog of OsCLC1 accounts for the transport of nitrate from the vacuole to the cytosol (Geelen et al., 2000; De Angeli et al., 2006), these findings suggest an increased cytosolic nitrate concentration that fuels the predicted nitrate reduction in Si-treated leaves. Furthermore, the ammonium transporters *AMT2.2* and *AMT3.3* were downregulated by Si treatment in infected leaves. The localization of most ammonium and nitrate transporters in rice leaves remains to be elucidated (Suenaga et al., 2003; Martinoia et al., 2007; Gaur et al., 2012), but their differential expression suggests that Si induces a shift in nitrogen transport in Si-treated plants.

Influence of *C. miyabeanus* (co,I vs. co,m) on central metabolism

The transcriptional changes in central metabolic genes after brown spot infection (co,I vs. co,m, Fig 3.7 B) indicated a downregulation of photosynthetic genes involved in light-dependent reactions and Calvin cycle (Table S2). Furthermore, GO analysis suggested that brown spot infection decreased photorespiration. Many orthologs of the photorespira-

tory enzyme *Rubisco* were downregulated in brown spot infected leaves. Even though Rubisco plays an essential role in carboxylation during photosynthesis, transient increases in the expression of *Rubisco* genes are often attributed to photorespiration during which Rubisco incorporates oxygen into the Calvin cycle in stead of carbon dioxide (Wang et al., 2012; Lakshmanan et al., 2013). Several photorespiratory marker genes characterized by Lakshmanan et al. (2013) were significantly downregulated although the level of downregulation was not very pronounced. The decrease in photosynthetic genes seemed to be accompanied by alterations in nitrogen metabolism. Brown spot infection led to a decreased expression of *nitrate* (*NR*) and *nitrite reductases* (*NiR*) and an upregulation of cytosolic *glutamine synthetase 1.3* (*GS1.3*) which is associated with senescence-mediated nitrogen remobilization (Pageau et al., 2006; Tabuchi et al., 2007).

Influence of Si on infected leaves (si,I vs. co,I) on central metabolism

There are many similarities between the influence of Si in uninfected leaves and leaves inoculated with *C. miyabeanus*. First, the beneficial effect of Si on photosynthesis is also apparent after brown spot infection (Fig 3.5, Fig 3.7 c). These results can be linked to the fact that Si-treatment reduces the level of chlorosis in rice leaves infected with *C. miyabeanus* (Fig 3.2 A and B). Second, even though plant defense responses are energy-demanding (Bolton, 2009; Bauwe et al., 2012), mitochondrial ATP synthesis was significantly lower in Si-treated leaves after infection. Together with the fact that Si application did not seem to induce defense-related processes (Fig 3.6), these findings suggest that Si application might not confer brown spot resistance via the activation of typical defense responses. Finally, Si induced the expression of nitrate and nitrite reductases and nitrate transporters *NRT1.1C*, *NRT1.4* and *CLC1*, whereas the ammonium transporters *AMT2.2* and *AMT3.3* were downregulated in Si-treated leaves infected with *C. miyabeanus*.

On the other hand, the microarray experiment also showed differential expression of genes that are specific for the influence of Si on rice leaves infected with *C. miyabeanus* and might play a significant role in conferring Si-induced brown spot resistance. These findings indicated that Si-induced photorespiration might lead to increased resistance towards *C. miyabeanus*. Photorespiration is a complex and energy-consuming interorganellar process that is usually initiated by stomatal closure that leads to low CO_2 and/or high O_2 levels *in planta* (Foyer et al., 2009; Bauwe et al., 2012). In C3-plants like rice, these conditions give rise to the incorporation of O_2 instead of CO_2 in the Calvin cycle by Rubisco. The resulting glycolate is conversed in the peroxisomes to glycine which is transported to the mitochondria where it is catalyzed, releasing CO_2 and NH_3 which are recycled

in the chloroplasts. Photorespiratory CO_2 feeds back into the Calvin cycle and excess NH_3 is transported to the chloroplasts and reassimilated in the cellular nitrogen cycle (Wingler et al., 2000; Linka and Weber, 2005; Nunes-Nesi et al., 2010). Si-application led to an increased expression of several photorespiratory genes: *Rubisco*, *ribose-5-phosphate isomerase* (*RPI*), *phosphoglycerate kinase* (*PGK*) and *glycine dehydrogenase* (*GDC*). Accordingly, Si also up-regulated several photorespiratory marker genes including *glutamate:glyoxylate aminotransferase*(*GGAT*), *phosphoglycolate phosphatase* (*PGLP*), plastidic *glycolate/glycerate translocator* (*PLGG*), *phosphoribulokinase* (*PRK*), *serine:glyoxylate aminotransferase* (*SGAT*). Furthermore, Si application also seems to influence nitrogen metabolism in brown spot infected leaves. The Si-mediated upregulation of the ammonium transporter *AMT1.3* is hypothesized to be responsible for transporting the considerable amounts of ammonium that are released during photorespiration (Tanaka et al., 2004; Foyer et al., 2009; Li et al., 2012). Microarray analysis showed that chloroplastic *GS2* and *Fd-GOGAT* are upregulated in Si-treated rice leaves infected with *C. miyabeanus*. These findings suggest that the potential increases in nitrate reduction and photorespiratory ammonium transport increase the ammonium concentration in the chloroplast that in turn need to be re-assimilated by GS2 and Fd-GOGAT.

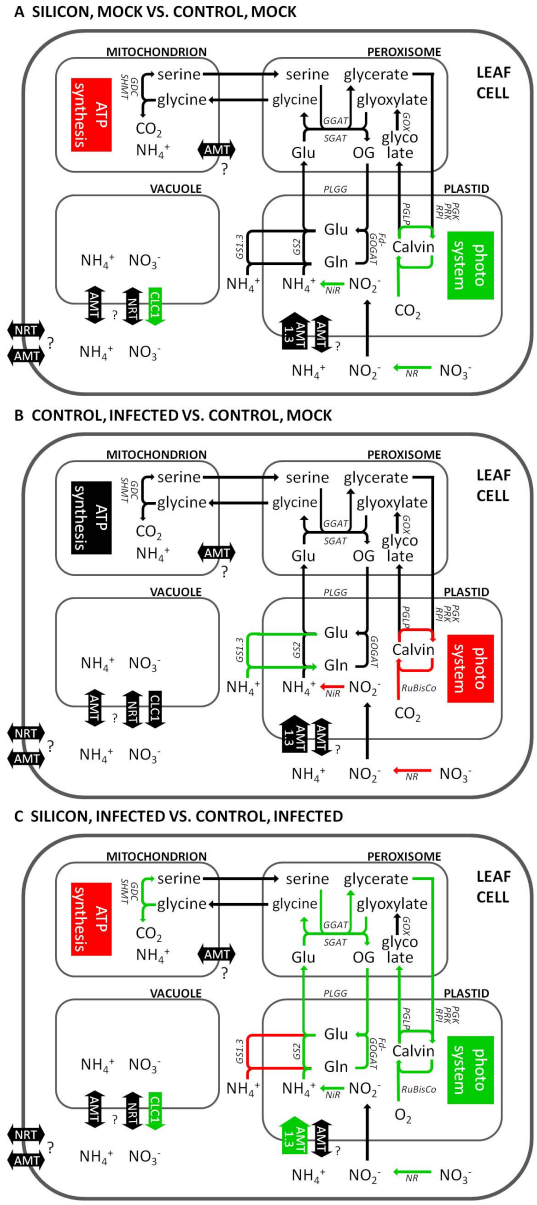


Figure 3.7: Influence of A) Si application on uninfected rice leaves (Si, mock vs. control, mock), B) *Cochliobolus miyabeanus* (control, infected vs. control, mock) and C) Si application on rice leaves infected with *C. miyabeanus* (Si, infected vs. control, infected) on the expression of genes involved in central metabolism. Arrows in red or green resp. indicate a down- or upregulation of gene expression (GEO accession GSE55330). Abbreviations: AMT, ammonium transporter, CLC1, nitrate transporter, NRT, nitrate transporter, GDC, glycine dehydrogenase, GGAT, glutamate:glyoxylate aminotransferase, NADH- OR Fd-GOGAT, NADH- or Ferredoxin-dependent glutamate synthase, GOX, glycolate oxidase, GS, glutamine synthetase, NR, nitrate reductase, NiR, nitrite reductase, PGK, phosphoglycerate kinase, PGLP, phosphoglycolate phosphatase, PLGG, plastidic glycolate/glycerate translocator, PRK, phosphoribulokinase, RPI, ribose-5-phosphate isomerase, Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase, SGAT, serine:glyoxylate aminotransferase, SHMT, serine hydroxymethyltransferase

3.3 Discussion

3.3.1 Effect of Si application on rice plants

The application of Si has a very diverse influence on plants, not only conferring broad spectrum disease resistance, but also increasing tillering, growth and yield. Microarray data revealed that Si enhances the expression of photosynthetic-related and nitrate reductive proteins.

Promoter analysis of the TFs that are differentially expressed due to Si-treatment (Table 3.2) showed that the promoters of the upregulated TFs were enriched in binding sites for MYB, MADS and GAMYB TFs. These binding sites are overrepresented in promoters of genes that play a role in gibberellin signaling and flower development (Riechmann et al., 1996; Gocal et al., 2001; Yang et al., 2001; Kaneko et al., 2004; Tsuji et al., 2006), which is in accordance with the fact that Si application has been reported to increase gibberellin synthesis in rice (Hwang et al., 2007). Given the link between gibberellins and flower development (Mutasa-Göttgens and Hedden, 2009), increased levels of gibberellins might explain the earlier induction of flower development in Si-treated rice plants (see Chapter 3.2.1). On the other hand, the promoters of downregulated TFs were enriched in TGA, ERF, ABF and DREB binding sites (Table 3.2). The TGA binding site normally occurs in the promoters of salicylic acid responsive genes whereas the ERF binding site are overrepresented in the promoters of ethylene sensitive genes (Hao et al., 2002; Kesarwani et al., 2007). Even though SA and ET are well-described stress hormones in rice (De Vleesschauwer et al., 2013), no convincing link between lower SA/ET signaling and broad spectrum disease resistance could be found. The abscisic acid responsive and drought responsive element binding sites ABF and DREB occurred more frequent in the promoters of downregulated TFs. These binding sites are associated with promoters of abiotic stress-responsive genes (Choi et al., 2000; Wickramasinghe and Rowell, 2005; Kesarwani et al., 2007; Roychoudhury et al., 2008; Srivasta et al., 2010). These findings suggest that Si-induced suppression of biotic and abiotic stress is responsible for the the promotion of plant growth in Si-treated plants.

In literature, a positive effect of Si on the growth of wheat, oats and rice has been described (Heinai et al., 2005; Toledo et al., 2012). Moreover, other reports clearly showed a growth-promoting effect of Si, even in the absence of stress (Tripathi et al., 2011; Detmann et al., 2012; Dallagnol et al., 2013). However, the growth promoting effect of Si is not always apparent. Several articles claim that Si has a positive effect on plant growth only during stress conditions (Agarie et al., 1992; Rodrigues, 2001; Hwang et al., 2007; Nwugo and Huerta, 2008; Sun et al., 2010; Nwugo and Huerta, 2011; Perez et al., 2014). Agarie et al.

(1992) found that under slightly suboptimal light conditions, Si treatment led to a higher growth increase than under optimal light conditions. In agreement with these findings, many transcriptional analyses on the effect of Si in rice, wheat, tomato and Arabidopsis show that Si only has very little impact on the metabolism of non-stressed plants (Fauteux et al., 2006; Brunings et al., 2009; Chain et al., 2009; Ghareeb et al., 2011). The apparent phenotypical difference between untreated and Si-treated rice plants used in this study therefore suggest that these plants have been grown under suboptimal conditions.

3.3.2 Influence of *C. miyabeanus* on rice metabolism

Brown spot infection in rice leaves is characterized by the occurrence of large necrotic lesions surrounded by chlorosis which cause premature senescence and cell death (Ahn et al., 2005; Dallagnol et al., 2011a). Microarray data suggests that *C. miyabeanus* actively inhibits the expression of photosynthetic genes at 12hpi when disease symptoms are not visible yet. These findings indicate that photosynthetic impairment might not be the consequence, but rather the cause of infection, as a strategy to induce premature senescence in rice leaves. There are two virulence factors of *C. miyabeanus* that might play a role in mediating pathogen mediated senescence. First, *C. miyabeanus* is known to produce the plant hormone ethylene (ET) which induces senescence in rice leaves and facilitates the infection process (see Chapter 4). Second, the manifold phytotoxins produced by *C. miyabeanus* are known to induce cell death and senescence (Xiao et al., 1991; Ahn et al., 2005). The complete array of toxins and their exact functions have not yet been characterized, but ophiobolins are the main group of toxins produced by Cochliobolus species (Au et al., 2000; Condon et al., 2013). The best-described toxins produced by *C. miyabeanus* are ophiobolin A and B which have been reported to disrupt the plasma membrane integrity (Chattopadhyay and Samaddar, 1976; Tipton et al., 1977) leading to the impairment of metabolic processes such as photosynthesis and protein and nucleic acid synthesis eventually leading to cell death (Chattopadhyay and Samaddar, 1980; Kim and Hyeon, 1984; Yun et al., 1988; Krizsán et al., 2010).

A similar role for the phytotoxins produced by *C. miyabeanus* is plausible, especially since these toxins are also known to induce senescence in rice leaves (Xiao et al., 1991; Ahn et al., 2005). The resulting pathogen-induced, premature senescence is likely responsible for causing the typical chlorotic lesions around the site of infection (Ahn et al., 2005). Three different roles were hypothesized for photosynthetic inhibition as a virulence strategy by *C. miyabeanus*. First, inhibiting photosynthesis might deprive invaded rice leaves from the energy necessary to fuel energy-demanding defense responses. Second, uncoupling the light reactions from the Calvin cycle leads to overreduction of the electron transfer

and oxidative damage to chloroplast membranes and senescence which can promote disease incidence in some plant-pathogen interactions (Kangasjärvi et al., 2012). Finally, decreasing photosynthesis induces metabolic changes that might provide essential nutrients for the pathogen. In this light, the microarray data suggested a senescence linked remobilization of nitrogen in brown spot infected rice leaves. An upregulation of cytosolic *glutamine synthetases* (*GS1.3*) and downregulation of *nitrate* and *nitrite reductases* is commonly observed in senescent plant leaves (Masclaux et al., 2001; Pageau et al., 2006). *C. miyabeanus* is known to alter amino acid metabolism during infection, probably for utilization during infection (Chattopadhyay and Dickson, 1960). In view of these findings, it is tempting to speculate that *C. miyabeanus* triggers senescence and nitrogen remobilization in order to secure access to nutrients and facilitate the infection process.

3.3.3 Influence of Si on rice resistance against *C. miyabeanus*

The application of Si is a known strategy to protect rice plants against different pathogens, among which *C. miyabeanus* (Dallagnol et al., 2009, 2011b, 2013). Si-treated leaves infected with *C. miyabeanus* showed significantly smaller necrotic lesions and less chlorosis surrounding the site of infection (Fig 3.2 A and B). The protective effect of Si in plants is generally assumed to be the result of two different phenomena (see Chapter 2.5). The hard and insoluble subcuticular silica layer grants certain vigor to rice leaves which is often believed to passively prevent pathogen penetration. However, we observed that short-term Si application rendered rice plants as resistant as plants that had been continuously treated with Si (Fig 3.2 D). Moreover, Si-treated plants already showed reduced levels of resistance three days after Si deprivation. These findings not only suggest that Si has a rather short-term mode of action, but also demonstrates that the active effect of Si application is dominant over the passive effect.

The differential expression due to Si application on infected leaves (Si, infected vs. control, infected) indicated that Si does not have a major influence on defense-related signaling pathways, such as salicylic acid, jasmonic acid and ethylene pathway. It seems that in stead of inducing biotic stress responses like the activation of the phenylpropanoid pathway or cell wall modification (Fig 3.6), Si application had an apparent influence on the central metabolism. Where *C. miyabeanus* is hypothesized to induce premature senescence and cell death as a virulence strategy, Si seemed to prevent pathogen-induced senescence and subsequent brown spot susceptibility by redirecting primary metabolic processes. In accordance with the fact that *C. miyabeanus* had little effect on the expression profile of Si-treated plants (Si, infected vs. Si, mock), these microarray data suggested that Si application does not induce brown spot resistance as such but rather

prevents pathogen-induced susceptibility. Based on the more detailed analysis of the effect of Si on the central metabolism of brown spot infected rice leaves (Si, infected vs. control, infected), several mechanisms that might be responsible for preventing Si-mediated resistance were suggested. Si application seemed to reverse most metabolic processes that are associated with *C. miyabeanus*-induced senescence. First, the decreased expression of the cytosolic glutamine synthetase, *GS1.3* and the upregulation of nitrate reduction suggests that Si prevents senescence-mediated nitrogen remobilization. The upregulated expression of the chloroplastic *GS2* and *Fd-GOGAT* suggest an augmented incorporation of ammonium molecules in the chloroplast. Furthermore, Si treatment protected infected rice leaves from *C. miyabeanus*-mediated downregulation of photosynthetic genes. Safeguarding the photosynthesis machinery appears to be a common aspect of Si-induced broad spectrum stress tolerance, as many reports unanimously show that Si protects and promotes photosynthesis during stress-situations (Nwugo and Huerta, 2008; Chen et al., 2011; Nwugo and Huerta, 2011; Dallagnol et al., 2013; Perez et al., 2014). However, the mechanisms that govern the Si-mediated protection of the photosynthesis pathway during stress have not been described yet. Our findings suggest that one of the driving forces behind the protective role of Si on photosynthesis might be photorespiration. Although photorespiration consumes more energy than it produces, it also prevents overreduction of photosynthetic electron transport and subsequent damage to the photosynthesis apparatus during stress-situations (Bauwe et al., 2012). Interestingly, an increase in photorespiration might also explain the increased nitrate reduction (*CLC1, NR, NiR*), ammonium transport towards the chloroplast (*AMT1.3*) and chloroplastic ammonium reassimilation (*GS2* and *Fd-GOGAT*) (Rachmilevitch et al., 2004; Naik, 2006; Foyer et al., 2009; Bloom et al., 2010). Mounting evidence indicates that photorespiration confers tolerance towards drought, salt, cadmium and chilling stress (Hoshida et al., 2000; Cheng et al., 2007; Rivero et al., 2009; Cai et al., 2011; Wang et al., 2012). In addition, photorespiration may also contribute to pathogen defense, an effect which is mostly linked to the occurrence of hypersensitive response due to accumulation of photorespiration-induced reactive oxygen species (Wingler et al., 2000; Kangasjärvi et al., 2012; Sørhagen et al., 2013; Rojas et al., 2014). Since reactive oxygen species and hypersensitive response are ineffective against *C. miyabeanus* (Ahn et al., 2005), it is likely that the potential role of photorespiration in Si-induced resistance against *C. miyabeanus* is due to the safeguarding of the photosynthesis apparatus.

The fact that brown spot infection downregulated the expression of photorespiratory genes, also led to the hypothesis that preventing photorespiration might be a virulence strategy of *C. miyabeanus*. In analogy with victorin, a toxin produced by *Cochliobolus*

victoriae, which inhibits the photorespiratory enzyme glycine decarboxylase (GDC) in oat plants as a strategy to induce susceptibility (Navarre and Wolpert, 1995, 1999; Tada et al., 2005), a similar role for *C. miyabeanus* toxins is proposed. Moreover, exogenous ET seems to induce stomatal opening (data not shown) which suggests that fungal ET by *C. miyabeanus* might play a role in preventing photorespiration. It might not be inconceivable that next to photosynthesis, photorespiration might be another target on the path of *C. miyabeanus* to infection.

3.3.4 Si-induced broad spectrum disease

In the past decade, a number of transcriptomic and proteomic studies have been conducted to explain the protective effects of Si in various pathosystems (Watanabe et al., 2004; Fauteux et al., 2006; Chain et al., 2009; Zargar et al., 2010; Ghareeb et al., 2011; Nwugo and Huerta, 2011). One of the most salient results of these studies is that Si negates many of the transcriptional changes induced by pathogen inoculation. For instance, in one of the first studies aimed at investigating the molecular basis of Si-induced plant resistance, (Fauteux et al., 2006) found that inoculation of Arabidopsis with the powdery mildew fungus *Erysiphe cichoracearum* resulted in transcriptional reprogramming of an extensive gene set of nearly 4,000 genes. Remarkably, comparing control and Si-treated plants, no major changes were found in either the number or expression level of up-regulated genes. In contrast, many of the down-regulated genes were less severely affected when plants were treated with Si, a clear indication of stress alleviation. Similar findings were obtained by (Chain et al., 2009) when studying the effect of Si application on the transcriptome of wheat plants inoculated with the biotrophic pathogen *Blumeria graminis* f. sp. *tritici*. In this case, Si-treated plants displayed an almost perfect mirror image of the expression changes seen in inoculated control plants. Moreover, contrary to the nearly 900 genes responding to *Blumeria* infection in control leaves, very few genes were regulated by the pathogen in Si-treated wheat plants, indicating that Si almost eradicated the stress imposed by the pathogen (Chain et al., 2009). In rice, the microarray study by (Brunings et al., 2009) and the microarray data presented in this thesis on the effect of Si on respectively rice blast and brown spot resistance, similarly show that the effect of infection on the transcriptome is diminished by Si treatment. Hence, instead of mounting resistance by directing massive transcriptional reprogramming of defense-related genes, Si appears to nullify the impact of pathogen inoculation on the plant's transcriptome. In light of our results, a minimal transcriptomic response suggests that Si prevents the exploration of pathogen virulence factors. Whether such impairment of pathogen virulence is due to Si boosting the plant perception of and/or response to so-called pathogen-associated

molecular patterns, or alternatively, results from inhibition of the production and/or delivery of specific virulence factors remains to be elucidated.

3.4 Conclusion

The beneficial effect of Si on both rice growth and resistance against *C. miyabeanus* is due Si-induced broad spectrum stress tolerance. In order to unravel the molecular mechanisms responsible for the prophylactive effect of Si, a microarray experiment was conducted which revealed that *C. miyabeanus* might induce premature senescence in order to impose susceptibility in rice leaves. Si application, on the other hand, seemed to confer brown spot resistance by redirecting the central metabolism in a photorespiration-dependent manner. Together with other microarray studies on Si-induced disease resistance in different plant species, our data suggest that rather than acting as an initiator of induced defense responses, Si might confer disease resistance by preventing pathogen's virulence strategies.

3.5 Material and methods

3.5.1 Plant material and growth conditions

All rice plants (*Oryza sativa* L.) were *japonica* cultivar Nipponbare. The rice seeds were surface sterilized with 70% ethanol for 1 min and 1% sodium hypochlorite solution for 10 min, rinsed three times with sterile distilled water and germinated at 28°C for five days on wet sterile filter paper in Petri dishes sealed with parafilm ($\geq$ 92% relative humidity). The seedlings were transplanted on vermiculite in half-strength modified Hoagland solution (Hewitt and Smith, 1975). Five days later the plantlets are transferred to a hydroponic system in full strength modified Hoagland solution (pH 6.5). The Hoagland solution was replaced every 7 days. The rice plants were grown in a growth chamber (28°C, 12h/12h light regime) for five weeks until they reached the 7-leaf stage.

3.5.2 Leaf gas exchange and fluorescence measurements

Gas exchange and fluorescence parameters were measured concurrently with a portable photosynthesis system (LI-6400; LiCor Biosciences, Lincoln, NE, USA) fitted with a fluorescence head (6400-40 LCF; LiCor Biosciences) and a CO_2 mixer (6400-01; LiCor Biosciences). Net photosynthesis (A_{net}), stomatal conductance (g_s), leaf transpiration (E), intracellular CO_2 concentration (C_i) and fluorescence parameters were measured on 13 plants per treatment in two biological repeats. The temperature of the leaf cuvette was

set to match the temperature in each treatment chamber at the start of the measurements. The light source of the fluorescence head was maintained at 1500 $\mu mol_{photons}.m^{-2}.s^{-1}$ while $[CO_2]$ in the cuvette was maintained at 380 $\mu mol_{CO_2}.l^{-1}$. Fluorescence parameters quantum yield of photosystem II (PSII) (ΦPSII) and maximum quantum yield of light saturated PSII (F_v'/F_m') were calculated as described in valentini et al. (1995) and Maxwell and Johnson (2000).

3.5.3 Pathogen inoculation and disease rating

Cochliobolus miyabeanus isolate Cm988, kindly provided by IRRI, was grown on potato dextrose agar (PDA, Difco) at 28°C in darkness. After a week the mycelium was put under 12h/12h blue light (Philips TLD 18W/08 and Philips TLD 18W/33) until sporulation. Conidia were harvested as described by Thuan et al. (2006) and suspended in a 0.5% gelatine (type B from bovine skin; Sigma-Aldrich G-6650) in a concentration of 10^4 conidia ml^{-1}. Plants in the 7-leaf stage (5 weeks old) were inoculated by spraying the conidial suspension on the leaves till run off (1ml per plant) using a compressor-powered airbrush gun. The plants were kept in a humid and warm infection chamber (28°C ± 4°C, 92% or greater relative humidity) to promote fungal penetration. After 18h the plants were transplaced to greenhouse conditions (28°C ± 4°C, 16h light/8h dark regime) for disease development. The symptoms were scored three days after inoculation using APS ASSESS 2.0 software (APS, St Paul, Minnesota, USA). All infection trials were repeated at least three times with similar results.

3.5.4 RNA extraction and quantitative RT-PCR

Total RNA was extracted from frozen leaf tissue using the spectrum plant total RNA kit (Sigma-Aldrich) and subsequently Turbo DNase treated according to the provided protocol (Ambion). First-strand cDNA was synthesized from 2 mg of total RNA using Multiscribe reverse transcriptase (Applied Biosystems) and random primers following the manufacturer's instructions. Quantitative PCR amplifications were conducted in optical 96-well plates with the Mx3005P real-time PCR detection system (Stratagene), using Sybr Green master mix (Fermentas) to monitor dsDNA synthesis. The expression of each gene was assayed in duplicate in a total volume of 25 µl including a passive reference dye (ROX) according to the manufacturer's instructions (Fermentas). The thermal profile used consisted of an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 57°C for 30 s, and 72°C for 30 s. To verify amplification of one specific target cDNA, a melting-curve analysis was included according to the thermal profile suggested by the manufacturer (Stratagene). The amount of plant RNA in each sample

was normalized using elongation factor eIFα (LO_Os03g08020) as internal control and mock treated samples were selected as calibrator. For analysis of the CmEFE (BK008840) expression in fungal and plant samples using the fungal ITS gene as normalizer. The data were analyzed using Stratagene's Mx3005P software. Nucleotide sequences of all primers used are listed in Table 3.3.

3.5.5 Expression profiling of rice TFs and *in silico* promoter analysis

The high-throughput expression profiling of rice TFs was performed according to Caldana et al. (2007) in three biological repeats on pooled samples containing the two youngest fully developed leaves of 12 seven week-old plants per sample. The resulting gene expression data were statistically analyzed with a (α=0.01, parametric). The TF genes that were significantly up- or downregulated (paired t-test, Si- vs. control-treatment, α=0.01) showing a three fold change in expression (-1.58 $>\log_2$ fold change $>$1.58) were selected for *in silico* promoter analysis. The 1kb upstream promoter region of the selected TF genes (Kawahara et al., 2013) were analysed using TRANSFAC software (Matys et al., 2006; Guo et al., 2008; Park et al., 2010). Overrepresented TF binding site motifs (α = 0.05) that occur uniquely in 75% of the promoters of respectively up- or downregulated TFs were identified as overrepresented motifs. The motif sequences and their function were substracted from the TRANSAC and PLACE databases (Higo et al., 1999).

3.5.6 Microarray analysis and data processing

Rice plants (cultivar Nipponbare) were grown and infected with the *C. miyabeanus* isolate Cm988 as described before. The microarray study setup consisted of a 2x2 factorial design (mock and infected; untreated and Si-treated). Samples were taken 12h post inoculation, each sample representing a pool from at least 6 individual plants in three biological repetitions. For microarray analysis, we used a previously described two-dye method allowing direct comparison between two samples on the same microarray (Satoh et al., 2010). Signal intensities among all arrays were normalized according to the quantile method for standardization (global scaling) using EXPANDER 6 (Shamir et al., 2005). Significance analysis was performed using a fixed linear model (LIMMA) implemented in MeV (Saeed et al., 2003). Differentially expressed genes were defined as genes with a false discovery rate of less than 0.05 and a 3-fold difference in signal ratio. The microarray data is available in the Gene Expression Omnibus (GEO accession GSE55330). Hierarchical linkage clustering was performed (complete linkage, euclidian distance) using MeV.

3.5.7 Validation of microarray results

Quantitative real-time polymerase chain reaction (PCR) was performed on 8 selected genes (Table 3.3) that were differentially regulated among the different comparisons. Quantitative PCR and microarray results were linearly correlated ($R^2 = 0.92$) (Fig. 3.8).

Table 3.3: Randomly picked primers for microarray validation

identifier	forward primer	reverse primer
LOC_Os03g08020	TTTCACTCTTGGTGTGAAGCAGAT	GACTTCCTTCACGATTTCATCGTAA
LOC_Os08g44270	GGATCGTCAGGAACTCGTGG	AATCGCCTCCAAACGGTAGC
LOC_Os11g41680	CCAGGAGGACTTGGACTTGG	TGAACAAGTCTCCGATTGTTGTC
LOC_Os10g21670	GTGTTGTCTCTGCGTGCATC	GGCAAAGCAGACACTGTGC
LOC_Os04g09900	GGATATCCCAGGCGAGGTTG	TGTGCAGGACCTTCCCAATC
LOC_Os07g48050	CTTGCTTGTGGTCGTGGCTC	TCCCGACAACAGAACAGACG
LOC_Os09g23530	ATCACGCCCAGATTCCTGAG	TCCTCCTGAACCCTTCTTGTG
LOC_Os04g48930	TTGCCGACCAGGACATTCAG	AGATCACGATTCTCGTCCCTTG
LOC_Os09g36680	AGTGGAACAGCTATGGCGTC	TAGTCCGGCTTGATGCCTTG

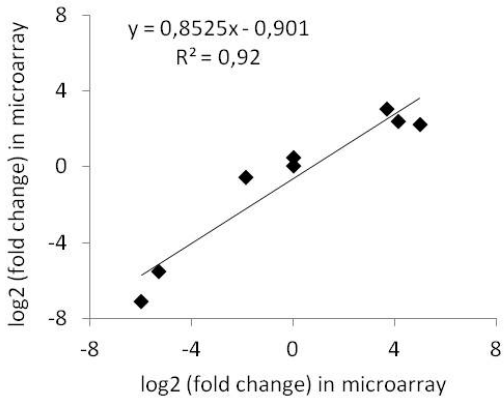


Figure 3.8: Volcano plot of microarray data of brown spot infected leaves vs. mock treated leaves 12h post inoculation (GEO accession GSE55330).

4

Ethylene production by *Cochliobolus miyabeanus* as a virulence strategy during rice infection

Authors

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Abstract

The plant hormone ethylene (ET) is a well-studied modulator of plant immune responses. Although ET is widely believed to condition resistance against necrotrophic pathogens, recent advances suggest a more complex reality with both positive and negative effects being reported depending not only on the pathogen lifestyle, but also on specialized features of each interaction. Here, we have analyzed the impact and dynamics of ET during progression of rice brown spot disease caused by the necrotrophic fungus *Cochliobolus miyabeanus*. We show that the pathogen tricks the plant into producing copious amounts of ET at least in part by synthesizing ET itself, a strategy that is especially important for less virulent *C. miyabeanus* strains. Moreover, our findings unmask ET as a key signal in the activation of gene expression by *C. miyabeanus* and demonstrate that host ET signaling compromises brown spot resistance via a two-pronged mechanism involving the amplification of senescence and inhibition of phenylpropanoid-driven defenses. Collectively, these data support a model whereby *C. miyabeanus* produces ET as a decoy to rewire the rice signaling circuitry and antagonize host immunity. Our results provide novel insights into the multifaceted role of ET in plant innate immunity and highlight the importance of microbial ET production in molding pathological outcomes.

4.1 Introduction

As sessile organisms, plants are constantly threatened by a wide variety of microbial pathogens, including fungi, viruses, bacteria and oomycetes. Many of the defense responses employed to counteract these attackers are regulated by intertwined signal transduction pathways, within which plant hormones fulfill central roles. Salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are the archetypal defense hormones and their importance in the regulation of the plant defense signaling network is well established. Upon pathogen attack, plants produce a complex blend of SA, JA and ET, which varies greatly in quantity, timing, and composition depending on the plant-attacker combination. It is thought that this so-called signal signature plays a pivotal role in the orchestration of the plant's immune response and eventually determines the specific nature of the defense response triggered (Rojo et al., 2003; De Vos et al., 2005; Mur et al., 2006).

In the model plant species *Arabidopsis thaliana*, SA is widely believed to act against biotrophic pathogens, which colonize and derive nutrients from living plant tissues whereas JA/ET-driven defenses are commonly accepted to trigger resistance against necrotrophic pathogens that kill host tissue and feed on the remains (Pieterse et al., 2012). In many other plant-pathosystems, however, the role of SA, JA and ET is not as clear-cut. For instance, in the model monocot plant rice (*Oryza sativa*), disease resistance seems to be controlled by a highly complicated signaling network that does not support a dichotomy between the effectiveness of the SA, JA and ET pathways and the lifestyle of a given pathogen (De Vleesschauwer et al., 2010, 2013).

Recently, various other hormones, including abscisic acid (ABA), gibberellins (GA), auxins, cytokinins and brassinosteroids have emerged as important regulators of plant-microbe interactions. Although their significance is less well characterized, evidence is accumulating that these hormones influence disease outcomes by feeding into the SA-JA/ET backbone of the plant immune system (Asselbergh et al., 2008; De Vleesschauwer et al., 2008; Robert-Seilaniantz et al., 2011; Xu et al., 2013; De Bruyne et al., 2014). Such interplay or crosstalk among defense pathways is thought to enable the plant to tailor its inducible defense arsenal to the type of attacker encountered and use its limited resources in a cost-efficient manner (Pieterse et al., 2012; Sharma et al., 2013). However, exciting new developments indicate that crosstalk may also be targeted by pathogens to tap into the plant defense signaling circuitry and redirect the host immune response. A classic example is the ability of the bacterial leaf speck pathogen *Pseudomonas syringae* pv. *tomato* to promote disease development by injecting effector proteins into the host cell that alter the plant's auxin and ABA physiology (Chen et al., 2007; de Torres-Zabala

et al., 2007; de Torres Zabala et al., 2009). In a complementary strategy, *P. syringae* also deploys the phytotoxin and JA-mimic coronatine to hyperactivate JA signaling and counteract effective SA-dependent defenses that normally serve to limit pathogen growth (Brooks et al., 2005; Cui et al., 2005; Melotto et al., 2006). Moreover, multiple other plant pathogens have been reported to produce plant hormones or their functional mimics, suggesting that manipulating host hormone signaling and hijacking crosstalk mechanisms is a common virulence strategy among plant attackers (Arshad and Frankenberger, 1992; Denancé et al., 2013; Grant et al., 2013).

In the past decades, the role of ET in regulating plant immune responses has attracted considerable interest. Analyses of plant mutants and transformants impaired in ET biosynthesis, perception or signaling have demonstrated the function and central importance of ET in the establishment of a broad array of local and systemic immune responses (Van der Ent and Pieterse, 2012). Although the widely held belief is that ET cooperates with JA in necrotroph resistance and antagonizes SA-mediated biotroph resistance, this is likely an oversimplified model (Broekaert et al., 2006; van Loon et al., 2006; Pieterse et al., 2009). Indeed, accumulating evidence in various plant species indicates that the effectiveness of the ET pathway is not always correlated to the lifestyle of the invading pathogen. For example, contrary to the classic binary defense model, disruption of ET signaling was found to render Arabidopsis both hypersusceptible to the biotrophic nematode *Heterodera schachtii* (Wubben et al., 2001) and more resistant to the necrotrophic pathogens *Ralstonia solanacearum*, *Verticillium dahliae* and *Fusarium oxysporum* (Pantelides et al., 2010, 2013). Similar findings were obtained in other plant-pathosystems. In rice, ET signaling increases resistance and susceptibility to the hemibiotrophic pathogens *Magnaporthe oryzae* and *Xanthomonas oryzae* pv. *oryzae*, respectively, (Iwai et al., 2006; Seo et al., 2011; Shen et al., 2011; Helliwell et al., 2013), while other studies implicated ET in inducing susceptibility to, among others, the necrotrophic fungi *F. oxysporum* f. sp. *lycopersici* and *Alternaria alternata* sp. *lycopersici* in tomato and the grey mold pathogen *Botrytis cinerea* in petunia (Lund et al., 1998; Lin et al., 2008; Jia et al., 2013). These observations suggest that ET plays a complex and ambiguous role in the plant immune system, the effect of which may depend not only on the infection mode of the attacking pathogen, but also on the plant species and specialized features of each interaction. Moreover, several plant pathogens have been demonstrated to autonomously produce ET *in vitro* and *in planta* (Valls et al., 2006; Van der Ent and Pieterse, 2012). However, the role of ET production for the pathogen as well as its significance in host-microbe interactions remains unclear, adding yet another layer of complexity in developing a coherent view of ET-modulated immunity.

Aiming to further dissect the immune-regulatory role of ET, we have analyzed its dynamics and function during progression of brown spot disease, caused by the necrotrophic fungal pathogen *Cochliobolus miyabeanus* (anamorph: *Bipolaris oryzae*). Particularly prevalent in rain-fed ecosystems, *C. miyabeanus* is known to produce a varied arsenal of phytotoxins, some of which are involved in suppression of plant phenol production (Vidhyasekaran et al., 1997; Condon et al., 2013). Previously, we demonstrated that exogenously administered ET renders rice hyper-susceptible to brown spot infection, whereas reduction of ET perception by the application of silver ions or disruption of ET signaling in *EIN2a* antisense plants resulted in a substantial reduction in disease severity (De Vleesschauwer et al., 2010). Moreover, gene expression experiments revealed a strong activation of ET signaling in susceptible but not in resistant rice plants, raising the hypothesis that *C. miyabeanus* exploits ET as a virulence factor and co-opts the rice ET signaling route to suppress other effectual defense pathways. However, how the pathogen is able to tap into the rice ET machinery and how increased ET signaling favors disease development remained unclear.

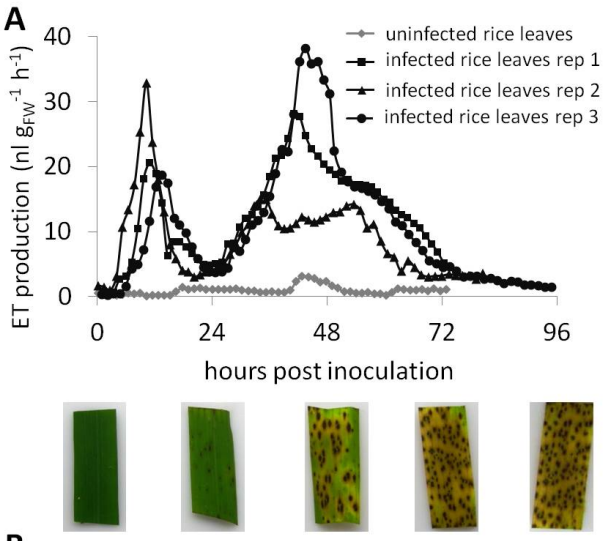
Here, we reveal that *C. miyabeanus* produces ET itself via a biosynthetic pathway that is distinct from plant ET biosynthesis and provide evidence that this fungal ET enhances susceptibility in rice both directly and indirectly via the activation of plant ET biosynthesis. Moreover, we show that the virulence of *C. miyabeanus* isolates is at least partly correlated with their ET producing abilities and describe how increased plant ET signaling compromises brown spot resistance by interfering with the phenylpropanoid pathway.

4.2 Results

4.2.1 *C. miyabeanus* infection promotes ET emission in rice leaves

Previously, we demonstrated that exogenously administered ET promotes susceptibility of rice to *C. miyabeanus* (De Vleesschauwer et al., 2010). To study the role of ET in orchestrating rice-*C. miyabeanus* interactions, we first assessed the temporal dynamics of ET production throughout the course of infection. Real-time ET monitoring of detached rice leaves inoculated with the virulent *C. miyabeanus* strain Cm988 revealed two distinct phases in ET production. A first rapid ET peak occurred within 24 hours post inoculation (hpi) and coincided with the development of small necrotic disease lesions (hpi), whereas a second more pronounced ET burst corresponding to advanced chlorotic disease lesions was observed from 48 hpi onwards (Fig. 4.1 A). Starting 72 hpi, ET emission levels decayed to near basal levels, a reaction that was associated with advanced necrosis of the inoculated leaves (Fig. 4.1 A, bottom).

The strong boost in ET production in *C. miyabeanus*-infected leaves was also reflected in a microarray experiment in which we analyzed the transcriptome of Cm988-inoculated rice leaves at 12 hpi (Fig S5.1, GEO accession GSE55330). Corroborating our ET measurements, brown spot infection significantly altered the expression of various ET biosynthesis genes including the ACC synthetase *OsACO7* and ACC oxidases *OsACS2* (Fig. 1B). Moreover, the transcriptome of *C. miyabeanus*-infected plants at 12 hpi displayed strong similarities to that of rice seedlings treated with the ET precursor ACC (Garg et al., 2012). Hierarchical clustering of all 699 genes significantly differentially expressed in both studies revealed that 66% (225 out of 341 genes; cluster I) of the transcripts induced upon brown spot infection are also upregulated in response to ACC treatment (Figure 4.1C). Similarly, genes downregulated by *C. miyabeanus* infection shared a 67% overlap with ACC-repressed genes (240 out of 358 genes; cluster III). Gene ontology (GO) enrichment analysis revealed an over-representation of genes involved in photosynthetic light harvesting among the transcripts down-regulated by both ACC-treatment and brown spot infection. In contrast, GO analysis of genes showing non-conserved expression (clusters II and IV) revealed no significantly enriched biological or cellular processes. In conjunction with the strong disease-promoting effect of exogenously administered ET (De Vleesschauwer et al., 2010), these data confirm ET as a crucial regulator of rice brown spot resistance and uncover ET as an important signal in the activation of gene expression in brown spot infected leaves.



B

ET biosynthesis genes	Identifier	Expression level (log_2)	p-value
OsACO7	LOC_Os01g39860	3.52	0.001
OsACS2	LOC_Os04g48850	2.72	0.000
OsACO5	LOC_Os05g05680	0.70	0.003
OsACO6	LOC_Os05g05670	0.89	0.001
OsACO3	LOC_Os09g27750	-0.25	0.198
OsACS1	LOC_Os03g51740	-0.88	0.127
OsACS5	LOC_Os01g09700	-0.90	0.163
OsACS6	LOC_Os06g03990	-0.09	0.541

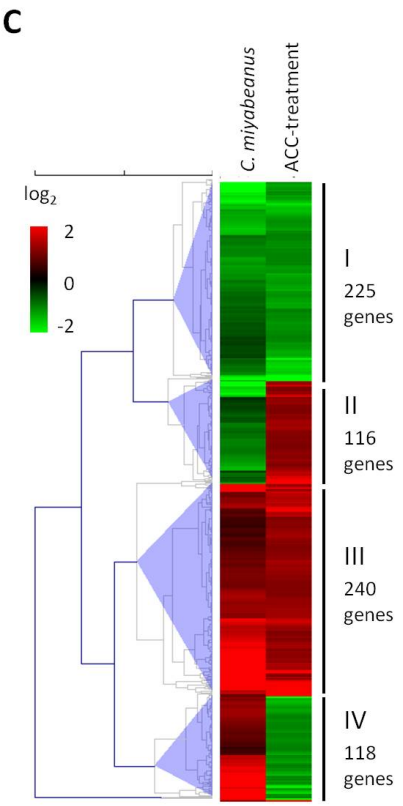


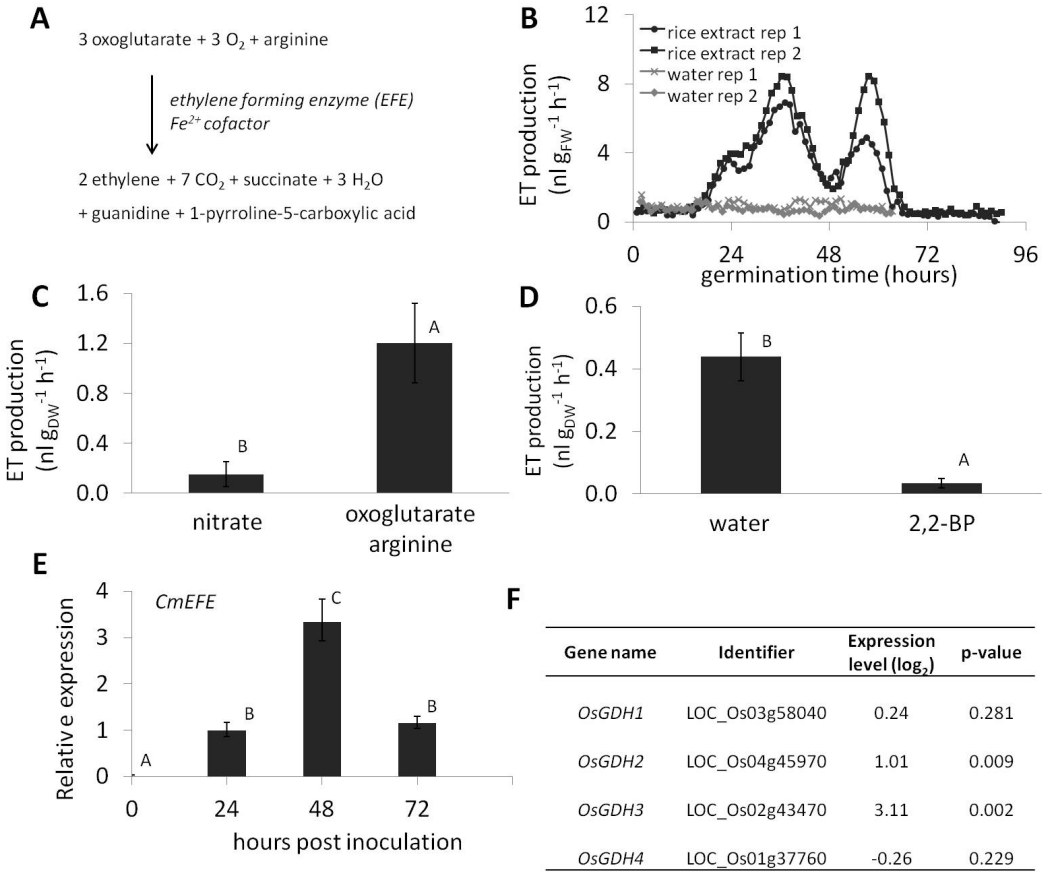
Figure 4.1: A, Real-time ET detection in detached leaves infected with *C. miyabeanus* (Cm988, 1 x 10^4 conidia.ml^{-1}) using laser photoacoustic detection (LPAD). Data presented are means ± SE of three independent biological repetitions are shown. Photographs depict representative disease development at the indicated time points post inoculation. B, Expression data of rice ET-biosynthesis genes from a microarray experiment on *C. miyabeanus* inoculated vs. non-inoculated leaves 12 h post inoculation. C, Hierarchical cluster analysis of 699 genes differentially expressed in *C. miyabeanus*- and ACC-treated rice compared with non-treated controls. ACC data was extracted from (Garg et al., 2012), while pathogen inoculation data originates from a publicly available microarray experiment on Cm988-inoculated rice leaves (GEO dataset GSE55330). Genes were clustered with The Institute for Genomic Research (TIGR) multi-experiment viewer using Euclidean distance and complete linkage. Gene subclusters of interest that are discussed in the text are indicated by labeled vertical bars to the right of the image.

4.2.2 *C. miyabeanus* produces ET *in vitro* via the 2-oxoglutarate pathway

Recent advances in plant immunity research have provided fascinating insights into the ingenious ways by which microbial pathogens modify plant hormone homeostasis. Besides influencing plant hormone synthesis, pathogens often manipulate the plant's signaling infrastructure by producing plant hormones or functional mimics thereof themselves. In fungi, ET is most commonly synthesized from the TCA cycle intermediate 2-oxoglutarate. This reaction is catalyzed by a single multifunction ethylene forming enzyme (EFE), and requires additional amino acids, arginine or histidine, and Fe^{2+} as cofactors (Fukuda et al., 1992; Chagué et al., 2006; Fig. 4.2 A). To test whether a similar pathway is operative in *C. miyabeanus*, we monitored the ET emission of Cm988 spores germinating on solid medium containing oxoglutarate and arginine. As shown in Fig. 4.2 B, Cm988 spores produced ET from oxoglutarate and arginine, but only when a sterile extract of rice leaves was added to the medium. Moreover, Cm988 emitted significantly more ET when grown on media containing arginine and oxoglutarate in comparison with medium containing an equimolar amount of nitrogen in the form of nitrate (Fig. 4.2 C). In contrast, application of 2,2-bipyridyl (2,2-BP), a strong iron chelator and excellent inhibitor of oxoglutarate-dependent ET biosynthesis in *Fusarium oxysporum* f.sp. *tulipae* (Hottiger and Boller, 1991), almost completely abolished fungal ET emission in liquid cultures, further demonstrating the ability of *C. miyabeanus* to produce ET in an oxoglutarate-dependent manner (Fig. 4.2 D).

Over the past few years, various EFE enzymes have been identified and characterized. EFEs typically contain two conserved domains, DIOX_N (pfam14226) and 2OG-FeII_oxy (pfam03171), both of which are specific for iron-dependent oxoglutarate dioxygenases Fukuda et al., 1992; Marchler-Bauer et al., 2011; Fig. S5.2 and S5.3). Blasting the amino acid sequences of these domains against the draft genome of *C. miyabeanus* (Condon et al., 2013) revealed the presence of a putative EFE, which we designated CmEFE (BK008840). Interestingly, gene expression analyses of Cm988-inoculated plants revealed that *CmEFE* transcript levels respond strongly to plant infection and peak at 48 hpi, concomitant with the strong ET burst observed in Cm988-inoculated plants (Fig. 4.2 E). However, when *C. miyabeanus* was grown on rice leaves that had been flash-frozen and thawed to inhibit active cell metabolism, the fungus failed to produce ET (data not shown). Given that most of the nutrients used by microbial pathogens originate from their host and that rice leaves are low in oxoglutarate (Yuan et al., 2007a), these findings suggest that *C. miyabeanus* may need to induce plant oxoglutarate accumulation in order to produce ET.

Consistent with this hypothesis, our microarray data showed a significant upregulation at 12 hpi of the glutamate dehydrogenases *OsGDH2* and *OsGDH3*, two out of the four oxoglutarate-forming enzymes in rice (Fig. 4.2 F).



Gene name	Identifier	Expression level (log_2)	p-value
OsGDH1	LOC_Os03g58040	0.24	0.281
OsGDH2	LOC_Os04g45970	1.01	0.009
OsGDH3	LOC_Os02g43470	3.11	0.002
OsGDH4	LOC_Os01g37760	-0.26	0.229

Figure 4.2: A, Microbial ET biosynthesis in an oxoglutarate-dependent manner (Fukuda et al., 1992). B, Real-time ET detection of germinating *C. miyabeanus* spores using laser photoacoustic detection (LPAD). The conidia (Cm988, 1 x 10^4 conidia.ml^{-1}) were sprayed on solid Czapek medium rich in oxoglutarate and arginine and complemented or not with sterile rice leaf extract. Data of two representative biological repetitions are shown. The experiment was repeated two more times with similar results. C, ET accumulation in *C. miyabeanus* isolate Cm988 grown for five days in liquid medium containing rice extract and equimolar amounts of nitrogen (4 mM) supplied in the form of nitrate or a mixture of oxoglutarate and arginine. Data presented are means ± SE of four independent biological replicates (Paired t-test, n=24, $\alpha = 0.05$). Different letters indicate statistically significant differences. D, Influence of 2,2-bipyridyl (2,2-BP) (1 mM) on ET accumulation in 7-days old liquid cultures of *C. miyabeanus* (Cm988). Data presented are means ± SE of three independent biological replicates (Paired t-test; n=148; $\alpha = 0.05$). Different letters indicate statistically significant differences. E, Relative expression of fungal ethylene forming enzyme, *CmEFE* in rice leaves infected with Cm988 (1 x 10^5 conidia.ml^{-1}). Data presented are mean relative expression levels of three independent experiments ± SE (Mann-Whitney; n=18; $\alpha = 0.05$). Different letters indicate statistically significant differences. F, Relative expression of rice *glutamate dehydrogenases* (*GDH*) in *C. miyabeanus*-infected leaves at 12hpi. Data were extracted from a publicly available microarray experiment

4.2.3 *C. miyabeanus*-produced ET boosts rice ET synthesis and acts as a virulence factor

The ability of *C. miyabeanus* to produce ET *in vitro* and the strong upregulation of *CmEFE* transcript levels during progression of brown spot disease strongly suggested that fungal ET might play an important role in shaping disease outcomes. To address this hypothesis, we first probed the effect of exogenously applied Ethephon, an ET-releasing growth regulator, on ET production in detached rice leaves treated or not with the plant ET biosynthesis blockers aminooxyacetic acid (AOA) and cobalt chloride ($CoCl_2$) (Fig. 4.3 A). Importantly, both chemicals significantly reduced ET levels in control-treated leaves but failed to lower ET in fungal cultures, demonstrating the effectiveness and specificity of these inhibitors under our experimental conditions. Furthermore, despite having no significant effect on the ET emission of rice leaves treated with low levels of Ethephon (10μM), co-application of AOA and $CoCl_2$ significantly reduced ET emissions triggered by higher Ethephon concentrations (100 μM), indicating that high doses of exogenously administered ET amplify endogenous ET production.

To test whether fungal ET might have a similar impact on plant ET synthesis during *C. miyabeanus* infection, we next quantified the effect of plant and fungal ET biosynthesis blockers on ET emissions in inoculated leaves (Fig. 4.3 B), on one hand, and brown spot resistance, on the other (Fig. 4.3 C). As expected, uninfected water-treated rice leaves produced almost no ET. Following infection, however, ET levels started to increase rapidly from 6 hpi onwards and peaked around 12 hpi at approximately 20 times the levels found in non-inoculated samples. Blocking plant ET production by treating leaf pieces with 500 μM of AOA and $CoCl_2$ partially inhibited ET emission levels, but failed to exert a significant effect on disease resistance. In contrast, blocking fungal ET production by adding 2,2-BP to the spore solution not only strongly reduced total ET titers but also significantly restricted subsequent disease development, while co-application of plant and fungal ET biosynthesis blockers mirrored the effect of single 2,2-BP treatments. Importantly, application of 2,2-BP had no significant impact on ET levels in non-infected rice tissues, demonstrating the specificity of this inhibitor for fungal ET synthesis. Together these findings indicate that production of ET by *C. miyabeanus* is an important virulence strategy and suggest that fungal ET at least partly acts by amplifying plant ET synthesis.

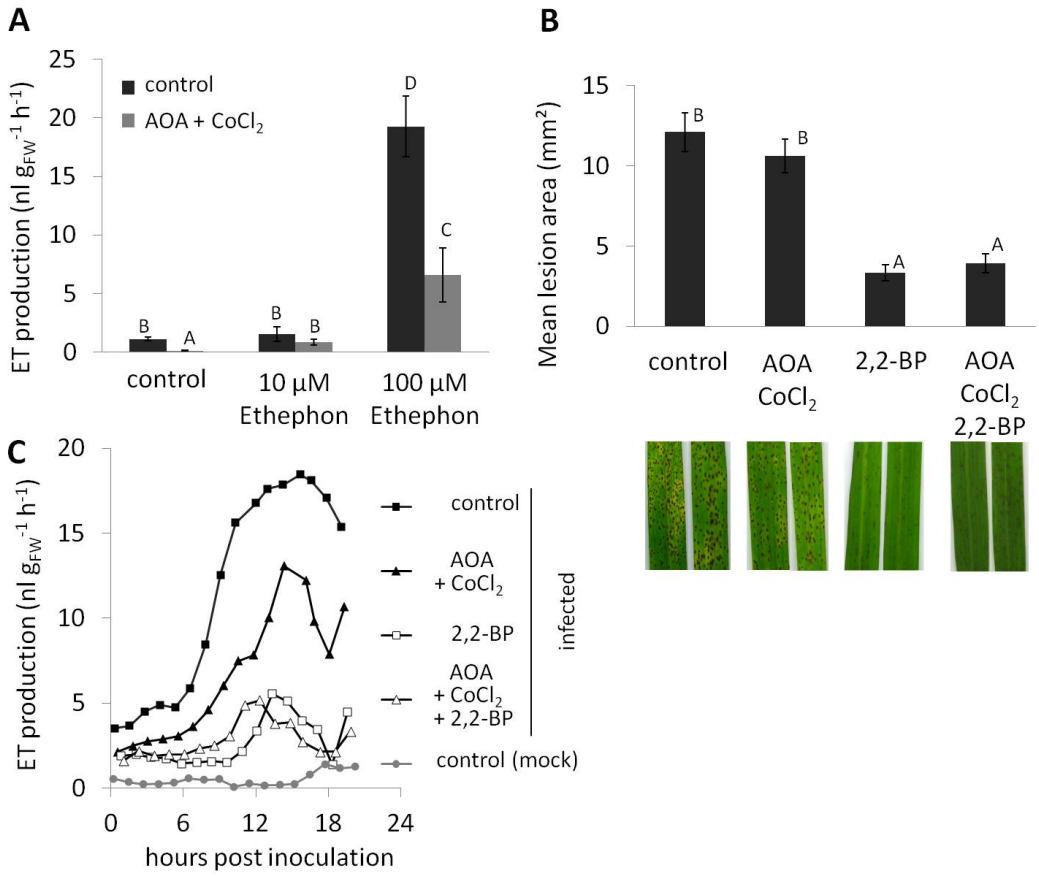


Figure 4.3: A, Influence of Ethephon on *in planta* ET accumulation in rice leaves treated with water or the ET biosynthesis inhibitors AOA and $CoCl_2$ (500μM each) 24h after treatment. Data presented are means ± SE of five independent biological replicates (Mann-Whitney; n=30; $\alpha = 0.05$). Different letters indicate statistically significant differences.

B, Effect of the fungal ET biosynthesis inhibitor 2,2-BP (2mM in spore solution) and the plant ET biosynthesis blocker (AOA and $CoCl_2$, 500μM each) on brown spot disease severity. Data presented are means ± SE of five independent biological repeats (Bonferoni; n=40; $\alpha = 0.05$). Different letters indicate statistically significant differences.

C, ET accumulation detected in detached leaves infected with *C. miyabeanus* (Cm988, 1 x 10^4 conidia.ml^{-1}) using laser photoacoustic detection (LPAD). Leaf segments were floated for 24h on aqueous solutions containing the plant ET biosynthesis blockers AOA and $CoCl_2$ (500μM each) and subsequently infected with conidial solution containing 0 or 2mM fungal ET biosynthesis blocker (2,2-BP). Data presented are from a representative experiment. The experiment was repeated twice with similar results.

4.2.4 Rice ET signaling but not ET biosynthesis is essential for brown spot development

The finding that chemical disruption of plant ET production only weakly affected disease severity whereas Ethephon application rendered plants hyper-susceptible (De Vleesschauwer et al., 2010; Fig. S5.6), suggested that ET action, rather than de novo ET biosynthesis, is a crucial factor modulating *C. miyabeanus* pathogenicity. Supporting this hypothesis, inhibition of ET signaling with silver thiosulfate (STS), a well-characterized inhibitor of ET action, almost completely abrogated brown spot development (Fig. 4.4 A and B). Given the senescence-inducing properties of ET on rice leaves (Fig. S5.4) fungal ET production might account for the induction of chlorotic lesions around the site infection. Despite inducing substantial levels of resistance, blocking fungal ET with 2,2-BP was significantly less effective in reducing mean lesion size as compared to STS application (Fig. 4.3 C). Along with the inability of AOA and $CoCl_2$ to mount resistance, these data not only highlight the central importance of plant ET signaling in the establishment of brown spot disease, but also suggest that *C. miyabeanus* has evolved virulence factors other than fungal ET to trick the plant into activating downstream ET action.

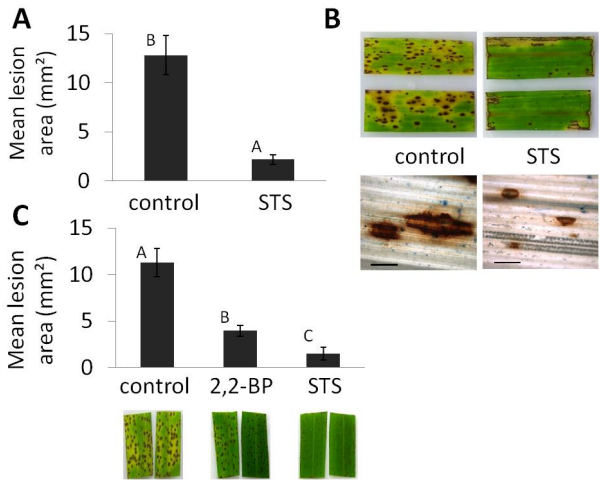


Figure 4.4: A, Positive influence of plant ET signaling blocker STS (1mM) on brown spot resistance. Detached leaves were treated 24h prior inoculation. Data presented are means ± SE of five independent biological repeats (Bonferoni; n=40; α = 0.05). Different letters indicate statistically significant differences. B, Pictures showing representative disease development in control and STS-treated plants at three days post inoculation. Scale bars are 500μm. C, Effect of STS (1 mM) and the fungal ET biosynthesis inhibitor 2,2-BP (2 mM) on brown spot resistance in detached leaf assays. Data presented are means ± SE of three independent biological repeats (Tukey; n=36; α = 0.05). Different letters indicate statistically significant differences.

4.2.5 ET signaling compromises phenylpropanoid-driven defenses against *C. miyabeanus*

Although the role of ET in the regulation of plant-microbe interactions has been intensively studied, it remains largely unclear how ET contributes to microbial virulence. To further decipher how ET signaling increases rice susceptibility to infection with *C. miyabeanus*, we studied the cytological alterations associated with fungal colonization using the intact leaf sheath method. Contrary to leaf blades, leaf sheath tissue is relatively flat and optically clear, which facilitates live cell imaging. Microscopic analysis of leaf sheaths sampled 48h after *C. miyabeanus* infection and stained with Evan's blue revealed massive cell death of both invaded and non-penetrated surrounding cells, whereas blocking ET signaling with STS strongly reduced the occurrence of pathogen-induced tissue necrotization (Fig. 4.5 A). Moreover, STS-treatment increased the accumulation of phenolic compounds around the site of penetration as visualized by an intense green autofluorescence under blue light excitation (Fig. 4.5 B). Quantification of the concentration of soluble phenolic

compounds in a detached leaf assay showed that neither Ethephon nor STS treatment significantly affected phenolics accumulation in non-inoculated samples (Fig. 4.5 C). However, substantial differences among treatments were seen following pathogen challenge, with ET and STS respectively enhancing and reducing the level of pathogen-induced phenolics as compared to inoculated controls. Similar effects were also observed in the activity pattern of two key enzymes of the phenylpropanoid pathway, namely phenylammonia lyase (PAL) and polyphenol oxidase (PPO) (Fig. 4.5 D and E). Exogenous application of different phenolics demonstrated the effectiveness of these compounds in reducing brown spot severity (Fig. S5.5). Collectively, these findings argue that ET promotes susceptibility to *C. miyabeanus* at least in part by antagonizing phenylpropanoid metabolism, leading to reduced deposition of phenolic compounds at the site of fungal penetration.

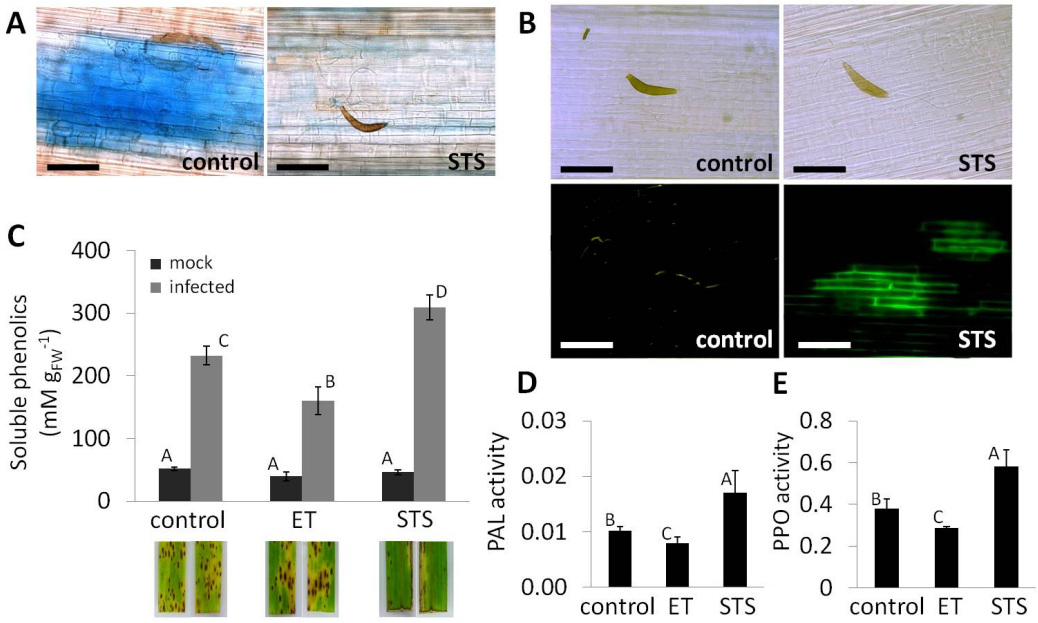


Figure 4.5: A, Microscopic analysis of the influence of ET signaling blocker STS (1 mM) on brown spot infection (Cm988, 1 x 10^4 conidia ml^{-1}) in rice sheaths. Micrographs depict representative fungal development at 48hpi. Plant cells were stained with Evan's blue to visualize cell death. Scale bar is 50 µm. B, Representative white light (upper panel) and epifluorescence (lower panel) images of control and STS-treated rice cells at 12 hpi. Scale bars are 50 µm. C, Positive influence of ET signaling blocker STS (1 mM) on accumulation of phenolic compounds in in mock- and Cm988-inoculated rice leaves at 24hpi. Data presented are means ± SE of three independent biological repeats (Tukey; n=18; $\alpha = 0.05$). Different letters indicate statistically significant differences. D, Phenylalanine ammonia lyase (PAL) activity ($\Delta abs_{290nm}.mg^{-1}{}_{protein}.min^{-1}$) and E, polyphenol oxidase (PPO) activity ($\Delta abs_{420nm}.mg^{-1}{}_{protein}.min^{-1}$) in rice leaves (24hpi) treated with Ethephon (100 µM) and ET signaling inhibitor STS (1 mM). Data presented are means ± SE of three independent biological repeats (Tukey; n=18; $\alpha = 0.05$). Different letters indicate statistically significant differences.

4.2.6 *C. miyabeanus* isolates differ in virulence and ET production

All previous experiments were conducted with the highly virulent isolate Cm988. To address the question of how fungal ET production relates to microbial virulence, we included two other isolates, WK1C and C412, in our analyses. Like Cm988, both of these isolates were isolated from diseased rice in field plots at the International Rice Research Institute (the Philippines). Infection trials revealed that WK1C is least virulent, followed

by C412 and Cm988 (Fig. 4.6 D). In line with these results, liquid cultures of isolates WK1C and C412 also clearly emitted less ET compared to Cm988 (Fig. 4.6 A) and, accordingly, showed a lower expression of *CmEFE* (Fig. 4.6 B). As shown in Figure 4.6 C, these strain-specific differences in virulence and CmEFE expression correlated well with both total ET emission levels (control) and synthesis of fungal ET (as revealed by AOA and $CoCl_2$ treatment) in detached leaf assays.

To further investigate this putative link between ET metabolism and *C. miyabeanus* virulence, a parallel set of inoculation experiments was performed wherein we tested the significance of plant ET synthesis and signaling in response to all three isolates. Intriguingly, inhibition of plant ET synthesis by combined AOA and $CoCl_2$ application had no significant impact on Cm988 infection but enhanced resistance against the weakly virulent strains WK1C and C412. In contrast, STS-mediated disruption of ET signaling severely restricted disease development irrespective of the pathogen's virulence. In view of the central importance of plant ET signaling in *C. miyabeanus* pathogenicity, we interpret these data to suggest that pathogen-induced plant ET synthesis is especially important for less virulent strains of *C. miyabeanus* which may produce too little ET themselves to initiate plant ET signaling (Fig. 4.6 D).

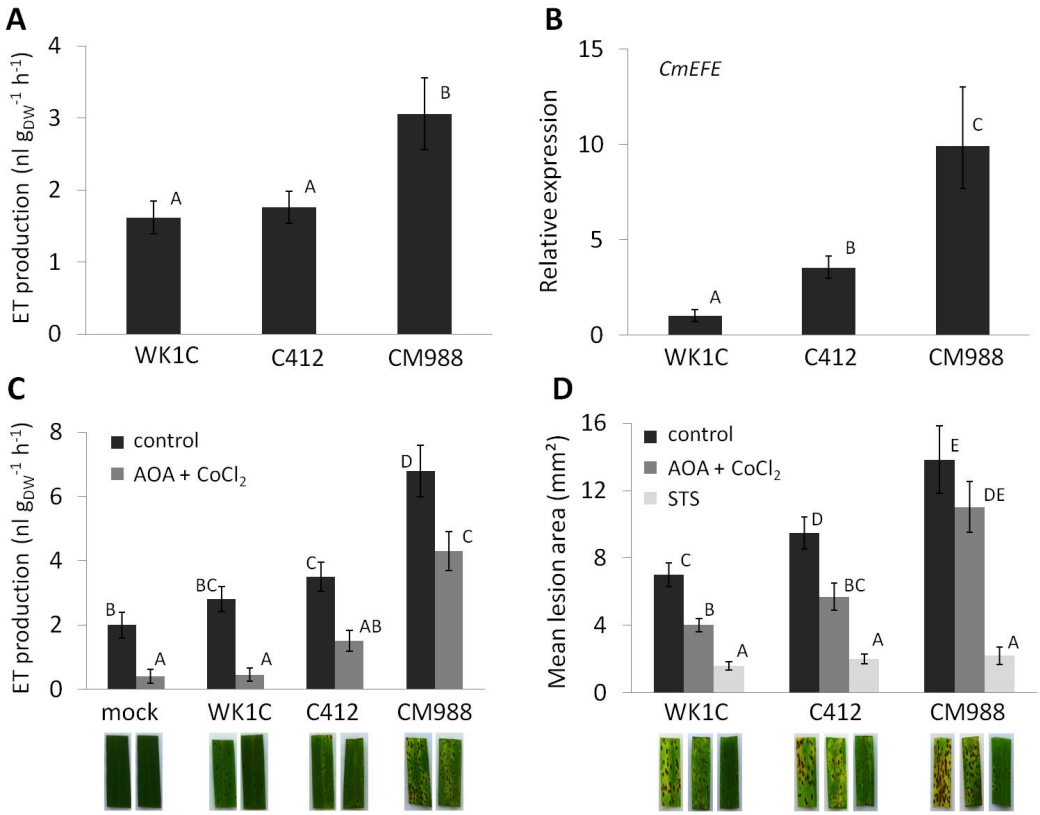


Figure 4.6: A,ET accumulation and B, relative expression of *CmEFE* in 7-day-old liquid cultures of *C. miyabeanus* isolates ranging in virulence from least (WK1C) to most virulent (Cm988). Data presented are means ± SE of at least three independent biological repeats (Mann-Whitney; $n \leq 12$; $\alpha = 0.05$). Different letters indicate statistically significant differences. C, Influence of the plant ET biosynthesis blockers AOA and $CoCl_2$ (500 µM each) on ET production in mock- and pathogen-inoculated leaves at 24hpi. Data presented are means ± SE of at least three independent biological repeats (Mann-Whitney; $n = 28$; $\alpha = 0.05$). Different letters indicate statistically significant differences. Photographs depict representative symptom development. D, Influence of the plant ET biosynthesis blockers AOA and $CoCl_2$ (500 μM each) and the ET signaling blocker STS (1 mM) on brown spot resistance at three dpi. Data presented are means ± SE of at least three independent biological repeats (Tukey; $n = 36$; $\alpha = 0.05$). Different letters indicate statistically significant differences.

4.3 Discussion

Since its discovery as the main defoliating component of coal gas by Neljubov, the gaseous hormone ET and its myriad effects on plant growth and development have been the subject of intense research. Fueled by the advent of large-scale -omics technologies and recent advances in computational biology, tremendous progress has lately been made in elucidating the molecular mechanisms by which ET is perceived and its signal transduced (for review see Merchante et al. (2013). Contrary to these paradigm-shifting advances, the precise role and function of ET in the regulation of plant-microbe interactions is less well understood, with both positive and negative effects being reported depending not only on the plant-attacker combination, but also on the hormone concentration, type of tissue, and even timing of infection (Van der Ent and Pieterse, 2012). Aiming to gain further insight into the defense-modulatory role of ET, we have analyzed its impact and dynamics during progression of rice brown spot disease caused by the necrotrophic fungus *Cochliobolus miyabeanus*. In keeping with the strong disease-enhancing effect of exogenously administered ET (De Vleesschauwer et al., 2010), our findings unmask ET as a key signal in the activation of gene expression by *C. miyabeanus*. Moreover, we show that the pathogen tricks the plant into producing copious amounts of ET at least in part by synthesizing ET itself, a strategy that is especially important for less virulent *C. miyabeanus* strains. Finally, we demonstrate that plant ET signaling compromises basal immunity to *C. miyabeanus* via a two-pronged mechanism involving disruption of phenylpropanoid metabolism.

Together with SA and JA, ET is a common component of the hormonal blend released by pathogen-infected plants. Real-time monitoring of ET levels during rice-brown spot interaction revealed a biphasic ET burst comprising an initial peak of ET around the time of necrotic lesion formation (i.e. 12 hpi), followed by a second more pronounced ET burst closely associated with further lesion expansion and spreading chlorosis. The first wave of ET production is only a small fraction of the magnitude of the second peak and possibly interferes with plant defense signaling, inducing a state of susceptibility. In contrast, the second peak around 48 hpi is so big that it likely initiates processes such as senescence and chlorosis, further amplifying disease development. Consistent with the strong rise in ET levels, microarray analysis of brown-spot infected leaves revealed substantial transcriptional reprogramming of several rice ET-biosynthesis genes, including OsACO7 and OsACS2. Moreover, the transcriptome of plants infected with *C. miyabeanus* displayed a strong overlap with that of rice leaves treated with the ET precursor ACC (Garg et al., 2012), suggesting that elevated ET concentrations *in planta*

are a primary signal in the activation of gene transcription by *C. miyabeanus*. These findings echoe previous findings in the *Arabidopsis-Pseudomonas syringae* pathosystem where effector proteins delivered by virulent bacterial stains were found to activate large suites of ABA-, auxin- and JA-responsive transcripts (Chen et al., 2007; de Torres-Zabala et al., 2007). Moreover, in conjunction with the strong disease-suppressive effect of the plant ET signaling inhibitor STS (Fig. 4 A and B), these results add weight to the concept that *C. miyabeanus* hijacks the plant ET pathway as a decoy strategy to rewire the rice immune signaling circuitry and suppress effectual defense pathways.

One of the outstanding questions in this regard is how does *C. miyabeanus* hijack the rice ET pathway? Naturally, plant pathogens have evolved a variety of strategies to overcome plant hormone-mediated immunity or induce host susceptibility by interfering with various hormonal processes. In many plant-pathogen interactions, hormone-based virulence strategies are based on the delivery into the host cell of proteinaceous virulence effectors that target hormone signaling components (Robert-Seilaniantz et al., 2011; Dou and Zhou, 2012). In tomato, for instance, the *Pseudomonas syringae* pv. *tomato* effectors AvrPto and AvrPtoB are well known to induce ET biosynthesis in order to trigger cell death and facilitate pathogenesis (Cohn and Martin, 2005). In addition, an increasing number of microbes are predicted to produce phytohormones or phytohormone mimics themselves, including SA, JA, ABA, CK, GA, and ET (Denancé et al., 2013). In plants, ET is produced from methionine via the intermediate ACC. In microorganisms, however, ET can be synthesized from different sources, including ACC, the methionine-derivative α-keto-γ-methylthiobutyric acid, and, as is the case in *C. miyabeanus* (Fig. 4.2) and most other fungi, the amino acids 2-oxoglutarate and arginine (Arshad and Frankenberger, 2002). Interestingly, *C. miyabeanus* only produced ET in response to plant extracts, suggesting that fungal ET production does not play a role in the development of the pathogen but rather is required for plant infection. Supporting this assumption, we found two glutamate dehydrogenase genes, namely OsGDH2 and OsGDH3, to be strongly upregulated in brown spot-infected leaves. Often activated under stress- and senescence-inducing conditions (Masclaux-Daubresse et al., 2005; Pageau et al., 2006; Qiu et al., 2009; Masclaux-Daubresse et al., 2010), glutamate dehydrogenases regulate the mitochondrial glutamate/oxoglutarate equilibrium by catalysing the formation of oxoglutarate from glutamate (Dubois et al., 2003; Miyashita and Good, 2008). Since oxoglutarate is not abundantly present in plant cells (Weber, 2002), it is tempting to speculate that *C. miyabeanus* activates its own ET production by inducing oxoglutarate synthesis in infected leaves.

In fungi, oxoglutarate-dependent ET production is commonly mediated by a single multi-

function ethylene forming enzyme (Arshad and Frankenberger, 2002). Blasting EFE protein sequences and pharmacological experiments using the EFE inhibitor 2,2-BP led us to identify a homologue in *C. miyabeanus*, designated CmEFE. To our interest, gene expression experiments revealed a strong induction of CmEFE during infection, suggesting that fungal ET acts as a virulence factor, attenuating the plant resistance response. Consistent with this hypothesis, blocking fungal ET synthesis using 2,2-BP not only abrogated ET production by *C. miyabeanus in vitro* but also significantly increased brown spot resistance. Although production of microbial ET has been described before as a virulence strategy for bacterial plant pathogens such as *P. syringae* pv. *glycinea* and *Ralstonia solanacearum* (Weingart et al., 2001; Valls et al., 2006; Macho et al., 2010), EFE enzymes are found in a wide range of micro-organisms, including post-harvest pathogens, plant beneficial micro-organisms and even micro-organisms that do not interact with plants (Eckert et al., 2014). Therefore, oxoglutarate-dependent microbial ET production may fulfill multiple roles, acting as either a positive or negative regulator of both microbial development and plant defense responses.

Early studies with ET-producing strains of *R. solanacearum*, *P. syringae* and *Xanthomonas citri* have shown that enhanced ET production in diseased plant tissues can be either of host or microbial origin, depending on the specific plant-attacker combination and possibly also the timing of infection (Crownshaw and Pegg, 1976; Goto et al., 1980; Lu et al., 1989; Weingart and Volksch, 1997). To determine the source of ET and its role in brown spot-infected rice leaves, we took advantage of the two different ET biosynthesis pathways in the plant and fungus. These experiments demonstrated that attenuation of fungal ET production by 2,2-bipyridil almost completely negated ET production in diseased leaves, whereas blocking plant ET biosynthesis using the chemical inhibitors AOA and $CoCl_2$ was significantly less effective in reducing ET levels. Although these results indicate *C. miyabeanus* is the prevalent source of ET in brown spot infected leaves, they also suggest that fungal ET amplifies plant ET production. In support of this assumption and consistent with previous findings (Barry et al., 2000; Alexander and Grierson, 2002), we found high concentrations of exogenously administered Etephon to increase endogenous ET production. Opposite to the auto-inhibitory system that generally operates during normal vegetative growth of the plant, ET biosynthesis in brown spot-infected tissues therefore seems to be controlled by a positive feedback mechanism similar to the one described in senescing tissues and ripening fruits (Iqbal et al., 2011).

Despite the apparent importance of ET as a fungal virulence factor, blocking plant ET signaling with the ET receptor inhibitor STS triggered significantly higher levels of resistance compared to the fungal ET blocker 2,2-BP. These findings may be reconciled

by considering that *C. miyabeanus* secretes additional virulence factors that impinge on the plant ET pathway and require ET signaling action for their mode of action. Like other *Cochliobolus* species, *C. miyabeanus* produces a broad palette of cell death-inducing phytotoxins, including ophiobolins A and B (Xiao et al., 1991; Kim et al., 1999; Ahn et al., 2005). Moreover, several lines of evidence point towards a close interconnection between phytotoxin-induced disease susceptibility and plant ET signaling. For instance, many toxins produced by phytopathogenic microorganisms, comprising both prokaryotes and eukaryotes, induce senescence and increase ET biosynthesis in a wide variety of plants (Mercado Vergnes et al., 2006). Moreover, ET-dependent signaling often participates in phytotoxin-triggered host cell death. In one of the best studied examples, AAL, a host-selective toxin produced by *Alternaria alternata* f. sp. lycopersici, has been shown to induce plant cell death by triggering a complex signaling cascade centered around the key ET transcription factor EIN3 (Crownshaw and Pegg, 1976; Moore et al., 1999; Chen et al., 2009; Mase et al., 2012, 2013). Finally, many toxin-producing pathogens contain EFE homologues and some of these pathogens, including *Verticillium dahliae* and *Fusarium oxysporum*, have been reported to produce ET as well (Tzima et al., 2010). Together these findings not only reinforce the contention that ET can function as a toxin synergist (Crownshaw and Pegg, 1976), but also suggest that the effectiveness of the ET pathway in triggering disease resistance could be predicted based on the toxin-secreting capacities of the pathogen. In this context, it may be no coincidence that most interactions for which ET has been shown to increase necrotroph susceptibility involve well-known toxin producers such as *Verticillium dahliae*, *A. alternata* f. sp. *lycopersici* and *R. solanacearum*) (Pantelides et al., 2010, 2013).

One particularly interesting finding in this study was the observation that the expression of CmEFE as well as ET production *in vitro* and *in planta* is correlated with the virulence of different *C. miyabeanus* isolates. Moreover, blocking plant ET biosynthesis decreased the total ET emission for all isolates and significantly increased disease resistance towards the less virulent isolates, despite being ineffective against the most virulent isolate. One possible interpretation of these findings is that highly virulent strains produce sufficient fungal ET to fully activate the plant ET signaling pathway such that plant-derived ET might not have an additional effect on susceptibility. Conversely, in case of more weakly virulent isolates that produce less fungal ET, pathogen-induced plant ET may still add to ET pathway activation, thereby further enhancing brown spot severity. The observation that STS-mediated disruption of ET signaling triggered high levels of resistance independent of the intrinsic level of pathogen virulence supports this concept. Strain-specific differences in disease severity, however, were still evident following exogenous ET applica-

tion, indicating that *C. miyabeanus* virulence is not only determined by the ability of the pathogen to manipulate plant ET signaling.

Over the past few years, ET has been implicated in the regulation of several structural and biochemical plant defense responses such as xylem occlusions, cell wall-strengthening hydroxyproline-rich glycoproteins and pathogenesis-related proteins (Adie et al., 2007). Based on our findings, two mechanisms are proposed that may explain how ET contributes to fungal virulence. First, given the limited amount of pathogen-triggered necrosis in STS-treated leaves (Fig. 4.4 B) and the well-established role of ET in the amplification of certain types of cell death (Moore et al., 1999; de Jong et al., 2002; Woltering et al., 2003; Mase et al., 2012), it is not inconceivable that ET increases brown spot severity by boosting pathogen-induced host cell death. In support of this assumption, gene ontology analysis of all transcripts commonly regulated by ET and brown spot infection revealed several processes that are linked to senescence, such as Figure S5.4. Moreover, senescence-associated amino acid metabolism in rice leaves was previously shown to be essential for *C. miyabeanus* proliferation inside diseased tissues, further supporting our hypothesis (Chattopadhyay and Bera, 1978; Matsubara and Kuroda, 1980). A second mode of ET action was borne out by the observations that the enhanced brown spot resistance in STS-treated leaf sheaths is associated with hyperactivation of the phenylpropanoid pathway and localized accumulation of phenolic compounds at the site of infection. Considering that production of phenolics is a well-studied defense strategy against *C. miyabeanus* (Shabana et al., 2008) and that some of the phytotoxins produced by the pathogen have been reported to interfere with phenol metabolism (Vidhyasekaran et al., 1992), these data thus suggest that ET-mediated brown spot susceptibility may also derive from suppression of effectual phenylpropanoid-driven defences.

4.4 Conclusion

In summary, our results favor a scenario whereby *C. miyabeanus* strains cause disease by hijacking the rice ET signaling pathway. Under our current model (Figure 4.7), *C. miyabeanus* rapidly starts synthesizing ET in penetrated rice cells via a reaction that requires 2-oxoglutarate and arginine as cofactors and is catalysed by a single Ethylene Forming Enzyme. In a self-amplification loop, fungal ET in turn triggers plant ET synthesis, the combined action of which leads to activation of the rice ET signaling pathway. Host ET signaling dictates transcriptional reprogramming of extensive gene sets in diseased leaves and closely interacts with fungal phytotoxins to compromise plant immunity via a dual mechanism comprising amplification of pathogen-induced senescence

and disruption of phenylpropanoid-driven defenses that normally serve to limit pathogen growth. While providing novel insights into the multifaceted role of ET in the plant's defense signaling circuitry, the findings presented in this study underscore the importance of microbial ET in modulating plant immunity and hint at a tight relationship between microbial phytotoxin production and ET-induced plant susceptibility.

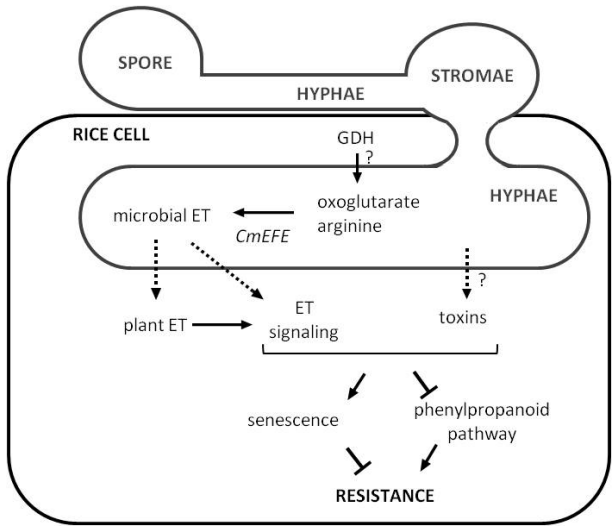


Figure 4.7: Hypothetical model illustrating the role of plant and fungal ET in triggering susceptibility against *C. miyabeanus* in rice

4.5 Addendum: Microbial 2-oxoglutarate-dependent ET biosynthesis in plant-pathogen interactions

Ethylene (ET) is primarily known as a plant hormone that modulates many developmental processes and plays an important role as mediator of defense responses in plants (Wang et al., 2002; van Loon et al., 2006). However, several microorganisms can also produce ET via pathways that are different from plant ET biosynthesis. To date three different microbial ET biosynthetic pathways have been described (Cristescu et al., 2006; Chagué, 2010). Two pathways produce ET from methionine with either 1-aminocyclopropane-1-carboxylic acid (ACC) or 2-keto-4-methylthiobutyric acid (KMBA) as intermediate. The third and best-known pathway describes the production of ET from 2-oxoglutarate and arginine, catalyzed by an ET forming enzyme (EFE) (Fukuda et al., 1992; Chagué, 2010; see Fig 4.2 A). Several plant-pathogens have been reported to produce ET following the 2-oxoglutarate dependent pathway (Table 4.1). To date, only the EFEs of *Pseudomonas syringae* pathovars, *Ralstonia solanacearum* and *Penicillium digitatum* have been identified and functionally characterized (Fukuda et al., 1989; Nagahama et al., 1991a; Sato et al., 1997; Genin and Boucher, 2002). These EFE protein sequences are characterized by the presence of two conserved domains, DIOX_N (Pfam14226) and 2OG-FeII_oxy (pfam03171) (see Chapter 4.2.2) which are specific for enzymes that show 2-oxoglutarate/Fe(II)-dependent dioxygenase activity (Aravind and Koonin, 2001; Hausinger, 2004; Hagel and Facchini, 2010; Marchler-Bauer et al., 2011).

Table 4.1: List of microorganisms that produce ET in a 2-oxoglutarate-dependent manner.

Micro-organism	**Reference**
Cochliobolus miyabeanus	see Section 4.2.2
Fusarium oxysporum	Hottiger and Boller (1991)
Penicillium cyclopium	Pazout and Pazoutova (1989)
Penicillium digitatum	Fukuda et al. (1986, 1988)
Pseudomonas syringae pv. *cannabina*	Weingart et al. (1999)
Pseudomonas syringae pv. *glycinea*	Watanabe et al. (1998); Weingart et al. (1999); Weingart and Volksch (1997); Weingart et al. (2001)
Pseudomonas syringae pv. *phaseolicola*	Weingart et al. (2001); Weingart and Volksch (1997); Fukuda et al. (1992); Nagahama et al. (1991b)
Pseudomonas syringae pv. *pisi*	Weingart et al. (2001); Weingart and Volksch (1997)
Pseudomonas syringae pv. *sesami*	Sato et al. (1997)
Ralstonia solanacearum	Valls et al. (2006); Genin and Boucher (2002); Macho et al. (2010)

Due to the availability of a fast growing assembly of microbial genome and proteome sequences, many EFEs have been identified and annotated based on the homology with characterized EFEs, mainly from *R. solanacearum* and *P. syringae* pathovars. In most cases however, ET producing abilities of the microorganism is still to be proven. Moreover,

there are manifold putative EFE proteins that are homologous with characterized EFEs with the same DIOX_N and 2OG-FeII_oxy domains (Eckert et al., 2014). A phylogenetic tree of all EFE protein homologs, both annotated and putative, shows two large subgroups, one containing fungi and the other bacteria, and a smaller subgroup containing mainly plant-pathogenic fungi from the *Pleosporaceae* family (Fig 4.8). In order to gain more insight on the role of potential microbial ET biosynthesis in this diverse group of microbes containing a putative EFE homolog, a relevant sub selection of these microorganisms was grouped according to their lifestyle and function (Table 4.2). Some of these organisms are known to produce ET via an annotated EFE, others contain an annotated EFE but have not yet been proven to produce ET and the rest only contains a putative EFE homolog (Eckert et al., 2014).

Table 4.2: Selection of microorganisms that contain a putative EFE-homolog with accession numbers from GenBank

type of microorganism	microorganism	accession number	protein function
plant pathogenic bacterium	*Pseudomonas cannabina*	AAD16441.1	ET producer (EFE)
	Pseudomonas savastanoi	WP_004661945.1	putative EFE
	Pseudomonas syringae pv. *glycinea*	ZP_11566673.1	ET producer (EFE)
	Pseudomonas syringae pv. *phaseolicola*	BAA02477.1	ET producer (EFE)
	Pseudomonas syringae pv. *pisi*	AAD16443.1	ET producer (EFE)
	Pseudomonas syringae pv. *sesami*	AAD16442.1	ET producer (EFE)
	Ralstonia solanacearum	YP_006031629.1	ET producer (EFE)
	Ralstonia solanacearum	YP_006061415.1	ET producer (EFE)
	Streptomyces bottropensis	WP_005474170.1	putative EFE
	Streptomyces sviceus	ZP_06914214.1	annotated EFE
	Xanthomonas oryzae pv. oryzicola	YP_005628789.1	annotated EFE
plant pathogenic fungus	*Claviceps purpurea*	CCE32144.1	annotated EFE
	Cochliobolus heterostrophus	EMD86143.1	putative EFE
	Cochliobolus miyabeanus	BK008840	ET producer (EFE)
	Cochliobolus sativus	EMD69871.1	putative EFE
	Colletotrichum graminicola	EFQ36771.1	putative EFE
	Colletotrichum higginsianum	CCF31757.1	putative EFE
	Colletotrichum orbiculare	ENH88987.1	putative EFE
	Colletotrichum orbiculare	ENH88987.1	annotated EFE
	Fusarium oxysporum f. sp. *cubense*	EMT67163.1	annotated EFE
	Magnaporthe oryzae	XP_366046.1	annotated EFE
	Neofusicoccum parvum	EOD48827.1	annotated EFE
	Pyrenophora teres f. teres	XP_003302061.1	putative EFE
	Pyrenophora tritici-repentis	XP_001935406.1	putative EFE
	Setosphaeria turcica	EOA91625.1	putative EFE
	Verticillium alfalfae	XP_003000034.1	annotated EFE
	Verticillium dahliae	EGY19158.1	annotated EFE
post harvest fungus	*Colletotrichum gloeosporioides*	ELA32715.1	annotated EFE
	Neurospora crassa	XP_957496.2	annotated EFE
	Neurospora tetrasperma	EGZ73504.1	annotated EFE
	Penicillium digitatum	EKV19239.1	annotated EFE
beneficial soil microbe	*Aspergillus clavatus*	XP_001270369.1	putative EFE
	Laccaria bicolor	XP_001884879.1	putative EFE
	Metarhizium anisopliae	EFY99197.1	putative EFE
	Pseudomonas fluorescens	WP_017528495.1	putative EFE
	Trichoderma virens	EHK24268.1	putative EFE
nitrogen-fixating bacterium	*Frankia* sp.	WP_007510697.1	putative EFE
insect pathogen	*Beauveria bassiana*	EJP64077.1	putative EFE
	Cordyceps militaris	EGX93620.1	putative EFE

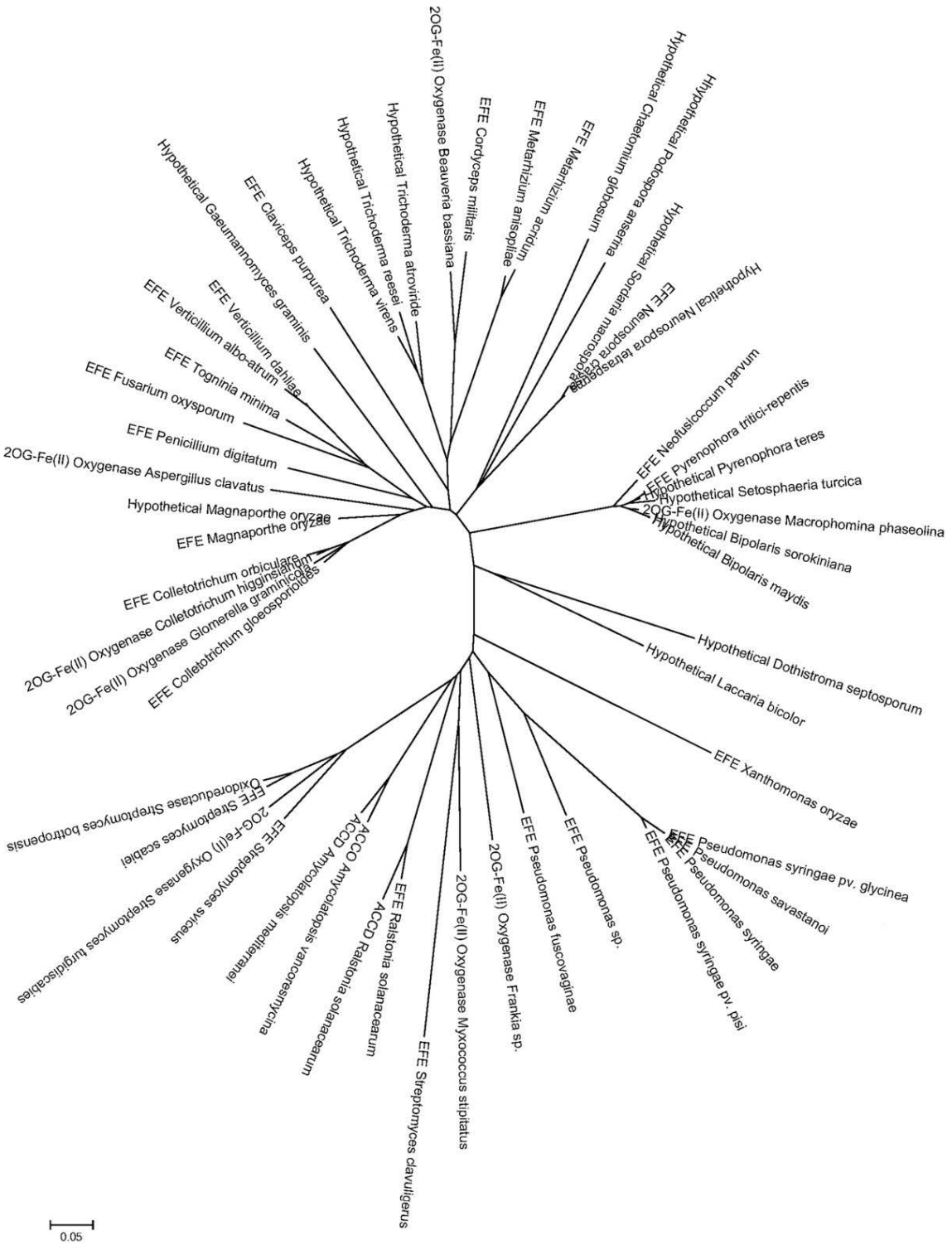


Figure 4.8: Phylogenetic study of Ethylene-Forming Enzyme (EFE) and EFE-homologous sequences The tree could be divided into two large group (fungi and bacteria) and one small group (mostly *Pleosporaceae* fungi) (Eckert et al., 2014).

4.5.1 Role of 2-oxoglutarate-dependent ET biosynthesis during plant-microbe interactions

The prevalence of plant-pathogenic microorganisms in Table 4.2 indicates that microbial ET might have different modes of action during the infection process. First, microbial 2-oxoglutarate dependent ET biosynthesis might serve as a virulence factor for some microorganisms in order to cause susceptibility in their host plants. This is the case for the plant pathogenic bacteria *R. solanacearum* and *P. syringae* pathovars (Weingart et al., 1999; Valls et al., 2006; Baltrus et al., 2011) as well as the necrotrophic leaf fungus *C. miyabeanus* (see Chapter 4) and the hemibiotrophic wilting fungus *Verticillium dahliae* (Crownshaw and Pegg, 1976; Pegg, 1981; Tzima et al., 2010). Moreover, *V. dahliae* mutants that are impaired in the cyclic AMP-dependent protein kinase A (cAMP/PKA) pathway, produce less ET and are less virulent (Tzima et al., 2010, 2012). These data suggest that the cAMP/PKA pathway, which is a known fungal signaling pathway (Borges-Walmsley and Walmsley, 2000), regulates fungal ET biosynthesis in *V. dahliae*.
Secondly, microbial ET might serve as a signaling component that could regulate the fungal infection process which could be the case for different *Neurospora* and *Colletotrichum* species and *Magnaporthe oryzae*. None of these pathogens show a convincing link between ET production and host susceptibility, so it is unlikely that ET production by these fungi acts as a virulence factor. However, spore germination and appressorium formation in these fungi are mediated by the cAMP/PKA pathway that regulates different fungal developmental processes (Borges-Walmsley and Walmsley, 2000). This has been reported for *Neurospora crassa* (Pall and Robertson, 1987; D'Enfert, 1997), *M. oryzae* (Xu et al., 1997; Kulkarni et al., 2005), *C. orbiculare* (Kubo and Takano, 2013) and *C. gloeosporioides* (Barhoom and Sharon, 2004; Priyatno et al., 2012). The germination of spores and appressorium formation of the post-harvest fungus *C. gloeosporioides* also seems to be induced by ET (Flaishman and Kolattukudy, 1994; Flaishman et al., 1995; Kim et al., 2000). Given that *C. gloeosporioides* occurs on climacteric fruits like tomato and avocados, ET produced during ripening might induce the infection process, although the spores also germinate in the absence of ET. These findings led to the hypothesis that ET, either host-derived or produced by *C. gloeosporioides*, is necessary to initiate the fungal development at the start of the infection process.
In conclusion, it seems that several plant pathogenic bacteria and fungi can produce ET in a 2-oxoglutarate-dependent manner as a virulence strategy. However, ET might also function as a fungal signaling component that possibly mediates different developmental processes during infection probably downstream of the important cAMP/PKA signaling

pathway. Given the widespread occurrence of putative EFE homologs in plant pathogens (Table 4.2), the role of microbial ET in plant-pathogen interactions promises to be an interesting research topic in the future.

4.5.2 Role of microbial ET biosynthesis for beneficial soil microorganisms

Since the function of 2-oxoglutarate-dependent microbial ET biosynthesis has been almost exclusively investigated in host-pathogen interactions, the current knowledge on microbial ET is mostly associated with plant-pathogen interactions. However, manifold soil microorganisms are known to produce and/or degrade ET, depending on soil type and conditions (Zechmeister-Boltenstern and Smith, 1998). Especially the ET degradation by microbial ACC deaminases is a well-described mechanism (Glick, 2005; Glick et al., 2007; Glick, 2014). A myriad of soil microorganisms show ACC deaminases (EC 3.5.99.7) and their activity is generally asociated with plant growth promotion and nitrogen fixation. Furthermore, under aerobic soil conditions, ET is used as a carbon and nutrient source by several groups of microorganisms (Shennan, 2006). On the other hand, anaerobic conditions seem to raise ET levels as a result of fermentation processes that increase the levels of microbial ET precursors along with a decreased O_2-dependent ET degradation (Arshad and Frankenberger, 2002; Mundle et al., 2012).

ET plays an important role during the establishment of symbiotic relations between microorganisms and their host plants, but the outcome is different depending on the ET concentration (Guinel and Geil, 2002; Khatabi and Schäfer, 2012). Intermediate levels of ET promote root colonization by beneficial soil microbes such as mycorrhizal fungi and nitrogen-fixating rhizobacteria (Wood, 2001; Riedel et al., 2008; López-Ráez et al., 2010), whereas high ET concentrations trigger root defense responses that prevent root colonization (Guinel and Geil, 2002; Khatabi and Schäfer, 2012). For putative ET producing beneficial soil microorganisms, moderate ET biosynthesis might therefore serve as a strategy to facilitate root colonization.

Microbial 2-oxoglutarate-dependent ET production might also play a role in mediating induced systemic resistance (ISR) against pathogen invasion. Arabidopsis root colonization with *Pseudomonas fluorescens* WCS417r leads to increased resistance against various pathogens like *P. syringae* pv. *tomato, Erwinia carotovora* pv. *carotovora, Botrytis cinerea* and *Hyaloperonospora parasitica* (Pieterse et al., 1996; Ton et al., 2002). *P. fluorescens* WCS417r had the same positive influence on rice plants infected with *Magnaporthe oryzae* (De Vleesschauwer et al., 2008). The protective effect of *P. fluorescens* WCS417r is dependent on the activation of the jasmonic acid and ET signaling in roots,

without altering the plant biosynthesis (Pieterse et al., 2000; Hase et al., 2003; De Vleesschauwer et al., 2008). These findings suggest that potential 2-oxoglutarate-dependent ET production by *P. fluorescens* might play a role in mounting ISR and protecting plants against pathogen attacks.
The role of microbial ET in mutualistic symbiosis between plants and soil microorganisms has not received sufficient attention in the past. However, numerous reports on the role of ET on the interaction between plants and non-pathogenic soil microbes indicate that microbial ET might be a key player in modulating different processes that are at the base of the beneficial effect of different soil microorganisms on growth and pathogen resistance.

4.5.3 A broad mode of action for microbial 2-oxoglutarate dependent ET biosynthesis?

The widespread occurence of putative EFE homologs in a diverse group of microorganisms which contains microbes that are not directly associated with plants, such as insect pathogens (Table 4.2) suggests a broad mode of action for microbial 2-oxoglutarate dependent ET biosynthesis. Since microbial ET can function as a fungal signaling component during plant-pathogen interactions, ET might serve as a general microbial signaling component. Although there is no convincing evidence for this assumption, the hypothesized novel role for microbial ET may shed new light on microbial signalling networks and the modulation of metabolic processes in various microorganisms.

4.6 Materials and methods

4.6.1 Plant material and growth conditions

All rice plants (*Oryza sativa* L.) were *japonica* cultivar Nipponbare. The rice seeds were surface sterilized with 70% ethanol for 1 min and 1% sodium hypochlorite solution for 10 min, rinsed three times with sterile distilled water and germinated at 28°C for five days on wet sterile filter paper in Petri dishes sealed with parafilm ($\geq$ 92% relative humidity). The seedlings were transplanted on vermiculite in half-strength modified Hoagland solution (Hewitt and Smith, 1975). Five days later the plantlets are transferred to a hydroponic system in full strength modified Hoagland solution (pH 6.5). The Hoagland solution was replaced every 7 days. The rice plants were grown in a growth chamber (28°C, 12h/12h light regime) for five weeks until they reached the 7-leaf stage.

4.6.2 Pathogen inoculation and disease rating

Cochliobolus miyabeanus isolates Cm988, C412 and WK1C kindly provided by International Rice Research Institute, were grown on potato dextrose agar (PDA, Difco) at 28°C in darkness. After a week the mycelium was put under 12h/12h blue light (Philips TLD 18W/08 and Philips TLD 18W/33) until sporulation. Conidia were harvested as described by Thuan et al. (2006) and suspended in a 0.5% gelatine (type B from bovine skin; Sigma-Aldrich G-6650) in a concentration of 1 x 10^4 conidia.ml^{-1}. Plants in the 7-leaf stage (5 weeks old) were inoculated by spraying the conidial suspension on the leaves till run off (1ml per plant) using a compressor-powered airbrush gun. The plants were kept in a humid and warm infection chamber (28°C $\pm$ 4°C, 92% or greater relative humidity) to promote fungal penetration. After 18h the plants were transplaced to greenhouse conditions (28°C $\pm$ 4°C, 16-h-light/8-h-dark regime) for disease development. The symptoms were scored three days after inoculation using APS ASSESS 2.0 software (APS, St Paul, Minnesota, USA). All infection trials were repeated at least three times with similar results.

4.6.3 Microscopic analysis

Leaf sheaths of 5 week-old rice plants were peeled off until only the roots and sheath of the youngest fully developed leaf remained. This sheath was placed in a tray lined with wet paper towels with the adaxial side of the inner sheath epidermis facing upwards and filled with a conidial suspension of *C. miyabeanus* (1 x 10^4 conidia.ml^{-1}, 1ml per sheath). The tray was covered with transparent plastic and placed under greenhouse conditions (28°C $\pm$ 4°C, 16-h-light/8-h-dark regime). Samples for microscopy were taken at 0, 24, 48 and

72 hours post inoculation and at least six trimmed sheath sections originating from three plants were sampled per time point. The microscopic slides were cut according to Koga et al. (2004). Fungal hyphae were stained with trypan blue (Stone et al., 2000). Evan's blue staining was used to colour dead cells (Mergemann and Sauter, 2000). Phenolic compounds were visualized as autofluorescence under blue light epifluorescence (Olympus U-MWB2 GPF filter set; excitation, 450-480 nm; dichroic beam splitter, 500 nm; barrier filter BA515). Images were acquired digitally (Olympus Colorview II camera) and further processed with the Olympus analySIS cellF software.

4.6.4 Pharmacological Experiments

The ET releasing Etephon (2-chloroethylphosphonic acid), ET precursor ACC (1-amino cyclopropane-1-carboxylic acid), the ET biosynthesis inhibitor AOA (aminooxyacetic acid) and $CoCl_2$ were purchased from Sigma-Aldrich. Silver thiosulfate (STS), an inhibitor of ET signaling, was prepared by mixing solutions of 0.1 M sodium thiosulfate with 0.1 M silver nitrate in a 4:1 ratio (Zhao et al., 2002; Shoresh et al., 2005). All chemicals were dissolved in water at the indicated concentrations and were applied by cutting the 2 youngest fully developed leaves in 7 cm segments and putting them in the chemical solutions 24h prior to inoculation. Afterwards, the leaf segments were placed in square Petri dishes (Greiner Bio-One, 688102) lined with moist paper towels, sprayed with *C. miyabeanus* conidial suspension or drop-inoculated with six 10 µl droplets of suspension (1 x 10^4 conidia.ml^{-1} in 0.25% gelatine). After 24 h, the droplets were removed with a laboratory tissue, and resistance was quantified by measuring lesion area at 72 hpi using APS assess 2.0 (APS, St Paul, Minnesota, USA). Unlike all other chemicals, the fungal ET biosynthesis inhibitor 2,2-BP and the phenolics catechol, trans-cinnamate, gallic acid were added to the spore solution at the time of inoculation.

4.6.5 Quantification of ET accumulation

The production of ET by *C. miyabeanus in vitro* was measured on mycelium grown on liquid and solid Czapek medium (Kubo et al., 1989) containing 40 mM sucrose, 5.6 mM KH_2PO_4, 0.14 mM KH_2PO_4, 2 mM $MgSO_4$, 0.04 mM $FeSO_4$, 6.7 mM KCl, 5 mM nitrogen and a sterile rice extract (pH 5.6). Nitrogen was supplied in the form of nitrate or a combination of 5 mM oxoglutarate and 1.25 mM arginine. Media were inoculated with conidial (1 x 10^4 conidia.ml^{-1}) or mycelial suspension and cultured in scintillation vials at 28°C and 120 rpm for 7 days. Afterwards, the vials were sealed with a rubber syringe cap allowing ET to collect in the vial for 24 h prior to quantification. To study ET emission rates in brown spot-infected plants, the two youngest fully developed leaves of five-week-

old plants were detached, cut into 3-cm pieces and spray-inoculated with virulent Cm988 (1 x 10^4 conidia.ml^{-1}). Three hours prior to the indicated time points, leaf pieces were transferred to 5-ml glass scintillation vials, which were subsequently sealed with a rubber syringe cap allowing ET to collect in the vial. At the indicated time points, ET production was quantified using either gas chromatography (Thermo Finnigan TRACE GC Ultra; 25m, 0.54 mm, CP-Parabond CP7354) or a more sensitive laser-based ethylene detector (Sensor Sense, ETD-300) in combination with a real time gas flow through system (LPAD) as described by Cristescu et al. (2013). All experiments were repeated at least three times with comparable results.

4.6.6 Quantification of soluble phenolics and enzymatic assays

Total soluble phenolics were extracted according to Zhang et al. (2013) and quantified using the Folin-Ciocalteu's method (Kováčik and Bačkor, 2007). Soluble proteins were extracted by resuspending the crushed tissue (150 mg FW) in 0.8 ml of 0.1 M potassium phosphate buffer (pH 8), containing 2% (w/v) polyvinylpyrrolidone, 0.1% (v/v) Triton X-100, 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF). Crude enzyme extracts were vortexed for 1 min and centrifuged at 10,000 x g for 10 min. PAL and PPO activities were measured exactly as described by Seifi et al. (2013) and Zhang et al. (2013), respectively. Total protein content was determined using the Bradford method (Bradford, 1976).

4.6.7 RNA extraction and quantitative RT-PCR

Total RNA was extracted from frozen leaf tissue using the spectrum plant total RNA kit (Sigma-Aldrich) and subsequently Turbo DNase treated according to the provided protocol (Ambion). First-strand cDNA was synthesized from 2 mg of total RNA using Multiscribe reverse transcriptase (Applied Biosystems) and random primers following the manufacturer's instructions. Quantitative PCR amplifications were conducted in optical 96-well plates with the Mx3005P real-time PCR detection system (Stratagene), using Sybr Green master mix (Fermentas) to monitor dsDNA synthesis. The expression of each gene was assayed in duplicate in a total volume of 25 µl including a passive reference dye (ROX) according to the manufacturer's instructions (Fermentas). The thermal profile used consisted of an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 57°C for 30 s, and 72°C for 30 s. To verify amplification of one specific target cDNA, a melting-curve analysis was included according to the thermal profile suggested by the manufacturer (Stratagene). The amount of plant RNA in each sample was normalized using elongation factor eIFα (LOC_Os03g08020) as internal control and

mock treated samples were selected as calibrator. For analysis of the CmEFE (BK008840) expression in fungal and plant samples using the fungal *ITS* gene as normalizer. The data were analyzed using Stratagene's Mx3005P software. Nucleotide sequences of all primers used are listed in Supplemental Table S5.1.

4.6.8 Identification of EFE homologs in different microorganisms

To reveal putative EFE proteins in different microorganisms the annotated EFE protein sequences of *Pseudomonas cannabina* (AAD16441.1), *P. syringae* pv. *glycinea* (ZP 11566673.1), *P. syringae* pv. *phaseolicola* (BAA02477.1), *Ralstonia solanacearum* (YP 006031629.1) and *Cochliobolus miyabeanus* (BK008840) were blasted against the NCBI (Johnson, 2008) and MycoCosm databases (Grigoriev et al., 2014) with similar outcome. All results with an E-value $\leq$ 1 x 10^{30} were retained. Table 4.2 shows a relevant selection of microorganisms that contain a EFE homolog arranged according to the type of microorganism.

4.6.9 Accession numbers

Accession numbers of the genes used in this study are *OsEBP89* (LOC_Os03g08460), *eIF*α (LOC_Os03g08020), *CmITS* (X78122.1) and *CmEFE* (BK008840).

4.6.10 Acknoledgements

We thank Dr. Marie-Christine Van Labeke for help with the gas chromatography, the people of Shoshi Kikuchi's lab for assistance during the microarray experiment, Dr. Tim De Meyer for supervising analysis of the microarray data and Denys Marchenko and Holger Danner for their much appreciated help during the LPAD.

4.7 Supplemental data

Table S5.1: Primers for expression analysis.

Gene name	Identifier	Forward primer	Reverse primer
CmITS	X78122.1	TCCTCCGCTTATTGATATGC	GGAAGTAAAAGTCGTAACAAGG
CmEFE	BK008840	ATGCCACCTGGTTACAGAGC	CAGAGATAGATCGCGAAGAC
OseIFα	LOC_Os03g08020	TTTCACTCTTGGTGTGAAGCAGAT	GACTTCCTTCACGATTTCATCGTAA
OsEBP89	LOC_Os03g08460	TGACGATCTTGCTGAACTGAA	CAATCCCACAAACTTTACACA

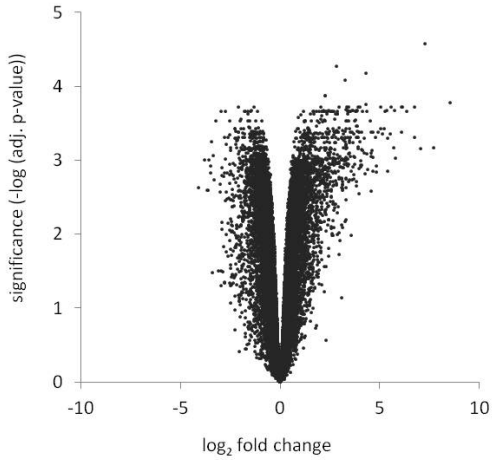


Figure S5.1: Volcano plot of microarray data of brown spot infected leaves vs. mock treated leaves 12h post inoculation (GEO accession GSE55330).

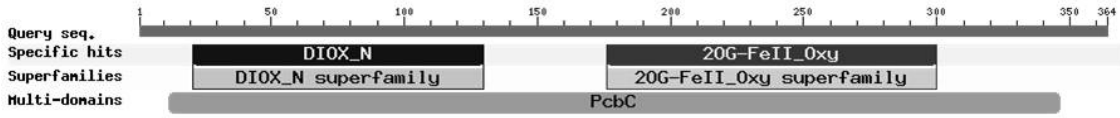
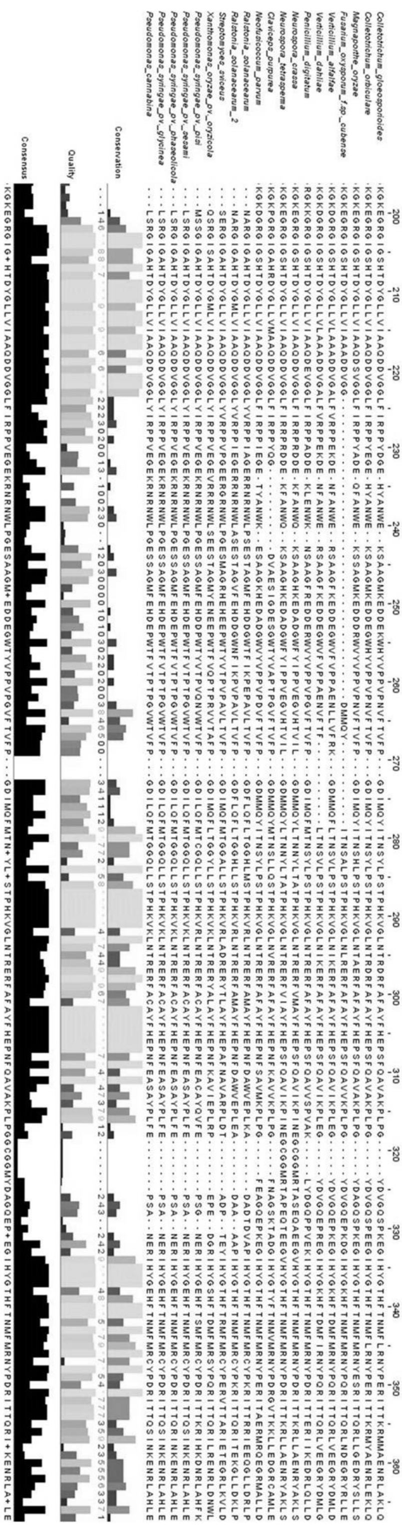


Figure S5.2: Conserved domains in CmEFE protein (Conserved Domain Database; Marchler-Bauer et al., 2011).

Colletotrichum_gloeosporioides
Colletotrichum_orbiculare
Magnaporthe_oryzae
Fusarium_oxysporum_f.sp._cubense
Verticillium_alfalfae
Verticillium_dahliae
Penicillium_digitatum
Neurospora_crassa
Neurospora_tetraspema
Glaviceps_purpurea
Neofusicoccum_parvum
Ralstonia_solanacearum
Ralstonia_solanacearum_2
Streptomyces_sviceus
Xanthomonas_oryzae_pv._oryzicola
Pseudomonas_syringae_pv._pisi
Pseudomonas_syringae_pv._oesani
Pseudomonas_syringae_pv._phaseolicola
Pseudomonas_syringae_pv._glycinea
Pseudomonas_cannabina
Conservation
Quality
Consensus

(a)



(b)

Figure S5.3: Alignment of annotated microbial oxoglutarate-dependent ethylene forming enzymes (EFE). All protein sequences are derived from NCBI (Jalview,Waterhouse et al., 2009).

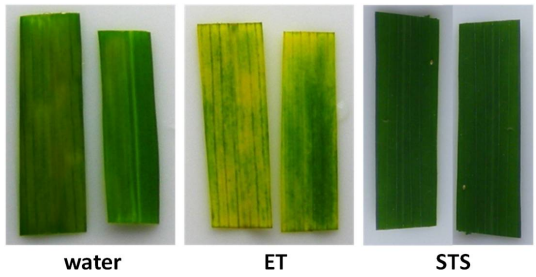


Figure S5.4: Leaf pieces after 7 days of floating in water, Etephon (100μM) or STS (500μM) for 7 days.

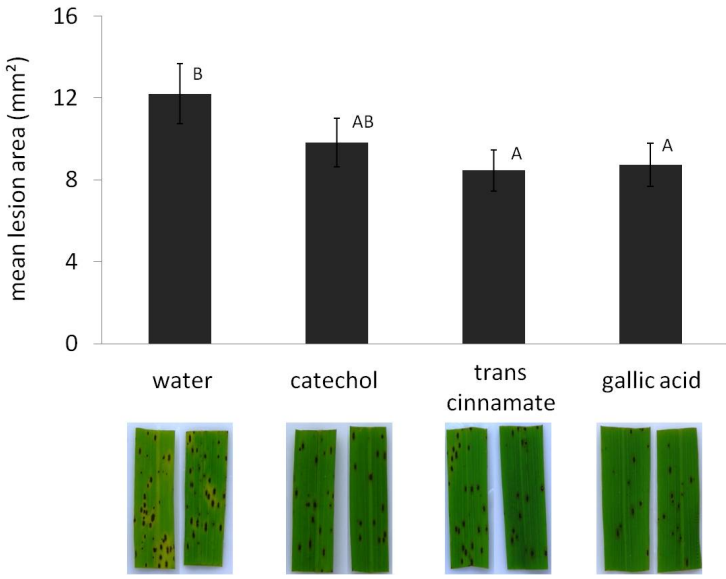


Figure S5.5: Effect of exogenous application of different phenolic compounds on rice leaves 48 h after infection with a spore solution (Cm988, 1 x 10^4 conidia.ml^{-1}) containing water or 1mM of catechol, trans-cinnamate or gallic acid. Data presented are means ± SE of a representative experiment.

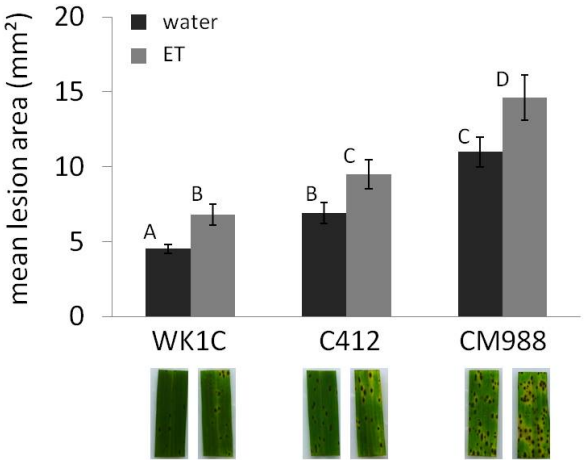


Figure S5.6: Influence of exogenous ET on resistance against *C. miyabeanus* isolates ranging in virulence from least virulent (WK1C) to the most virulent (Cm988). Detached leaves were treated 24h prior inoculation with water and etephon (100μM). The leaf pieces were inoculated with conidial solution (Cm988, 1 x 10^4 conidia.ml^{-1}) (Parametric test, Bonferoni correction, α=0.05). Data presented are means ± SE of a representative experiment.

5

Silicon-induced resistance to the rice brown spot pathogen *Cochliobolus miyabeanus* involves repression of pathogen-induced ethylene action

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Abstract

Over the past decades, various studies have shown the ability of silicon (Si) to mitigate a wide variety of abiotic and biotic stresses. However, despite this relative wealth of knowledge, much remains to be discovered about the mechanistic basis and regulation of Si-afforded stress protection. Aiming to shed further light onto the prophylactic effect of Si, we have investigated the role of hormone defense pathways in governing Si-induced resistance to the rice brown spot pathogen *Cochliobolus miyabeanus*. To delineate the involvement of multiple hormone pathways, we have pursued a multidisciplinary approach combining exogenous hormone applications, pharmacological inhibitor experiments, time-resolved hormone measurements, and bioassays with hormone-deficient and/or -insensitive mutant lines. Contrary to many other types of induced plant resistance, we found that Si-induced brown spot resistance functions independently of the archetypal stress hormones salicylic acid (SA), jasmonic acid (JA) and abscisic acid (ABA). Similarly, our data rule out a major involvement of the developmental hormones gibberellic acid (GA), auxin and cytokinin (CK). In contrast, several lines of evidence suggest that Si steers its positive effect on *C. miyabeanus* resistance through negative crosstalk with the rice ET pathway. Consistent with ET functioning as a virulence factor of *C. miyabeanus*, exogenous ET application increased susceptibility, whereas genetic and pharmacological disruption of ET signaling rendered plants less vulnerable to brown spot, thereby inducing a level of resistance similar to that observed on Si-treated wild-type plants. Moreover, ET emission levels and transcript levels of the ET-responsive marker gene *OsEBP89* were markedly lower following Si application. Moreover, Si failed to further increase the already high levels of resistance observed in ET-insensitive rice lines, suggesting that Si triggers brown spot resistance by preventing the fungus from hijacking the host ET machinery. Interestingly, rather than antagonizing rice ET signaling per se, Si likely interferes either directly or indirectly with production of ET by *C. miyabeanus*. In conclusion, our findings favour a scenario whereby Si induces rice brown spot resistance by disarming fungal ET and argue that impairment of pathogen virulence factors is a core resistance mechanism underpinning Si-induced plant immunity.

5.1 Introduction

To combat infection by microbial pathogens, plants have evolved a sophisticated immune system providing several strategic layers of constitutive and inducible defense mechanisms. Many of these defenses are regulated by a complex network of signal transduction pathways, within which plant hormones play key roles (De Vleesschauwer et al., 2013; Grant et al., 2013). Salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are the archetypal defense hormones and their significance in the hard wiring of the plant immune network is well-established (Rojo et al., 2003; De Vos et al., 2005; Mur et al., 2006). Upon infection, plants produce a highly specific blend of SA, JA, and ET, with the exact combination seemingly depending on the pathogen lifestyle. In the model plant species *Arabidopsis thaliana*, SA is predominantly active against biotrophic pathogens feeding on living tissues, whereas cell death-causing necrotrophic pathogens are usually deterred by JA- and ET-driven defenses. Moreover, interaction between these two pathways is often antagonistic, which has led many authors to suggest that plant immunity follows a binary model with SA and JA/ET having opposing influences (Bari and Jones, 2009).

Although valid for many plant-pathogen interactions, this traditional view is overly generalized, and accumulating findings in both dicot and monocot systems suggest a more complex reality (Pieterse et al., 2012). For instance, in rice *Oryza sativa*, one of the most important food crops worldwide and a model for molecular genetic studies in cereals, disease resistance seems to be controlled by a highly complicated signaling network that does not support a dichotomy between the effectiveness of the SA, JA and ET pathways and the lifestyle of a given pathogen (De Vleesschauwer et al., 2010, 2013; Riemann et al., 2013). Moreover, over the past few years various other hormones including abscisic acid (ABA), gibberellins (GA), auxins and cytokinins (CK) have emerged as important determinants of plant-microbe interactions. Although their significance is less well characterized, evidence is accumulating that these hormones influence disease outcomes at least in part by feeding into the SA-JA/ET backbone of the plant immune system (Asselbergh et al., 2008; De Vleesschauwer et al., 2008; Robert-Seilaniantz et al., 2011). This so-called crosstalk among defense pathways is thought to enable the plant to tailor its inducible defense arsenal to the type of attacker encountered and use its limited resources in a cost-efficient manner (Pieterse et al., 2012).

Recent developments indicate that crosstalk may also allow successful pathogens to manipulate the plant's immune signaling network for their own benefit by shutting down biologically effectual defenses through negative network connections (Robert-Seilaniantz et al., 2011). A classic example is the production by some *Pseudomonas syringae* strains

of a phytotoxin called coronatine that structurally resembles JA derivatives. Actively secreted in the host, coronatine is assumed to hyperactivate JA signaling, thereby counteracting SA- dependent defenses and facilitating bacterial invasion (Brooks et al., 2005; Cui et al., 2005; Melotto et al., 2006). Similarly, we previously demonstrated that the rice brown spot pathogen *Cochliobolus miyabeanus* exploits ET as a virulence factor and co-opts the rice ET signaling route to suppress effective defense pathways (De Vleesschauwer et al., 2008). Moreover, by taking advantage of the different ET biosynthesis pathways in the plant and the pathogen, we were able to show that fungal ET production is the main source of ET in inoculated rice leaves and an important determinant of pathogen virulence (see chapter 4). Given the economic importance of brown spot and the current lack of effective control measures (Savary et al., 2000), disease management strategies interfering with ET-induced susceptibility seem to hold great potential for durable and sustainable brown spot control in the future.

Over the last decade, various studies have shown the ability of silicon nutrition to mitigate a wide variety of abiotic and biotic stresses (see chapter 2.5). Silicon (Si) is a commonly available element in many soils and is absorbed by plant roots in the form of noncharged silicic acid [$Si(OH)_4$]. A specific transporter system transports silicon through the xylem into leaf cells where it is deposited into an insoluble, subcuticular silica layer that is believed to act as a physical barrier hampering pathogen penetration (Ma and Yamaji, 2006). Although important, a fast growing number of papers indicate that this passive role of Si is not solely determinant for the silicon-elicited resistance. Indeed, analyses of different plant species showed that Si can also boost the plant's inducible defense machinery, acting as a biological inducer of a large spectrum of immune responses (Fauteux et al., 2005; Ghareeb et al., 2011; Dallagnol et al., 2013 chapter 2.5).

Nevertheless, despite a multitude of studies demonstrating the prophylactic effects of Si, few reports have focused on understanding the mechanistic basis and regulation of Si-afforded disease control (see Chapter 2.5). Recent progress, however, suggests that Si may modulate stress responses by influencing plant hormone homeostasis. In soybean, for instance, Si treatment reportedly induces synthesis of gibberellic acid, while Si-treated rice accumulates slightly higher levels of gibberellin and JA and lower levels of ET (Fauteux et al., 2006; Hwang et al., 2007; Lee et al., 2010; Ye et al., 2013). Si also strongly interacts with JA in rice defense against insect herbivores (Ye et al., 2013), while recent data implicate Si in regulating wound-induced JA biosynthesis (Kim et al., 2014).

. Although much remains to be discovered, these findings clearly demonstrate the potential of Si to interfere at multiple levels with hormone biosynthesis and signaling pathways.

Aiming to gain novel insights into the molecular mechanisms of Si-afforded disease control, we have investigated the role of hormone signaling pathways in governing Si-induced brown spot resistance. By combining exogenous hormone applications and bioassays with hormone-deficient and/or -insensitive mutant lines, we found that Si triggers resistance to *C. miyabeanus* by preventing the fungus from hijacking the rice ET pathway. Moreover, our findings link this disruption of pathogen-induced host ET signaling to Si-mediated deactivation of fungal ET and argue that impairment of pathogen virulence factors may be a core resistance mechanism underpinning Si-induced plant immunity.

5.2 Results

5.2.1 Exogenously administered ET weakens Si-inducible brown spot resistance

Dallagnol et al. (2013) previously demonstrated the ability of Si to protect rice leaves from infection by *C. miyabeanus*. To test the effectiveness of Si under our experimental conditions, plants were grown in a gnotobiotic hydroponic system and, when five weeks old (7-leaf-stage), spray-inoculated with the *C. miyabeanus* strain Cm988. On control plants, Cm988 was highly virulent, producing typical ellipsoidal light- or dark-brown lesions with a grey sporulating center, surrounded by large zones of chlorotic tissue . These susceptible-type lesions often coalesced within 96 hpi, killing large areas of affected leaves. By contrast, on plants grown in the presence of 2mM Si, fungal development was severely restricted, producing a resistance phenotype characterized by the appearance of isolated, pinpoint-size necrotic spots without excessive chlorosis (Fig. 5.1 A).

In a first attempt to elucidate the hormone signaling network orchestrating Si-mediated resistance to *C. miyabeanus*, we tested the effect of exogenous hormone applications on brown spot development in control and Si-treated rice plants (Fig. 5.1 B). To this end, detached leaf segments were floated in aqueous solutions containing the respective hormones, and 24h later inoculated with a conidial suspension of Cm988. Disease severity was assessed three days post inoculation (dpi) by calculating the mean lesion area using APS assess imaging software. In agreement with previous findings (Ahn et al., 2005; De Vleesschauwer et al., 2010), neither SA (100μM) nor JA (100μM) treatment had a significant impact on the mean lesion area in control plants and both hormones also failed to significantly alter the level of resistance in Si-treated seedlings. Similar results were obtained when pretreating plants with either GA (GA3, 10μM) or indole-acetic acid (IAA, auxin, 100μM), the main auxin in rice, despite these concentrations being high enough to induce hormone marker gene expression (data not shown). Higher and

lower concentrations also failed to trigger increased resistance or susceptibility (data not shown), suggesting that GA and auxin are no major players in either basal or Si-inducible resistance. In contrast, topical application of the ET-releasing growth regulator Ethephon (100μM) significantly increased brown spot severity in both control and Si treatments, whereas supplying plants with 100μM ABA significantly reduced disease severity in control, water-treated plants only.

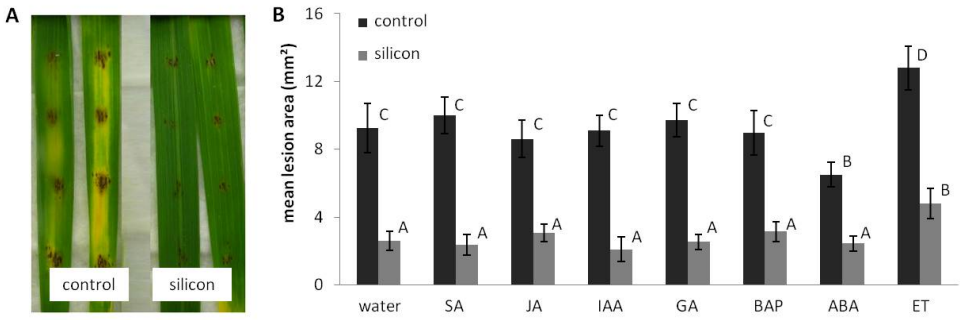


Figure 5.1: A) Si-induced brown spot resistance, 3 days after drop-inoculation (1 x 10^4 conidia.ml^{-1}). B Influence of exogenous hormone application on brown spot resistance in control and Si-treated rice leaf pieces (1 x 10^4 conidia.ml^{-1}). Leaf pieces were treated 24h prior inoculation with salicylic acid (SA, 100μM), jasmonic acid (JA, 100μM), auxin (IAA, 100μM), gibberellic acid (GA_3, 100μM), cytokinin (BAP, 100μM), abscisic acid (ABA, 100μM) and Ethephon (ET, 100μM). Data presented are mean lesion area (mm^2) $\pm$ SE of three independent experiments (Mann-Whitney, n=36, $\alpha = 0.05$). Different letters indicate statistically significant differences.

5.2.2 Si-induced resistance to *C. miyabeanus* is independent of cytokinin signaling

At the macroscopic level, the phenotype of Si-inducible brown spot resistance is best characterized by the almost complete lack of chlorosis surrounding the necrotic disease lesions. Phytohormones are well known to play important roles in regulating senescence and chlorosis with ET, SA, JA and ABA being reported as positive regulators of these processes. In contrast, cytokinin (CK) has long been shown to function in delaying senescence and chlorosis, a role often described as antagonistic to other hormones or signals (Gan and Amasino, 1995; Zwack et al., 2013). To investigate whether CK also contributes to the prevention of pathogen-induced chlorosis in Si-treated plants, we first assessed the impact of Cm988 inoculation on the cytokinin content of control and Si-

treated plants using highly sensitive ultra-performance liquid chromatography electrospray tandem mass spectrometry (UPLC-MS/MS, Novák et al., 2008). The CK-measurement (Table 5.1) showed that in susceptible control leaves the most strongly induced cytokinins were isopentenyl adenine (iP) and the iP-derivatives isopentenyl-adenine-riboside (iPR), isopentenyl-adenine-ribotide (iPRMP) and isopentenyl-adenine-N-glycoside (iPNG). Resistant Si-treated leaves, however, displayed a slightly different CK signature, these plants accumulating comparatively less iP and iP-derivatives but showing an almost twofold increase in the levels of trans-zeatin (tZ).

To test whether this shift in CK balance towards tZ may contribute to Si-induced resistance, detached leaves of control and Si-supplemented plants were treated with iP and tZ (100μM each) and, 24h later, drop-inoculated with Cm988 (Fig. 5.2 A). However, similar to what was observed for the synthetic aromatic CK 6-benzylaminopurine (BAP, 50 μM), neither iP nor tZ were able to interfere with either basal or Si-inducible resistance. The purine derivative Pi-55, a well-characterized inhibitor of CK action that blocks downstream CK signaling by competitive receptor binding (Spíchal et al., 2009), similarly failed to significantly affect disease severity in either control or Si-treated leaves (Fig. 5.2 B). Furthermore, intact plant bioassays revealed that Si was equally effective in reducing brown spot severity on *Gn1a*, a near-isogenic rice line displaying reduced overall CK activity due to over-accumulation of conjugated CK bases (Ashikari et al., 2005), and its parent line Koshihikari (Fig. 5.2 C). When considered together, these data strongly suggest that Si-induced resistance functions independently of CK signaling and, hence, that the alterations in CK content detected in Si-treated plants are not causally involved in the establishment of brown spot resistance.

Table 5.1: Concentration of cytokinin forms (pmol gFW^{-1}) in control and Si-treated rice leaves at different time points after inoculation with *C. miyabeanus*. Data presented are means ± SD from 6 pooled plants.

Control	0h pmol g_{FW}^{-1}		SD	6h pmol g_{FW}^{-1}		SD	12h pmol g_{FW}^{-1}		SD	24h pmol g_{FW}^{-1}		SD	48h pmol g_{FW}^{-1}		SD	72h pmol g_{FW}^{-1}		SD
iP	0.08	±	0.01	0.14	±	0.07	1.13	±	0.83	1.86	±	1.12	4.19	±	0.57	7.34	±	2.63
iPNG	1.29	±	0.09	1.26	±	0.10	2.64	±	1.10	5.47	±	2.67	28.82	±	17.40	42.76	±	23.19
iPR	0.69	±	0.18	2.16	±	0.27	1.46	±	0.65	2.14	±	0.44	4.81	±	0.27	6.17	±	0.53
iPRMP	0.00	±	0.00	0.00	±	0.00	61.44	±	6.57	72.45	±	34.66	94.23	±	9.86	95.14	±	19.36
tZ	11.92	±	1.44	4.28	±	1.41	5.66	±	1.59	4.91	±	1.64	5.93	±	1.84	8.32	±	1.86
tZ9G	7.93	±	1.08	7.10	±	0.23	7.49	±	0.66	8.04	±	1.36	7.82	±	0.48	9.38	±	0.84
tZOG	9.62	±	0.53	9.79	±	2.06	9.08	±	1.01	8.64	±	1.37	8.98	±	1.99	9.18	±	2.37
tZR	0.09	±	0.03	0.07	±	0.01	0.09	±	0.02	0.09	±	0.03	0.09	±	0.01	0.10	±	0.02
tZRMP	0.34	±	0.12	0.38	±	0.10	0.30	±	0.07	0.23	±	0.08	0.25	±	0.06	0.23	±	0.09
cZ	0.21	±	0.08	0.26	±	0.06	0.21	±	0.05	0.17	±	0.05	0.35	±	0.11	0.26	±	0.02
cZNG	0.74	±	0.10	0.84	±	0.08	0.89	±	0.19	1.18	±	0.19	2.21	±	0.50	2.71	±	0.69
cZOG	21.52	±	2.04	23.27	±	2.94	24.27	±	2.01	25.72	±	3.80	23.64	±	3.95	21.84	±	2.42
cZR	1.92	±	0.30	1.34	±	0.21	1.57	±	0.40	1.37	±	0.51	2.45	±	0.30	3.87	±	1.10
DHZ	0.03	±	0.00	0.01	±	0.00	0.02	±	0.00	0.02	±	0.00	0.05	±	0.00	0.05	±	0.00
DHZNG	0.11	±	0.03	0.12	±	0.03	0.13	±	0.02	0.17	±	0.04	0.20	±	0.06	0.24	±	0.04
DHZOG	2.10	±	0.34	2.15	±	0.11	2.06	±	0.18	2.51	±	0.37	2.18	±	0.43	2.71	±	0.05

Silicon	0h pmol g_{FW}^{-1}		SD	6h pmol g_{FW}^{-1}		SD	12h pmol g_{FW}^{-1}		SD	24h pmol g_{FW}^{-1}		SD	48h pmol g_{FW}^{-1}		SD	72h pmol g_{FW}^{-1}		SD
iP	0.06	±	0.02	0.13	±	0.05	0.42	±	0.32	0.41	±	0.25	1.15	±	0.86	3.28	±	1.96
iPNG	2.24	±	0.84	2.13	±	0.24	2.74	±	0.84	3.40	±	1.32	6.62	±	3.67	10.29	±	6.57
iPR	1.39	±	0.19	1.88	±	0.34	1.76	±	0.24	2.41	±	1.12	2.31	±	0.47	3.56	±	0.54
iPRMP	0.00	±	0.00	0.00	±	0.00	11.12	±	1.39	31.15	±	0.89	19.31	±	1.25	33.95	±	27.15
tZ	13.41	±	3.07	8.46	±	2.30	11.85	±	2.98	12.39	±	1.52	17.74	±	2.50	17.92	±	1.64
tZ9G	11.48	±	1.15	11.08	±	0.53	9.87	±	0.87	11.55	±	0.64	10.84	±	0.51	9.34	±	0.77
tZOG	11.08	±	2.08	10.22	±	1.68	9.96	±	1.81	9.10	±	1.65	9.94	±	1.45	9.13	±	1.55
tZR	0.08	±	0.02	0.06	±	0.01	0.09	±	0.03	0.11	±	0.04	0.19	±	0.03	0.21	±	0.02
tZRMP	0.23	±	0.05	0.18	±	0.01	0.18	±	0.04	0.44	±	0.12	0.66	±	0.10	0.55	±	0.05
cZ	0.18	±	0.05	0.30	±	0.09	0.28	±	0.05	0.27	±	0.07	0.40	±	0.14	0.55	±	0.07
cZNG	1.32	±	0.09	1.16	±	0.09	1.25	±	0.12	1.34	±	0.07	1.75	±	0.30	1.78	±	0.47
cZOG	21.08	±	1.12	21.74	±	1.22	21.83	±	2.12	23.65	±	2.91	24.88	±	4.24	24.20	±	2.74
cZR	1.40	±	0.23	1.57	±	0.46	2.15	±	0.59	2.32	±	0.62	1.56	±	0.36	2.15	±	0.77
DHZ	0.02	±	0.00	0.03	±	0.01	0.03	±	0.00	0.04	±	0.00	0.03	±	0.01	0.03	±	0.00
DHZNG	0.21	±	0.04	0.14	±	0.03	0.16	±	0.02	0.15	±	0.03	0.16	±	0.04	0.17	±	0.06
DHZOG	2.70	±	0.40	2.68	±	0.11	2.53	±	0.14	2.71	±	0.27	2.62	±	0.30	1.97	±	0.14

cZ, cis-zeatin, cZNG, cis-zeatin-N-glycoside, cZOG, cis-zeatin-O-glycoside, cZR, cis-zeatin-O-glycoside, DHZ, dihydro-zeatin, DHZNG, dihydro-zeatin-N-glycoside, DHZOG, dihydro-zeatin-O-glycoside, iP, isopentenyl-adenine, iPR, isopentenyl-adenine-riboside, iPRMP, isopentenyl-adenine-ribotide, tZ, trans-zeatin, tZNG, trans-zeatin-N-glycoside, tZOG, trans-zeatin-O-glycoside, tZR, trans-zeatin-riboside, tZRMP, trans-zeatin-ribotide

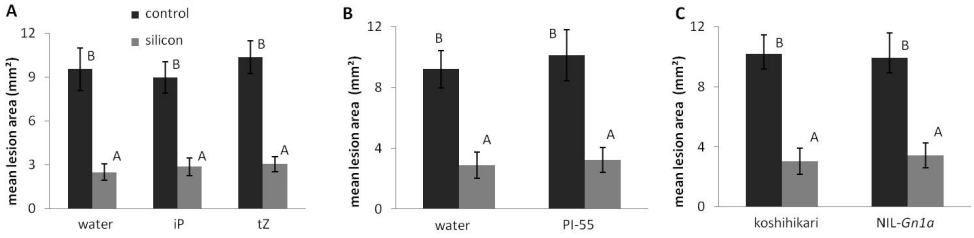


Figure 5.2: Influence of A) exogenous cytokinins and C) CK-signaling blocker, PI-55 on brown spot resistance in control and Si-treated rice leaf pieces. Leaf pieces were treated 24h prior inoculation with water, isopentenyl adenine (iP, 100μM) and trans-zeatin (tZ, 100μM). Data presented are mean lesion area (mm^2) $\pm$ SE of four independent experiments (ANOVA, n=12, $\alpha = 0.05$). D) Influence of Si on brown spot resistance in *Gn1a*, a near-isogenic rice line (parent line: *japonica* cv Koshihikari) that displays a reduced overall CK activity. Data presented are mean lesion area mm^2) $\pm$ SE of three independent experiments (ANOVA, n=6, $\alpha = 0.05$).

5.2.3 ET but not SA, JA or ABA is a key player in Si-induced brown spot resistance

Given the well-documented role of SA, JA, ET and ABA in orchestrating plant immune responses, additional bioassays were performed with a number of transgenic and mutant rice lines that are either deficient in or insensitive to these hormones. All lines and the respective wild-types were routinely treated with 2 mM Si and, when five weeks old, inoculated with virulent Cm988. Rice lines that are either deficient in SA (*NahG*) (Fig. 5.3 A) or JA (*hebiba*) (Fig. 5.3 C) or show an altered expression of the SA master regulator, *OsNPR1* (*OsNPR1* ox and *OsNPR1* RNAi, Fig. 5.3 B), all retained the high level of Si-induced resistance observed in the respective wild-types, indicating that neither *de novo* synthesis of SA and JA nor SA signaling is an essential prerequisite for Si-induced resistance to *C. miyabeanus*. Similar findings were obtained when quantifying the level of basal and Si-inducible resistance in plants silenced for the ABA-inducible MAP kinase OsMPK6. One of the better studied MAP kinases in rice, OsMPK6 (previously referred to as OsMPK5) functions as a positive regulator of ABA signaling. Accordingly, *OsMPK6* RNAi plants are partially ABA-insensitive and display reduced expression of ABA-responsive genes (Bailey et al., 2009). However, as shown in Figure 5.3 D, we were unable to detect any reproducible or significant differences in overall disease severity between wild-type and *OsMPK6* RNAi plants, whether grown in the presence or absence of Si. Therefore, even though exogenous ABA confers brown spot resistance, ABA signaling does not appear to be essential for either basal or Si-inducible brown spot

resistance.

Different results, however, were obtained when testing the effectiveness of Si in plants silenced for the central ET signal transducer Ethylene Insensitive 2a (*OsEIN2a*). Consistent with previous results (De Vleesschauwer et al., 2010) and corroborating the disease-promoting effect of exogenously administered Ethephon, ET-insensitive *OsEIN2a* antisense plants were significantly more resistant towards brown spot than wild type plants, confirming the negative role of ET in basal resistance to *C. miyabeanus* (Fig. 5.3 E). Moreover, while the level of resistance of non-treated *OsEIN2a* antisense plants mirrored that of Si-treated wild-type plants, application of Si failed to further increase resistance in the *OsEIN2a* background. In conjunction with the results from the exogenous hormone applications, these results suggest that Si-inducible brown spot resistance is not reliant on SA, JA, ABA, GA, IAA or CK signaling, but rather involves suppression of pathogen-triggered ET action.

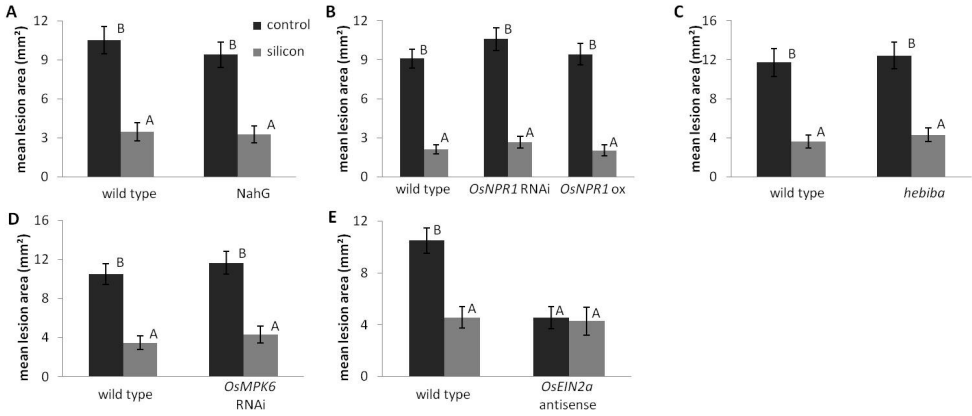


Figure 5.3: Influence of Si on brown spot resistance in different transgenic and mutant rice lines. A) SA-deficient *NahG* transgenic rice line (wild type: *japonica* cv Nipponbare) B) *NH1* ox and *NH1* RNAi rice lines (wild type: *japonica* cv Taipei 309) C) JA-deficient mutant *hebiba* (wild type: *japonica* cv Nihonmasari) D) ABA-insensitive MPK6 RNAI rice line (wild type: *japonica* cv Nipponbare) E) ET-insensitive *EIN2a* antisense rice line (wild type: *japonica* cv Dongjin). Data presented are mean lesion area three days after inoculation (mm^2) ± SE of three independent experiments (ANOVA, n=12, $\alpha = 0.05$). Different letters indicate statistically significant differences.

5.2.4 Si prevents *C. miyabeanus* from hijacking the rice ET pathway

To address the hypothesis that Si-induced resistance is associated with suppression of the plant ET pathway, we first monitored ET emission rates in control and Si-treated plants at various times after brown spot inoculation. Consistent with its role as a fungal virulence factor, infection with *C. miyabeanus* isolate Cm988 caused a strong and continuous increase in ET production in control leaves, whereas Si-treated plants emitted significantly less ET in response to pathogen attack (Fig. 5.4 A). Quantitative reverse-transcription PCR (qRT-PCR) analysis revealed a similar trend for the ET-responsive transcription factor gene *OsEBP89*, suggesting that Si antagonizes not only pathogen-induced ET synthesis but also alleviates downstream ET signaling (Fig. 5.4 B). Consistent with this hypothesis, feeding detached control leaves with the ET signaling inhibitor silver thiosulfate (STS) induced the same level of brown spot resistance as observed upon Si treatment, resulting in the development of small, pinpoint size lesions with very limited development of chlorosis (Fig. 5.4 C and D). Moreover, STS failed to exert an additive effect in Si-treated plants, further supporting our hypothesis. On the other hand, exogenous ET-application (100μM Ethephon) significantly increased disease severity in both control and Si-treated rice leaves (Fig. 5.4 D), whereas application of the plant ET biosynthesis blockers aminooxyacetic acid (AOA) and cobalt chloride ($CoCl_2$, 500μM each) failed to significantly affect disease development, irrespective of Si treatment. Together, these findings infer i) that ET action, rather than *de novo* plant ET biosynthesis, is an important factor contributing to *C. miyabeanus* pathogenicity, and ii) that Si prevents the fungus from co-opting the rice ET pathway as a decoy strategy to suppress other more effectual immune responses.

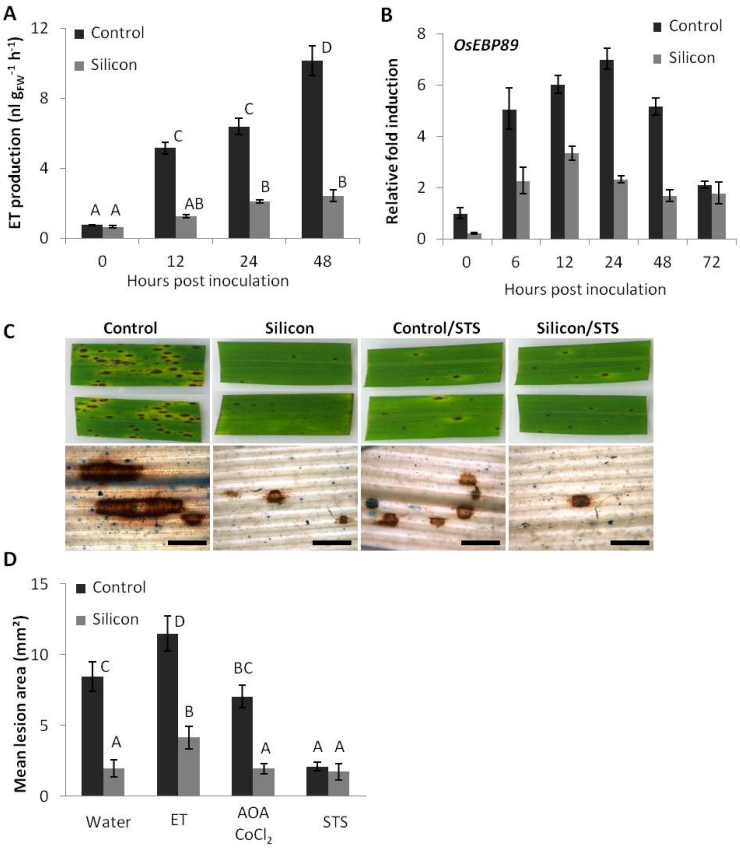


Figure 5.4: A) Ethylene production in infected rice leaves at different time points after infection (1 x 10^4 conidia.ml^{-1}). Data presented are mean values ± SE of three independent experiments (ANOVA, n=6, $\alpha = 0.05$). Different letters indicate statistically significant differences. B) Relative expression of the ET-responsive *EBP89* gene in control and Si-treated rice leaves at various time points after infection (1 x 10^4 conidia.ml^{-1}). C) Influence of blocking ET signaling with STS (1mM) and Si-treatment on brown spot incidence on rice leaves three days post inoculation (1 x 10^4 conidia.ml^{-1}). The lower part show magnification of brown spot lesions on the same leaves cleared in boiling ethanol under the light microscope, scale bars are 500 μM. D) Influence of ET (100μM), ET biosynthesis blockers AOA and $CoCl_2$ (500μM each) and ET signaling blocker STS (1mM) on rice leaves infected with *C. miyabeanus* (1 x 10^4 conidia.ml^{-1}). Data presented are mean lesion area (mm^2) ± SE of at least three independent experiments (ANOVA, n $\leq$ 36, $\alpha = 0.05$). Different letters indicate statistically significant differences.

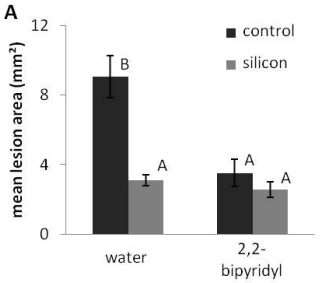
5.2.5 Blocking fungal ET production mimics Si-inducible brown spot resistance

Previously, we demonstrated that *C. miyabeanus* strain Cm988 is equipped with an Ethylene Forming Enzyme (EFE) and can produce copious amounts of ET *in vitro* when grown on medium containing plant extracts and the EFE substrates 2-oxoglutarate and arginine (Van Bockhaven et al. unpublished). Moreover, pharmacological experiments revealed that fungal ET is the prevalent source of ET in brown spot infected plants (Van Bockhaven et al. unpublished). In view of these findings and given the observations that infected leaves emitted significantly less ET following Si treatment (Fig. 5.4 A) while blocking plant ET synthesis did not compromise Si-induced resistance, we asked whether application of Si might interfere with fungal ET production. To this end, detached leaves were fed with the fungal ET biosynthesis blocker 2,2-bipyridyl and subsequently inoculated with virulent Cm988. Interestingly, similar to what we observed for the plant ET signaling inhibitor STS, blocking fungal ET production with 2,2-bipyridyl mimicked the resistance-inducing effect of Si application, while co-application of both compounds had no additive effect on the level of brown spot resistance (Fig. 5.5 A).

Two different, not mutually exclusive, scenarios can be hypothesized to explain these findings with Si either alleviating fungal ET production *per se* or, alternatively, mitigating the effect of fungal ET by rendering inoculated plants ET-insensitive. In this context, it is noteworthy that Cm988 only produces ET in response to plant extracts, which suggests that *C. miyabeanus* must reprogram the plant's metabolism to ensure sufficient precursors for fungal ET production (Van Bockhaven et al. unpublished). Taking into account that oxoglutarate is indispensable for EFE-mediated microbial ET production (Fukuda et al., 1992; Chagué et al., 2006) and that rice leaves are low in oxoglutarate (Yuan et al., 2007b), we hypothesized that *C. miyabeanus* may induce plant oxoglutarate accumulation to initiate ET synthesis. In support of this assumption, transcriptome analysis of Si- and control-treated plants responding to Cm988 inoculation (GEO accession GSE55330) showed that in control plants, *C. miyabeanus* significantly up-regulated the expression of the oxoglutarate-forming glutamate dehydrogenases *OsGDH2* and *OsGDH3* (co,I vs. co,m) (Dubois et al., 2003; Miyashita and Good, 2008) (Fig. 5.5 B). Moreover, although brown spot infection also induced *OsGDH2* and *OsGDH3* transcript accumulation in Si-treated plants (si,I vs. si,m), a clear repressive effect of Si application on expression of *OsGDH1*, *OsGDH2* and *OsGDH3* was evident in both uninfected and infected leaves (si,m vs. co,m and si,I vs. co,I). In view of these findings, it is tempting to speculate that downregulation of GDH expression in Si-treated rice leaves alleviates fungal ET

production by *C. miyabeanus* through lowering the plant oxoglutarate pool.

A



B

Gene name	Identifier	si,m vs. co,m	p-value	si,l vs. co,l	p-value	co,l vs. co,m	p-value	si,l vs. si,m	p-value
GDH1	LOC_Os03g58040	-2.10	0.000	-1.64	0.000	-	0.281	-	0.012
GDH2	LOC_Os04g45970	-3.28	0.000	-3.15	0.000	1.01	0.009	1.14	0.007
GDH3	LOC_Os02g43470	-2.43	0.003	-3.68	0.000	3.11	0.002	1.86	0.008
GDH4	LOC_Os01g37760	0.72	0.003	0.43	0.229	-	0.408	-0.55	0.000

Figure 5.5: A) Mean lesion area on leaves infected with solution (1 x 10^4 conidia.ml^{-1}) containing water or 2,2-bipyridyl (2mM) 3 days after spray-inoculation. Data presented are mean lesion area (mm^2) ± SE of three independent experiments (Mann-Whitney, n=36, $\alpha = 0.05$). Different letters indicate statistically significant differences. B) Microarray data in a 2x2 factorial design: control (co) and Si-treated (si), mock-treated (m) and infected (I) 12h after infection showing differential expression of *glutamate dehydrogenases* (*GDH*) (GEO accession GSE55330).

5.2.6 Si-induced resistance against *C. miyabeanus* is based on restriction of fungal progression in the mesophyll and involves priming for enhanced deposition of phenolic compounds

Over the past few years, Si has been shown to modulate a wide variety of physical and biochemical immune responses, including expression of pathogenesis-related proteins, production of phytoalexins, and formation of pathogen-triggered callose deposits and cell wall fortifications (see Chapter 2.5). To further study how Si counteracts brown spot infection, we studied fungal development and plant defense responses in control and Si-treated leaf sheaths at various times after inoculation with virulent Cm988. Contrary to leaf blades, leaf sheath tissue is relatively flat and optically clear, which facilitates live cell imaging. Quantitative recording of attempted brown spot infections revealed no significant differences in the number of unsuccessful penetration events, indicating that Si does not impede pre-penetration development of *C. miyabeanus* (data not shown). Moreover, rather than showing a rapid epidermis-based resistance reaction, Si-treated plants were characterized by severe restriction of fungal spreading in the mesophyll, similar to what we previously observed for ABA-inducible brown spot resistance (De Vleesschauwer et al., 2010). Starting 12 hpi, Si-treated plants also displayed strong accumulation of phenolic compounds in the anticlinal walls of both penetrated and sur-

rounding cells as indicated by strong blue light-induced autofluorescence (Fig. 5.6). Moreover, consistent with Si suppressing pathogen-triggered ET action, applying the ET signaling blocker STS phenocopied Si-inducible brown spot resistance with a similar restriction of mesophyll spreading and increased accumulation of phenolic compounds near the site of infection. Given the importance of phenolic compounds in basal defense of rice to *C. miyabeanus* (Vidhyasekaran et al., 1992; Shabana et al., 2008), it is not inconceivable that Si-mediated accumulation of phenolic compounds hampers hyphal growth, preventing further pathogen ingress and curtailing symptom development.

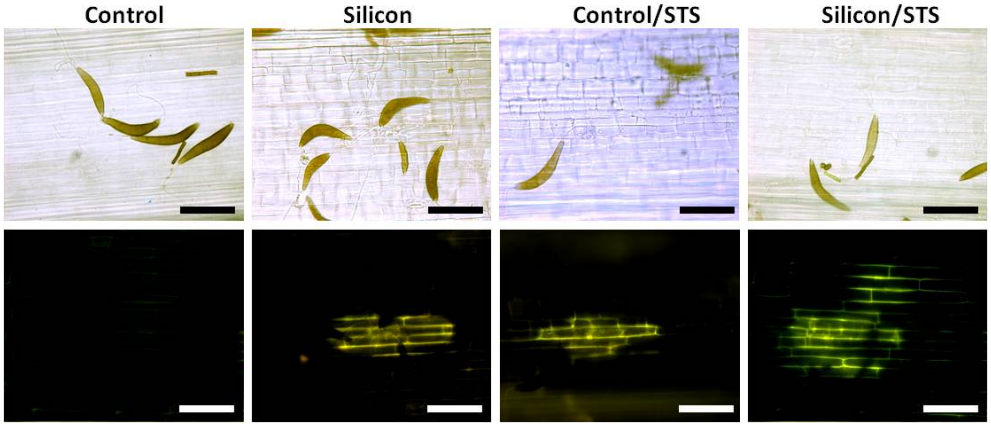


Figure 5.6: Microscopic analysis of the influence of the ET signaling blocker STS (1mM) on brown spot infection in rice sheaths of control or Si-treated plants. Microscopic slides of pretreated rice sheaths were cut 24h after brown spot infection (1 x 10^4 conidia.ml^{-1}). Photographs in the upper section show microscopic slides under normal light. The photographs in the lower section show accumulation of phenolic compounds around the site of infection, visualized as yellow autofluorescence under blue light excitation. Scale bar is 50μM.

5.3 Discussion

To date, dozens of reports have documented the ability of Si to alleviate biotic and abiotic stress. Despite this relative wealth of knowledge, the exact mechanisms by which Si exerts its beneficial effects are still poorly understood, a situation that hinders the widespread application of Si for agricultural purposes. In an attempt to unravel the molecular underpinnings of Si-afforded disease control, we investigated the hormone signaling circuitry governing Si-mediated resistance against the rice brown spot fungus *Cochliobolus miyabeanus*.

Using a combination of exogenous hormone treatments, pharmacological inhibitor experi-

ments and bioassays with hormone-deficient and -insensitive rice lines, we found that Si-induced resistance to *C. miyabeanus* functions independently of either GA, ABA, auxin and CK signaling, and also does not rely upon boosted expression of SA- and JA-dependent defenses. By contrast, several lines of evidence indicate that Si-induced brown spot resistance is based on antagonistic crosstalk with the ET signaling pathway. First, in accordance with previous results (De Vleesschauwer et al., 2010), exogenous Ethephon application rendered plants hypersusceptible to brown spot infection, confirming the negative impact of ET on basal rice immunity to *C. miyabeanus*. Second, inhibition of ET signaling, either by antisense suppression of *OsEIN2a*, a central signal transducer in the rice ET pathway (Jun et al., 2004) or by infiltration of the ET antagonist STS, induced levels of resistance similar to those found in Si-supplemented plants. Moreover, Si treatment of the *OsEIN2a* transgenics or co-application of Si and STS had no additive effect on brown spot resistance, suggesting that Si specifically targets the ET signaling pathway to condition resistance. Third, ET emission levels and transcript levels of the ET-responsive marker gene *OsEBP89* were markedly lower in Si-treated plants than in controls upon *C. miyabeanus* attack. Taken together, these results highlight the importance of ET homeostasis in determining rice-brown spot interactions and favour a model whereby Si protects rice from *C. miyabeanus* attack at least in part by antagonizing pathogen-induced ET signaling.

5.3.1 Priming of plant hormone pathways is an important facet of Si-triggered immunity

In keeping with our findings, plant hormones are well known to play pivotal roles in orchestrating different types of induced resistance, including pathogen-triggered systemic acquired resistance (SAR). One of the most archetypal induced resistance responses, SAR requires endogenous accumulation of SA and is tightly associated with the transcriptional reprogramming of a battery of SA-inducible genes, including those encoding pathogenesis-related proteins (Durrant and Dong, 2004). In addition, there is also ample evidence for induced disease resistance conditioned by signal molecules other than SA, as illustrated by induced systemic resistance (ISR). Opposite to SAR, ISR is a type of inducible resistance that is triggered upon colonization by beneficial rhizobacteria or plant growth-promoting fungi and classically involves JA- and ET-regulated defenses. Recent evidence, however, also point to a role of GA, auxin and ABA signaling in ISR (De Vleesschauwer and Höfte, 2009; Petti et al., 2012; Chowdappa et al., 2013; Vos et al., 2013), while still other variations are indicated by the ability of some ISR-eliciting strains to trigger SA-dependent resistance mechanisms (De Meyer et al., 1999; Domenech et al., 2006;

De Vleesschauwer and Höfte, 2009). Therefore, it seems there is not one definitive pathway for induced resistance, but that various hormone-dependent signaling conduits may govern the resistance phenotype depending on the inducing agent and plant-attacker combination.

Interestingly, evidence is accumulating that Si-treated plants display a similar flexibility in the hormone circuitry that is deployed to counteract pathogen invasion. For instance, whereas our results clearly indicate that Si-inducible brown spot resistance functions independently of either SA, JA and GA, other studies demonstrated increased levels of these hormones in Si-treated plants responding to pathogen attack or herbivory (Fauteux et al., 2006; Hwang et al., 2007; Lee et al., 2010; Ye et al., 2013). Similarly, a recent study revealed that Si primes JA-induced tolerance to herbivory (Ye et al., 2013), while earlier findings linked Si to activation of SA and JA-mediated defenses in various plant species (Fauteux et al., 2006). A similar scenario is evident for the ET pathway with Si boosting the expression of ET-responsive transcripts upon infection of rice with the hemibiotrophic blast pathogen *Magnaporthe oryzae* (Brunings et al., 2009), but repressing ET biosynthesis and signaling following attack by *C. miyabeanus*.

These apparently conflicting findings support the view that Si does not impose continuous changes in hormone homeostasis, but rather sensitizes or primes hormone biosynthesis and signaling processes, thereby creating a flexible signaling network that enables plants to fine-tune its immune response to the type of invader encountered. In compliance with this concept, there is ample evidence demonstrating the ability of Si to boost a wide variety of pathogen-specific immune responses (see Chapter 2.5). For example, in addition to the enhanced deposition of phenolic compounds observed in this study (Fig. 5.6), Si treatment was previously shown to restrict pathogen proliferation by accelerating and/or intensifying distinct defense responses such as papillae formation, cell wall fortification, phytoalexin production and expression of pathogenesis-related proteins (Kauss et al., 2003; Rodrigues et al., 2005; Rémus-Borel et al., 2009; Dallagnol et al., 2011b; Shetty et al., 2011). By analogy with a similar phenomenon in metazoan systems, this enhanced capacity to mobilize cellular defenses is often referred to as 'priming' (Conrath, 2011).

Because priming initiates a state of readiness that does not confer resistance *per se* but rather allows for accelerated induced resistance once an attack occurs, one presumed benefit is that it entails less fitness costs than direct induction of defense (van Hulten et al., 2006). Moreover, priming is thought to confer flexibility to adapt the defense response to a specific challenge, leading to a less costly and broader spectrum of resistance (Van der Ent et al., 2008; Conrath, 2011). As such, priming may not only explain the broad-spectrum character of Si-afforded stress alleviation, but also could offer a mechanistic

framework for how Si is able to trigger resistance against a multitude of stresses without inducing resistance trade-offs and/or growth and yield penalties. Although the molecular aspects of priming are still poorly understood, the induction of priming is increasingly associated with accumulation of dormant signaling components such as MAP kinases and transcription factors as well as chromatin modifications and alterations of primary metabolism (Conrath, 2011). Assessing whether similar mechanisms are operative in Si-mediated defense priming is a major challenge ahead.

5.3.2 Si antagonizes *C. miyabeanus*-produced ET

The predicted role of ET repression in Si-inducible brown spot resistance echoes previous findings in rice where exogenously administered ABA was shown to enhance basal resistance to *C. miyabeanus* through negative crosstalk with the rice ET pathway (De Vleesschauwer et al., 2010). One important player orchestrating this ABA-ET antagonism is the MAP kinase OsMPK6 (previously known as OsMPK5). Compared with wild-type plants, *OsMPK6* RNAi lines exhibit higher levels of ET and a lower level of ABA (Bailey et al., 2009). Furthermore, OsMPK6 directly interacts with and phosphorylates OsEIL1, an ortholog of Arabidopsis EIN3, implicating OsMPK6 in regulation of both ABA and ET synthesis and their signal transduction (Bailey et al., 2009). However, contrary to ABA-inducible brown spot resistance, Si treatment proved to be equally effective in wild-type and OsMPK6 RNAi plants, ruling out a major role of OsMPK6 in Si-mediated inhibition of pathogen-triggered ET action. Hence, despite both targeting the ET signaling conduit, ABA and Si seem to employ different strategies to counteract pathogen-induced ET signaling. The observation that blocking fungal ET production with the inhibitor 2,2-bipyridyl mimicked the resistance-inducing effect of Si suggested that other than repressing plant ET signaling, Si might also induce brown spot resistance by interfering with microbial ET. One possible mechanism hitherto would be for Si to render plants insensitive to pathogen-produced ET. However attractive, such a concept is hard to reconcile with the ability of exogenously administered Ethephon to alleviate Si-induced brown spot resistance. Therefore, rather than mitigating the effect of fungal ET, Si likely interferes either directly or indirectly with the biosynthesis of microbial ET. Like other fungi, *C. miyabeanus* produces ET from the TCA cycle intermediate 2-oxoglutarate in a reaction catalyzed by a single multifunction Ethylene Forming Enzyme (EFE) (Fukuda et al., 1992; Chagué et al., 2006; Van Bockhaven et al. unpublished). Particularly noteworthy in this respect is the up-regulation of several glutamate dehydrogenases (*GDH*) in control plants responding to brown spot infection. GDHs are a small protein family controlling the equilibrium between glutamate and 2-oxoglutarate in the mitochondria.

Although the enzyme can work in both directions, it is believed that during stress conditions GDH mainly converts glutamate into 2-oxoglutarate (Masclaux-Daubresse et al., 2005; Pageau et al., 2006; Qiu et al., 2009; Masclaux-Daubresse et al., 2010). Considering that *C. miyabeanus* only produces ET in response to plant extracts (Van Bockhaven et al. unpublished) and that rice plants are naturally low in oxoglutarate (Müller et al., 2001; Yuan et al., 2007b), we hypothesize that *C. miyabeanus* induces host *GDH* expression to increase the plant oxoglutarate pool and activate its own ET synthesis. In this scenario, down-regulation of *GDH* in Si-treated rice would prevent oxoglutarate accumulation, consequently lowering ET production by *C. miyabeanus* and attenuating ET-mediated induction of host susceptibility.

5.4 Conclusions

In summary, our results support a model whereby Si induces resistance to the rice brown spot pathogen *C. miyabeanus* at least in part by preventing the fungus from hijacking the rice ET pathway. Moreover, our results suggest that in addition to interfering with the synthesis and/or action of fungal ET, Si also primes naive rice leaves for enhanced accumulation of phenolic compounds at the site of fungal penetration. Together these results offer novel insights into the signaling circuitry governing Si-afforded disease control and argue that impairment of pathogen virulence factors might be a core mechanism accounting for the broad-spectrum disease resistance in Si-treated plants. Further elucidating how Si exerts its prophylactic effects will not only advance our fundamental understanding of how plants cope with their enemies in the context of induced resistance, but also may create new avenues for developing crops that are better able to withstand multiple attackers.

5.5 Material and methods

5.5.1 Plant material and growth conditions

All rice plants (*Oryza sativa* L.) were *japonica* cultivar Nipponbare and the corresponding SA-deficient *NahG* and ABA-insensitive *OsMPK6* RNAi rice lines (Xiong and Yang, 2003; Yang et al., 2004), *japonica* cultivar Taipei and *OsNPR1* ox and *OsNPR1* RNAi rice lines (Yuan et al., 2007c). The JA-deficient mutant hebiba (Riemann et al., 2003) and ET-insensitive *OsEIN2a* antisense transgenic line 471 (Jun et al., 2004) are in the background of *japonica* cultivars Nihonmasari and Dongjin, respectively. The rice seeds were surface sterilized with 70% ethanol for 1 min and 1% sodium hypochlorite solution for 10min, rinsed three times with sterile distilled water and germinated at 28°C for five days on wet

sterile filter paper in Petri dishes sealed with parafilm ($\geq$ 92% relative humidity). The seedlings were transplanted on vermiculite in half-strength modified Hoagland solution (Hewitt and Smith, 1975). Five days later the plantlets are transferred to a hydroponic system in full strength modified Hoagland solution (pH 6.5). The Hoagland solution was replaced every 7 days. The rice plants were grown in a growth chamber (28°C, 12h/12h light regime) for five weeks until they reached the 7-leaf stage.

5.5.2 Pathogen inoculation and disease rating

Cochliobolus miyabeanus isolate Cm988, kindly provided by International Rice Research Institute, was grown on potato dextrose agar (PDA, Difco) at 28°C in darkness. After a week the mycelium was put under 12h/12h blue light (Philips TLD 18W/08 and Philips TLD 18W/33) until sporulation. Conidia were harvested as described by Thuan et al. (2006) and suspended in a 0.5% gelatine (type B from bovine skin; Sigma-Aldrich G-6650) in a concentration of 10^4 conidia.ml^{-1}. Plants in the 7-leaf stage (5 weeks old) were inoculated by spraying the conidial suspension on the leaves till run off (1ml per plant) using a compressor-powered airbrush gun. The plants were kept in a humid and warm infection chamber (28°C $\pm$ 4°C, 92% or greater relative humidity) to promote fungal penetration. After 18h the plants were transplaced to greenhouse conditions (28°C $\pm$ 4°C, 16-h-light/8-h-dark regime) for disease development. The symptoms were scored three days after inoculation using APS ASSESS 2.0 software (APS, St Paul, Minnesota, USA). All infection trials were repeated at least three times with similar results.

5.5.3 Pharmacological Experiments

The ethylene (ET) releasing Etephon (2-chloroethylphosphonic acid), ET precursor ACC (1-amino cyclopropane-1-carboxylic acid), the ET biosynthesis inhibitor AOA (aminooxy-acetic acid) and $CoCl_2$, SA (salicylic acid), JA (jasmonic acid), ABA (abscisic acid), tZ (trans-zeatin), iP (isopentenyladenine) BAP (6-benzylaminopurine) and GA_3 (gibberellic acid 3) were purchased from Sigma-Aldrich, Duchefa or VWR. Silver thiosulfate (STS), an inhibitor of ET signaling, was prepared by mixing solutions of 0.1 M sodium thiosulfate with 0.1 M silver nitrate in a 4:1 ratio (Zhao et al., 2002; Shoresh et al., 2005). The CK-signaling blocker PI-55 was kindly provided by Dr. Lukas Spichal from Palacky University in the Czech Republic (Nisler et al., 2010). All chemicals were dissolved in water, unless indicated differently, at the indicated concentrations and were applied by cutting the 2 youngest fully developed leaves in 7 cm segments and putting them in the chemical solutions 24h prior to inoculation. Afterwards, the leaf segments were placed in square Petri dishes (Greiner Bio-One, 688102) lined with moist paper towels, sprayed with *C.*

miyabeanus conidial suspension or drop-inoculated with six μl droplets of suspension (1 x 10^4 conidia.ml^{-1} in 0.25% gelatine). After 24 h, the droplets were removed with a laboratory tissue, and resistance was quantified by measuring lesion area at 72 hpi using APS assess 2.0 (APS, St Paul, Minnesota, USA). Blocking fungal ET biosynthesis was done by adding 1 or 2 mM 2,2-bipyridyl (Hottiger and Boller, 1991) (Sigma Aldrich) to the spore solution before inoculation prior inoculation.

5.5.4 Microscopic analysis

Leaf sheaths of 5 week-old rice plants were peeled off until only the roots and sheath of the youngest fully developed leaf remained. This sheath was placed in a tray lined with wet paper towels with the adaxial side of the inner sheath epidermis facing upwards and filled with a conidial suspension of *C. miyabeanus* (1 x 10^4 conidia.ml^{-1}, 1ml per sheath). The tray was covered with transparent plastic and placed under greenhouse conditions (28°C ± 4°C, 16-h-light/8-h-dark regime). Samples for microscopy were taken at 0, 24, 48 and 72 hours post inoculation and at least six trimmed sheath sections originating from three plants were sampled per time point. The microscopic slides were cut according to Koga et al. (2004). Phenolic compounds were visualized as autofluorescence under blue light epifluorescence (Olympus U-MWB2 GPF filter set; excitation, 450-480 nm; dichroic beam splitter, 500 nm; barrier filter BA515). Images were acquired digitally (Olympus Colorview II camera) and further processed with the Olympus analySIS cellF software.

5.5.5 Quantification of ET accumulation

The production of ET by *C. miyabeanus in vitro* was measured on mycelium grown on liquid and solid Czapek medium with minor adjustments (Kubo et al., 1989) containing 40 mM sucrose, 5.6 mM KH_2PO_4, 0.14 mM KH_2PO_4, 2 mM $MgSO_4$, 0.04 mM $FeSO_4$, 6.7 mM KCl, 5 mM nitrogen and a sterile rice extract at a pH 5.6 inoculated with 1 x 10^4 conidia.ml^{-1} or with 1ml mycelial suspension. The nitrogen source was nitrate or a combination of 5 mM oxoglutarate and 1.25 mM arginine. The production on infected leaves was measured in leaf pieces infected with 1 x 10^4 conidia.ml^{-1} at indicated time points. Two different methods were used to detect ET production with a gas chromatograph (Thermo Finnigan TRACE GC Ultra; 25m, 0.54 mm, CP-Parabond CP7354) with manual injection or with a more sensitive laser-based ethylene detector in combination with a real time gas flow through system (LPAD) both described by Cristescu et al. (2013). All experiments were repeated at least three times with comparable results.

5.5.6 RNA extraction and quantitative RT-PCR

Total RNA was extracted from frozen leaf tissue using the spectrum plant total RNA kit (Sigma-Aldrich) and subsequently Turbo DNase treated according to the provided protocol (Ambion). First-strand cDNA was synthesized from 2 mg of total RNA using Multiscribe reverse transcriptase (Applied Biosystems) and random primers following the manufacturer's instructions. Quantitative PCR amplifications were conducted in optical 96-well plates with the Mx3005P real-time PCR detection system (Stratagene), using Sybr Green master mix (Fermentas) to monitor dsDNA synthesis. The expression of each gene was assayed in duplicate in a total volume of 25 µl including a passive reference dye (ROX) according to the manufacturer's instructions (Fermentas). The thermal profile used consisted of an initial denaturation step at 95°C for 10min, followed by 40 cycles of 95°C for 15 s, 57°C for 30 s, and 72°C for 30 s. To verify amplification of one specific target cDNA, a melting-curve analysis was included according to the thermal profile suggested by the manufacturer (Stratagene). The amount of plant RNA in each sample was normalized using elongation factor eIFα (LO_Os03g08020) as internal control and mock treated samples were selected as calibrator. For analysis of the CmEFE (BK008840) expression in fungal and plant samples using the fungal ITS gene as normalizer. The data were analyzed using Stratagene's Mx3005P software. Nucleotide sequences of all primers used are listed in Supplemental Table S5.1.

6

General conclusions and future perspectives

6.1 General conclusions

As mentioned in the problem statement (see Chapter 1), the main objective of this dissertation is to elucidate the mechanisms that underlie silicon (Si)-induced broad spectrum resistance. The data presented in previous chapters not only offered novel insights in the molecular underpinnings of the rice-*Cochliobolus miyabeanus* interaction, which was used as a model pathosystem, they also shed more light on the positive influence of Si on rice plants and brown spot resistance. Moreover, the improved molecular understanding on the effect of Si led to several hypotheses on how Si is able to mount broad spectrum disease resistance in plants.

6.1.1 Rice-*Cochliobolus miyabeanus* pathosystem

Despite its widespread occurrence and impact, relatively little is known about the interaction between rice and *C. miyabeanus*, the causal agent of brown spot disease. This necrotrophic rice leaf fungus affects millions of hectares of rice every growing season, mostly under suboptimal growing conditions (Barnwal et al., 2013). Rice leaves infected with *C. miyabeanus* show necrotic spots surrounded by chlorotic lesions eventually leading to premature leaf senescence and cell death. The virulence of *C. miyabeanus* is determined by many factors, among which the production of phytotoxic compounds (Condon et al., 2013). Many members of the *Cochliobolus* genus are known to produce a wide variety of toxins to facilitate the infection process. In case of *C. miyabeanus*, ophiobolin A and B are the best-characterized toxins which are known to promote pathogen-induced senescence and cell death in rice leaves (Xiao et al., 1991; Kim et al., 1999; Ahn et al., 2005; Condon et al., 2013). Another virulence factor is the production of ethylene (ET) by *C. miyabeanus* via a pathway that is distinct from plant ET biosynthesis (see Chapter 4). Exogenous ET is known to confer susceptibility towards *C. miyabeanus*, whereas suppressing plant ET perception due to application of silver ions that block ET receptors, disruption of ET signaling in *EIN2a* antisense plants or ABA-mediated repression of the plant ET signaling, results in a substantial increase in brown spot resistance (De Vleesschauwer et al., 2010; see Chapter 4). Moreover, *C. miyabeanus* emits substantial amounts of ET during early stages of the infection process via a reaction that requires 2-oxoglutarate and arginine as cofactors, catalysed by a single Ethylene Forming Enzyme (EFE). In this light, pathogen-induced upregulation of rice glutamate dehydrogenases might play an important role in supplying 2-oxoglutarate, which is one of the main precursors for fungal ET production. In turn, fungal ET induces plant ET biosynthesis in a positive feedback loop. The activation of the plant ET signaling pathway by a combination of

fungal and plant ET largely mediates the differential transcriptome of brown spot infected leaves resulting in induced senescence and disruption of phenylpropanoid-driven defenses that normally serve to limit pathogen growth. Furthermore, preliminary results show that exogenous ET aggravates the induction of cell death by *C. miyabeanus* crude toxin extract, whereas blocking the plant ET signaling with silver ions increases tolerance towards the toxin extract (data not shown). These data suggest a synergistic role between fungal toxins and ET in during brown spot infection.

Linking the mode of action of ET and toxins produced by *C. miyabeanus* with microarray data on the effect of brown spot infection on rice metabolic processes, identified potential plant targets of *C. miyabeanus* on its path to infection.

First, microarray data demonstrated that *C. miyabeanus* targets the photosynthesis apparatus even at an early stage of infection when no clear symptoms of disease development were visible, indicating that this is a cause rather than a consequence of brown spot infection. The production of toxins and/or ET by *C. miyabeanus* might be responsible for the observed decrease in photosynthesis, since both are known to impair photosynthesis and induce senescence in rice leaves (Xiao et al., 1991; Moore et al., 1999; Ahn et al., 2005; see Chapter 4). Photosynthetic impairment might have multiple purposes in the rice-*C. miyabeanus* interaction. On the one hand, impeding photosynthesis might interfere with the energy supply for energy-demanding defense responses. On the other hand, pathogen-mediated photosynthetic impairment also leads to overreduction of the photosynthesis apparatus and subsequent photooxidative damage (Bauwe et al., 2012), which might facilitate the infection process. Since *C. miyabeanus* is known to alter rice amino acid metabolism, probably for utilization during infection (Chattopadhyay and Bera, 1978; Matsubara and Kuroda, 1980), it is also possible that *C. miyabeanus* induces senescence in order to provide nutrients via the senescence-mediated nitrogen remobilization pathway (Pageau et al., 2006; Tabuchi et al., 2007). A second major plant metabolic change during brown spot infection is the downregulation of the phenylpropanoid pathway. The accumulation of fungitoxic phenolic compounds around the site of infection is a is well-studied defense mechanism against *C. miyabeanus* (Shabana et al., 2008). Since both phytotoxin and ET production by *C. miyabeanus* interfere with phenol metabolism (Vidhyasekaran et al., 1992; see Chapter 4), these findings indicate that pathogen-mediated abolishment the phenylpropanoid pathway is an important virulence strategy during the infection process. In conclusion, the necrotrophic leaf fungus *C. miyabeanus* seems to have evolved different virulence strategies that facilitate the infection process both by inducing senescence and by preventing plant defense responses. Although the data presented in this dissertation shed new light on the interaction between rice and *C. miyabeanus*, different

molecular aspects of brown spot infection remain to be elucidated.

6.1.2 Effect of Si on rice plants

In general, reports on Si-induced stress tolerance show that Si provides protection against different stress situations without the occurrence of growth trade-offs (see Chapter 2.5). However, there is no consensus on whether Si promotes plant growth under non-stress conditions. Even though several articles can be found that rule out a beneficial effect of Si on growth as such, most reports agree on the fact that Si has a positive influence on the plant's growth and development (Agarie et al., 1992; Rodrigues, 2001; Sun et al., 2010; Tripathi et al., 2011; Detmann et al., 2012; Dallagnol et al., 2013). However it is unsure whether Si-mediated growth promotion is the result of the protective effect of Si or whether these are two independent observations. In an attempt to provide more insight on the influence of Si, many transcriptional analyses have been performed in different plant species, concluding that Si has little influence on the transcriptome of non-stressed plants (Fauteux et al., 2006; Brunings et al., 2009; Chain et al., 2009; Ghareeb et al., 2011). However, the microarray data on Si-treated rice plants presented in this thesis, show that Si strongly influences the rice transcriptome (see Chapter 3). Furthermore, Agarie et al. (1992) showed that Si has a more pronounced growth-promoting effect under suboptimal conditions. These data are in accordance with increased levels of bioactive gibberellins (GAs) observed in Si-treated soybean and rice, which seems to be the result of decreased abiotic stress (Hwang et al., 2007; Hamayun et al., 2010; Lee et al., 2010; Colebrook et al., 2014). Higher GA homeostasis might explain the increased growth and earlier flower induction often observed in Si-treated plants (Heinai et al., 2005; Mutasa-Göttgens and Hedden, 2009; Toledo et al., 2012; Colebrook et al., 2014; see Chapter 4). These findings also coincide with the outcome of the promoter analysis of TFs that are differentially expressed in Si-treated rice leaves (see section 3.2.3) which suggest that Si-induced upregulation of several transcription factors might be mediated by a increased GA homeostasis in Si-treated plants. In conclusion, these findings favor a model where the accumulated effect of Si-induced tolerance towards omnipresent levels of minor stress during plant growth might result in an apparent growth increase.

6.1.3 Si-induced brown spot resistance

The positive influence of Si on rice resistance against the necrotrophic *C. miyabeanus* has been reported in different publications (Rezende et al., 2009; Dallagnol et al., 2009; Silva et al., 2012; Dallagnol et al., 2013). During brown spot infection, Si-treated plants are characterized by significantly smaller necrotic lesions and less chlorosis. This prophylactic

influence of Si most likely depends on the active role of water soluble silicic acid inside the cell, instead of the passive protection offered by the subcuticular silica layer. Moreover, the increase in resistance of Si-treated plants seems to be the result of the enhanced capacity of these plants to cope with the production of phytotoxins and ET by *C. miyabeanus*, which act as fungal virulence strategies. For instance, preliminary results suggest that Si application increases tolerance towards a crude toxin extract of *C. miyabeanus* (data not shown). Furthermore, inhibiting the plant ET signaling, either by exogenous silver ions that block ET receptors or by impairing the ET signaling in EIN2a antisense plants, mimicked Si-induced brown spot resistance (see Chapter 5). Similar findings were obtained by blocking the fungal ET biosynthesis with the inhibitor 2,2-bipyridyl. Together with the fact that Si treatment significantly decreased the ET emission during brown spot infection, these findings suggest that Si-mediated impairment of fungal ET biosynthesis confers brown spot resistance.

Interestingly, infection with *C. miyabeanus* does not lead to an increased recruitment of plant defense responses in Si-treated plants, but appears to redirect the central metabolism in rice leaves. Microarray data showed that Si reversed pathogen-induced decrease of photosynthetic processes (see Chapter 3), which confirms the findings by Dallagnol et al. (2013) who observed an increase in photosynthesis in infected leaves due to Si application. The observed impediment of *C. miyabeanus*-mediated senescence due to Si application might confer resistance by hampering the infection process and/or by depriving *C. miyabeanus* of essential nutrients for fungal growth and reproduction inside the rice leaves. The same microarray experiment also suggested a key role for photorespiration in Si-mediated protection of the photosynthesis apparatus (see Chapter 3). Si application also resulted in the strong accumulation of phenolic compounds around the site of infection (Dallagnol et al., 2011b, 2013; see Chapter 5) which is a well-described defense response against *C. miyabeanus* (Shabana et al., 2008). It seems that Si application prevents pathogen-mediated inhibition, rather than activating the phenylpropanoid pathway (Vidhyasekaran et al., 1992; see Chapter 3 and 5). In conclusion, the beneficial effect of Si on resistance against *C. miyabeanus* is not the result of a Si-mediated recruitment of defense responses, but is more likely associated with the impairment of *C. miyabeanus*' virulence strategies.

6.1.4 Si-induced defense mechanisms

The current knowledge on Si-induced resistance formed the starting point for the postulation of several mechanisms that might be responsible for the broad spectrum mode of action of Si in plant-pathogen interactions in Chapter 4. Based on the results obtained by studying the influence of Si on the rice-*C. miyabeanus* pathosystem, we elaborated on

different mechanisms that might explain Si-induced broad spectrum disease resistance.

Si-induced priming for enhanced defense

The most common hypothesis is that Si-induced broad spectrum disease resistance is due to priming of the defense responses. Defense priming renders the plant's immunity into a state of readiness that does not directly confer resistance, but allows for accelerated induced resistance after stress induction. This strategy not only confers flexibility to adapt the defense response to a specific challenge, it also leads to a less costly and broader spectrum of resistance (Van der Ent et al., 2008; Conrath, 2011). Si application appears to induce resistance in tomato plants towards the bacterial wilting pathogen *Ralstonia solanacearum* by priming the ET and jasmonic acid (JA)-signaling pathway (Ghareeb et al., 2011). In rice plants, Si primes JA-mediated defense responses resulting in increased resistance towards caterpillars of the rice leaf folder *Cnaphalocrocis medinalis* (Ye et al., 2013). Although the beneficial effect of Si on disease resistance is generally attributed to Si-induced defense priming, a central regulator of Si-induced priming has not been characterized. Given the association of priming with the accumulation of signaling components such as transcription factors (TFs) (Conrath, 2011), the observed differential expression of several transcription factors in Si-treated plants might be responsible for Si-induced priming (see section 2.6.1). Our observations on the role of Si-mediated expression of transcription factors as an inducer of broad spectrum disease resistance suggest that a Si-mediated increase in gibberellin homeostasis might be responsible for induced growth and flower initiation (see section 3.2.3). We could not find concrete evidence that Si-mediated accumulation is responsible for mounting broad spectrum disease resistance due to priming. However, all our results are based on existing databases and most of the transcription factors that were differentially expressed were not yet characterized. The possibility remains that in analogy with Pozo et al. (2008) one of the upregulation of one of the TFs from Table 3.2 is responsible for Si-mediated priming of plant defense responses. However, whereas priming generally protects plants over a slightly extended period of time, Si is only effective for a fairly short time span (see Chapter 3.2.1). Moreover, the multiple reports on Si-mediated disease resistance that suggest that Si prevents the exploitment of virulence factors, rather than leading to an increased recruitment of defense responses(see Chapter 3.3.4) not favor a scenario in which Si-mediated priming alone is responsible for triggering broad spectrum disease resistance. To conclude, the molecular aspects of priming are still poorly understood, therefore elucidating the mechanisms that are responsible for Si-induced resistance due to priming is not evident. However, future insights on the molecular base of defense priming might pave the way to unraveling the

possible role of Si-induced priming in mediating broad spectrum disease resistance.

Si-hormone interactions

Application of Si has been reported to alter hormone homeostasis and/or prime hormone pathways which might play an important role in broad spectrum disease resistance. In rice and soybean, Si imposes an increase of bioactive GAs, which is thought to be responsible for enhancing plant growth (Hwang et al., 2007; Hamayun et al., 2010; Lee et al., 2010). However, the protective role of Si during abiotic stress, herbivory or pathogen attack in different plant species is linked to the differential regulation of ABA, GA, JA, SA and ET pathways and can not be attributed to a fixed influence of Si on hormone pathways (Fauteux et al., 2006; Hwang et al., 2007; Brunings et al., 2009; Hamayun et al., 2010; Lee et al., 2010; Ghareeb et al., 2011; Ye et al., 2013; Kim et al., 2014). In rice, Si application conferred brown spot resistance by preventing the production of fungal ET, which is a known virulence strategy of the fungus (see Chapter 4). Rather than imposing a constitutive change in hormone homeostasis, Si seems to potentiate hormone pathways, creating an adaptable signaling network that enables plants to adapt to different stress-stimuli. These findings are also compatible with the hypothesis that Si confers increased stress tolerance by preventing the negative influence of stress-situations.

Linking Si-driven photorespiration to plant immunity

The beneficial effect of Si against pathogens and abiotic stress is generally characterized by the protection of photosynthetic abilities often in combination with an increased antioxidant capacity (Nwugo and Huerta, 2008; Tripathi et al., 2011; Resende et al., 2012; Chalmardi et al., 2013; Dallagnol et al., 2013; Shi et al., 2013; Perez et al., 2014). These finding are in accordance with our results on Si-mediated resistance against *C. miyabeanus*, during which Si-driven photorespiration is thought to be responsible for safeguarding the photosynthetic abilities of infected plants, thus leading to resistance (see Chapter 5). Moreover, induced photorespiration might be a common mechanism responsible for Si-mediated broad spectrum resistance by protecting photosynthesis during stress situations. Photorespiration is a process that consumes substantial amounts of energy and produces reactive oxygen species (ROS) in different cell organelles which need to be reduced in order to prevent oxidative damage (Foyer et al., 2009). The beneficial role of photorespiration during abiotic stress, generally lies in protecting the photosynthesis machinery, whereas an increase in antioxidant capacity quenches the subsequent ROS production (Wingler et al., 2000; Sørhagen et al., 2013). In general, photorespiration is reported to induce ROS-mediated cell death which can prevent infection (Kangasjärvi

et al., 2012; Sørhagen et al., 2013). We found that Si-driven photorespiration seems to induce brown spot resistance in rice, at least to some extent by safeguarding the photosynthesis apparatus during brown spot infection (see Chaper 3). Since Si-mediated disease resistance seems to be hallmarked by the protection of the photosynthesis machinery during infection, Si-driven phtotorespiration might be a common Si-mediated defense strategy against multiple pathogens.

Si-mediated impairment of pathogen's virulence strategies

One of the overall conclusions of Si-induced disease resistance is that rather than creating resistance by directing defense responses, Si appears to nullify the impact of pathogen inoculation. In this light the main mode of action of Si might be to rule out pathogen's virulence factors which are mainly toxins and effector proteins. In analogy with the role of Si in triggering enhanced heavy metal tolerance in Si-treated plans (Wu et al., 2013), two mechanisms were hypothesized that might prevent the activity of toxins and effectors. First, given the ability of silica to bind with various molecular groups (see Chapter 3), it might be possible that microbial effectors and toxins are immobilized by binding to colloidal silica in the cell, thus preventing their function in the infection process. An alternative hypothesis is that Si application might prevent the pathogen from influencing the host plant by embedding them in silica. Many articles report that Si application leads to the deposition of an amorphous matrix containing silica and aromatic components at the site of infection in rice, cucumber and Arabidopsis leaves (Kauss et al., 2003; Rodrigues et al., 2003; Ghanmi et al., 2004; Fauteux et al., 2006). Since silica deposition is often initiated by aromatic components (Carver et al., 1998; Epstein, 1999), the accumulation of phenolic compounds, which is a typical defense mechanism (Cheynier et al., 2013), might drive the deposition of silicic acid at the site of infection. This process could lead to the 'encapsulation' of the pathogen, which might not only impede further microbial progression, but also potentially prevents the exploitment of the pathogen's virulence strategies.

6.1.5 Mechanistics of Si-induced broad spectrum resistance

Even though several mechanisms that modulate Si-induced broad spectrum disease resistance have been hypothesized, the exact mode of action of Si in the plant remains to be elucidated. To date, there is no evidence for the existence of a plant Si signaling pathway. Therefore it seems more plausible that the role of Si as an initiator of disease resistance might be the result of the physical interaction between Si, both as silica as silicic acid, and plant metabolism. Based on our findings there are three different mechanistic hypotheses

that might link physical interaction of Si with its influence on plant defense responses. First, there is the continuous polymerization of Si that is thought to depend on photorespiration (see Section 2.6.4). The deposition of silica in plant cells might be the driving force behind Si-driven photorespiration and its suggested protective effect on disease resistance (see Section 6.1.4).

Second, the manifold reports on binding abilities of silica and silicic acid led to the hypothesis that the beneficial effect of Si might be the result of complexation / binding / chelating different components of the plant cell which might influence the plant defense system in a positive manner (see section 2.6.5). However, the binding abilities of Si are non-specific, only depending on the chemical characteristics of the molecules and since Si-induced broad spectrum resistance has been proven to be very flexible and dependent on the type of stress, it does not seem very plausible that the non-specific interaction between Si and plant molecules is the main initiator of Si-induced broad spectrum disease resistance. In the same light, immobilization of xenobiotic compounds due to adsorption by Si might be responsible for the increased levels of resistance in Si-treated plants (see Section 2.6.6). Nonetheless, given the non-specific binding abilities of Si, it is rather unlikely that Si inside the cytosol will immobilize xenobiotics without interfering with plant molecules. On the other hand, since the export of xenobiotics towards the apoplast is a common defense strategy of plants (see Section 2.6.6), inhibition of the action of xenobiotics due to adsorption by Si in the apoplast is a more plausible hypothesis.

A final approach is based on the fact that both influx and efflux of Si in plant cells is important for Si-treated plants, leading to the hypothesis that application of Si might be somehow deleterious because silica deposition inside the cytosol seems to prevent normal functioning of plant cells. Therefore rice plant leaves export cytosolic Si along with different plant molecules and xenobiotics either directly due to efflux of the molecules adsorbed by the exported Si.

Yet another option is that the constant efflux of negatively charged silicic acid ions from the cytosol to the apoplast might possibly activate other transporter proteins in order to maintain the ion strength of the cytosol and the plasma membrane potential. Based on microarray data on the effect of Si on rice leaves (see Chapter 3), Si-treatment leads to the differential regulation of many transporter genes, which strengthens this hypothesis. The Si-mediated hyperactivation of different transporters, might be responsible for the efflux of both plant molecules as xenobiotics.

6.2 Future perspectives

6.2.1 Microbial ethylene production by *C. miyabeanus*

Chapter 4 discusses fungal ethylene (ET) biosynthesis by *C. miyabeanus* catalyzed by Ethylene Forming Enzyme (EFE) and its role during brown spot infection as a virulence factor during infection of rice leaves. Even though there is strong evidence that ET production by *C. miyabeanus* serves as a virulence strategy in order to induce susceptibility in rice, there are different research topics that deserve more attention in the future.

First of all, the construction of a *C. miyabeanus* mutant that is impaired in the EFE gene would allow to further elucidate the role of fungal ET production during the infection process. Infection trials with this efe mutant will characterize the importance of fungal ET for the virulence of *C. miyabeanus*. Moreover, infecting Si-treated plants with the efe mutant could also confirm the hypothesis that Si-induced brown spot resistance is due to impairment of fungal ET biosynthesis.

Second, plant metabolism was hypothesized to be essential for ET biosynthesis by *C. miyabeanus*. Based on microarray data, the role of glutamate dehydrogenase (GDH) seems essential in both providing 2-oxoglutarate for ethylene (ET) biosynthesis by *C. miyabeanus* as a virulence strategy during infection (see Chapter 4) and in preventing fungal biosynthesis in Si-treated plants (see Chapter 3). A first step in elucidating the role of rice GDH during brown spot infection is to perform GDH-activity assays in untreated and Si-treated leaves after infection with *C. miyabeanus*. Furthermore, infection trials on rice mutants impaired in *OsGDH2* (LOC_Os04g45970) and *OsGDH3* (LOC_Os02g43470) would provide more insight on whether GDH plays an important role in fungal ET biosynthesis and in brown spot susceptibility.

Third, preliminary experiments revealed that ethylene increases the susceptibility of rice leaf pieces towards crude spore extracts containing *C. miyabeanus* toxins (data not shown). Follow-up studies on the effect of ET and toxin extract on rice leaf senescence should shed light on a potential synergism between toxins and ET produced by *C. miyabeanus*. Moreover, combining current understanding of the effect of ET on the transcriptome and proteome of stressed and non-stressed rice plants (Garg et al., 2012) might provide new insights into the action mechanism(s) of *C. miyabeanus* -produced phytotoxins.

6.2.2 Microbial ethylene as a common virulence factor for necrotrophic fungi related to the *Cochliobolus* genus

Several fungi related to the Cochliobolus genus harbor a putative ethylene forming enzyme (EFE) (Eckert et al., 2014). These fungi are mostly necrotrophic, toxin-producing pathogens that infect cereal plants. Moreover, ET has been described to negatively influence defense responses in cereals infected infected with *C. victoriae* (Shain and Wheeler, 1975; Navarre and Wolpert, 1999), *C. sativus* (Hodges, 1990), *C. heterostrophus* (Degani et al., 2004), *Pyrenophora tritici-repentis* (Ciuffetti et al., 2010). Together with the fact that *C. miyabeanus* produces ET *in planta* to induce a state of host susceptibility (see Chapter 4), these findings strongly suggest that EFE-mediated ET production is a common virulence strategy for fungi related to the *Cochliobolus* genus. The use of specific inhibitor compounds targeting EFE, *in vitro* and *in planta* ET measurements, and disease trials with ET-deficient and/or -insensitive plant mutants will help to further clarify the importance and function of microbial ET production in plant resistance and susceptibility. Moreover, given the essential role of EFE for *C. miyabeanus* and the homology of the putative EFEs among *Cochliobolus*-related fungi, EFE might constitute an ideal target for the development of novel highly specific fungicides.

6.2.3 The role of photorespiration in Si-induced brown spot resistance

Our microarray study of brown spot-infected leaves suggested a key role of photorespiration in the induction of brown spot resistance in Si-treated plants. In order to further validate this hypothesis, leaf gas exchange measurements on infected plants could distinguish whether the level of photorespiration is higher in Si-treated plants after infection. Also, infection trials with plants that have been treated with the photorespiration inhibitor pyrid-2-yl hydroxymethane sulfonate (Kumagai et al., 2011) could help to further elucidate the importance of photorespiration in Si-induced brown spot resistance. Preliminary results also showed that both exogenous ET and brown spot infection lead to stomatal conductance, which is known to prevent photorespiration (Foyer et al., 2009). In view of these findings, it is tempting to speculate that fungal ET production by *C. miyabeanus* might prevent photorespiration, thus leading to brown spot susceptibility. Time-resolved leaf gas exchange measurements and monitoring the expression of photosynthesis-associated marker genes can reveal the influence of ET on photorespiration, potentially shedding new light onto the role of fungal ET during brown spot infection. Recently, the positive role of photorespiration against abiotic and biotic stresses has received increasing attention (Kangasjärvi et al., 2012; Sørhagen et al., 2013). Attempts to improve photosynthesis in

C3 plants by impairing photorespiration should therefore pay special attention to the potential impact of such modifications on the plant's ability to withstand biotic and abiotic stress factors (Hanson et al., 2013; Peterhansel et al., 2013).

6.2.4 Mechanisms of Si-induced broad-spectrum disease resistance

Given the huge potential and value of Si nutrition in stress management, the application of a range of biotechnological strategies based on the modulation of Si content and its signaling effects could provide a unique tool for the genetic improvement of crop productivity in a sustainable manner. Classic genetic approaches and genom-wide transcriptional analyses are now beginning to unveil large numbers of Si targets, shedding light on the complexity and diverse activity of Si in plants.

Si-induced priming for enhanced defense

Chapter 3.6 revealed that Si application leads to the differential expression of several transcription factors (TF). Consistent with previous findings in BABA- and rhizobacteria-induced plants (Pozo et al., 2008), such accumulation of dormant TFs may contribute to the 'primed' state of Si-treated plants. However, this does not exclude the involvement of other types of signaling molecules in the establishment and/or maintenance of Si-induced priming. Indeed, accumulating evidence connects the primed state to subtle alterations in MAP kinases, chromatin modifications and alterations of primary metabolism such as MAP kinases, small RNAs and histone modifications. Using a combination of molecular, genetic and biochemical approaches, future work should therefore be focused on deriving a holistic picture of the signaling circuitry governing Si-inducing defense priming.
The fact that promoter analysis for rice gene is still in its infancy did not facilitate the research process. However, rice promoter analysis databases and software are developing fast and the list of up- and downregulated TFs (Table 3.2) can act as primer for future research on the regulation of Si-induced broad-spectrum resistance. For instance, evaluation of disease resistance and defense response activation in available T-DNA knockout mutants may help to decipher the possible role of these TFs in the Si-induced resistance. Alternati-vely, time-resolved hormone measurements and genome-wide expression profiling could provide additional insights into the cellular processes governing the gain- or loss-of-resistance phenotype.

Si as an inhibitor of pathogen virulence factors

One of the main conclusions of this research was that Si confers brown spot resistance by disarming the virulence factors of *C. miyabeanus*. Similar findings have been obtained

in other plant-microbe interactions, suggesting that Si-mediated impairment of microbial virulence factor is a core mechanism underpinning the prophylactic effects of Si in diverse plant species. Two different, not mutually exclusive, mechanisms can be hypothesized.
First, given the ability of orthosilicic acid to bind to certain sugars and hydroxyl-amino, it is not inconceivable that microbial effector proteins and toxins are immobilized by adsorption on silica. As for other molecular interactions, complexation and/ or interaction of pathogen virulence factors and silicic acid may alter the location, activity, transport and/or selectivity of the complexed molecules. In analogy with the fact that Cd-stress increases silica deposition in rice plants (Shi et al., 2005), microscopically measuring the silica layer of rice leaves that have been treated with pahtogenic effectors or phytotoxins compared to normal Si-treated rice leaves, might indicate whether adsorption of pathogenic virulence factors by Si is an important mechanism of Si-induced resistance. There are a number of fairly easy assays that can be performed in order to test the ability of *C. miyabeanus* toxins to bind to silica, for instance, by separating fungal toxin extract on a column packed with silica gel. If the toxins do bind silica, scanning electron microscopy could be used to localize the site of silica deposition and gain further insight into the underlying mechanism (Perry et al., 1990; Rodrigues et al., 2003; Ghanmi et al., 2004).
A second hypothesis is based on the observation that in several pathogens were found embedded in a matrix containing silica and phenolic compounds (Kauss et al., 2003; Rodrigues et al., 2003; Ghanmi et al., 2004; Fauteux et al., 2006). These findings support the hypothesis that pathogens might be encapsulated in silica, thereby preventing pathogens from exerting influence on their host plants. This process is potentially induced by the defense-related accumulation of phenolic compounds at the site of infection which is a known initiator of silica deposition (Carver et al., 1998; Epstein, 1999). Microscopical analysis of the presence of UV-fluorescent phenolic compounds in silica deposition in infected leaves might confirm this hypothesis.

Mechanistics of the effect of Si

Even though the underpinnings of the direct interaction of Si inside plants remains unknown, the hypotheses in Section 6.1.5 may guide future research.
First of all the microarray data in Chapter 3 showed that Si application led to the differential expression of different transporter genes among which several aquaporins. Since all plant Si transporters are aquaporins (Gomes et al., 2009), it is possible that one or more of the differentially expressed aquaporins transports Si from the cytosol of leaf cells towards the apoplast. The same methodology that is followed to characterize the rice root efflux transporter OsLsi2 (Ma et al., 2007) can be used to identify potential rice

leaf Si efflux transporters.

Furthermore, the expression of other transporter genes might be responsible for the efflux of plant molecules and/or xenobiotics towards the apoplast which is one of the hypothesized mechanisms via which Si might influence plant disease resistance 6.1.5. Thorough datamining will first lead to a selection of transporter proteins that might play a role in Si-induced resistance. Assessing the efficacy of Si in knockout mutants will futrther elucidate the potential role of these transporters.

Another hypothesis states that interaction between plant molecules and Si might mediate Si-induced broad spectrum resistance. In order to investigate this assumption, a Si-binding assay on plant proteins can be performed by separating rice leaf protein extracts on a column packed with silica gel as described in Section 6.2.4, followed by separation and identification of the Si-binding proteins using 2D-gel electrophoresis. Further characterization of the relevance of the binding capacity of these proteins can be analyzed in knockout mutants, whereas microscopical localization of the GFP-labeled proteins can be used to confirm whether and where inside the cell these proteins interact with Si.

Summary

Pathogen invasion can lead to vast yield losses and the demand for sustainable plant protection strategies has never been greater. Plants have developed an intricate network of constitutive and inducible defense mechanisms that enables them to cope with the continuous threat of a plethora of plant pathogens. Plant defense mechanisms often demand substantial amounts of the plant's energy and it is generally assumed that inducible defense mechanisms have evolved in order to save energy under stress-free conditions to reduce the defense-related fitness costs. The type and efficacy of plant defense responses that are induced after pathogen infection depends heavily on the lifestyle of the pathogen. Whereas biotrophic pathogens derive nutrients from living host tissues, necrotrophs extract nutrients from dead or dying cells. Chemical plant activators and selected strains of rhizobacteria can increase resistance against specific types of pathogens but these treatments are often ineffective or even cause susceptibility against others due to the occurrence of trade-offs. The application of silicon (Si) is one of the scarce examples of a treatment that effectively induces broad spectrum disease resistance, however, the underlying mechanisms that mediate the prophylactic effect of silicon on plant resistance remains to be elucidated. The main objective of this dissertation was to unravel the mechanisms that underlie Si-induced broad spectrum resistance, by using the interaction between rice and the necrotrophic rice leaf fungus *C. miyabeanus* as a model pathosystem.

In the first part we focused on the uptake of Si and its ability to protect plants against a broad spectrum of pathogens. Although several initiators of induced resistance have been described, Si application is one of the few disease control strategies that is able to induce broad spectrum disease resistance by boosting the plant's basal defense mechanisms. The prophylactic effect of Si is considered to be the result of both passive and active defenses. Although the phenomenon has been known for decades, the molecular basis of Si-induced disease control remains largely unknown. By combining knowledge on how Si interacts with cell metabolism in diatoms and plants, this part describes Si-induced regulatory mechanisms that might account for broad-spectrum disease resistance in plants. Priming of plant immune responses, alterations in phytohormone homeostasis, regulation of iron homeostasis, Si-driven photorespiration and interaction with defense signaling components are all potential mechanisms involved in regulating Si-triggered resistance responses. Further elucidating how Si exerts its beneficial properties may create

new avenues for developing plants that are better able to cope with multiple stresses.

In the second part of this dissertation, we aimed to further dissect the molecular mechanisms that govern the rice-*C. miyabeanus* interaction. Infection with *C. miyabeanus* is associated with the induction of premature senescence and cell death which promotes the infection process. We found that *C. miyabeanus* produces ethylene (ET) via a pathway that is distinct from plant ET biosynthesis, a process that requires 2-oxoglutarate and arginine and is catalyzed by a fungal ethylene forming enzyme (EFE). Fungal ET, both directly and indirectly via the activation of plant ET biosynthesis, induces ET signaling in rice. The pathogen-mediated onset of ET signaling impairs plant defense responses via the induction of senescence and inhibition of phenylpropanoid-driven defense responses against *C. miyabeanus*. Moreover, we also show that the virulence of *C. miyabeanus* isolates is at least partly correlated with their ET producing abilities. While providing novel insights into the multifaceted role of ET in the plant's defense signaling network, this part underscores that next to the well-described role of phytotoxins as a virulence factor of *C. miyabeanus*, fungal ET production also appears to be a main determinant of the virulence of *C. miyabeanus*. Furthermore, these findings highlight the importance of microbial ET in modulating plant immunity.

In the third part of this dissertation we investigated the prophylactic role of Si in the interaction between rice and *C. miyabeanus*. Genome-wide expression profiling suggested that *C. miyabeanus* uncouples photosynthesis from photo-harvesting with subsequent photo-oxidative damage being responsible for the induction of senescence. Application of Si, on the other hand, impaired pathogen-induced photo-oxidative damage and alleviated premature senescence, presumably by enhancing photorespiration activity. Together these findings support a scenario whereby the plant's central metabolism plays a pivotal role in the rice-*C. miyabeanus* pathosystem, leading to either susceptibility via pathogen-induced senescence or resistance through Si-induced prevention of senescence, possibly due to Si-driven photorespiration. Moreover, in accordance with earlier microarray studies, our results suggest that Si nullifies the impact of pathogen inoculation on the plant's transcriptome, rather than creating resistance where is none by directing massive transcriptional reprogramming of defense-related genes. Furthermore, we also found that ET is the main modulator of basal and Si-induced brown spot resistance in rice. Blocking the ET signaling pathway in rice mimics Si-induced brown spot resistance, producing a resistance phenotype hallmarked by small necrotic lesions due to the accumulation of fungitoxic phenolic compounds at the site of infection. Our data suggest that Si confers brown spot

resistance by preventing fungal ET biosynthesis. In conclusion, these observations not only reaffirm the significance of fungal ET biosynthesis as an important virulence factor for *C. miyabeanus*, but also coincide with earlier findings that Si application induces brown spot resistance by disarming fungal virulence factors.

In conclusion, the results denoted in this thesis have provided several novel insights into the molecular machinery governing the interaction between rice and *C. miyabeanus* and the prophylactic effect of silicon during brown spot infection. Even though we could not characterize a central Si-mediated mechanism that is responsible for mounting broad spectrum resistance in rice the findings presented in this dissertation provide an excellent primer for future studies on the beneficial role of silicon. Advances in the understanding of the molecular mechanisms that govern Si-induced broad spectrum resistance, will not only shed new light on the pathways underpinning broad spectrum resistance, it can also guide novel strategies to improve crop performance to ensure a sustainable and durable crop protection in the future.

Samenvatting

Infectie van de plant met ziekteverwekkers of pathogenen kan leiden tot enorme verliezen in opbrengst. Daarnaast neemt de vraag naar duurzame bestrijdingsstrategieën toe. Planten hebben een complex netwerk ontwikkeld van constitutieve en induceerbare afweermechanismen om zich te beschermen tegen een overvloed aan plantpathogenen. De afweermechanismen van de plant eisen vaak aanzienlijke hoeveelheden energie en algemeen wordt aangenomen dat induceerbare afweermechanismen zijn ontstaan om energie te besparen onder stressvrije condities. Het type en de werkzaamheid van de afweermechanismen van de plant na pathogeen infectie is sterk afhankelijk van de levensstijl van de pathogeen. Biotrofe pathogenen halen hun voedingsstoffen uit weefsels van levende gastheren terwijl necrotrofen voedingsstoffen extraheren uit dode of stervende cellen. Chemische plant activators en goedaardige wortelkoloniserende bacteriën kunnen de weerstand tegen bepaalde soorten ziekteverwekkers verhogen maar deze behandelingen zijn vaak ineffectief of veroorzaken zelfs gevoeligheid tegen andere pathogenen. De behandeling met silicium (Si) is een van de weinige voorbeelden van een behandeling die effectief breed spectrum ziekteresistentie induceert. Over de onderliggende mechanismen is echter nog weinig gekend. De belangrijkste doelstelling van deze thesis is het ontrafelen van de mechanismen die aan de basis liggen van breed spectrum Si-geïnduceerde plant resistentie. Als modelsysteem hebben we hiervoor gebruik gemaakt van de interactie tussen rijst en de necrotrofe schimmel *C. miyabeanus*.

In het eerste deel van deze thesis focussen we op de opname van Si en het vermogen om de plant te beschermen tegen een breed spectrum van ziekteverwekkers. Ondanks het feit dat verschillende types van geïnduceerde resistentie beschreven zijn, is het toedienen van Si één van de weinige ziektebestrijdingsstrategieën die breed spectrum ziekte resistentie kan induceren door het stimuleren van de basale afweermechanismen van de plant. Het profylactische effect van Si wordt beschouwd als het resultaat van zowel passieve en actieve verdedigingsmechanismen. Hoewel het fenomeen al decennia gekend

is, blijft de moleculaire basis van Si-geïnduceerde ziektebestrijding grotendeels onbekend. Voortbouwend op de kennis over de interactie tussen Si en het metabolisme van de cel in diatomeeën en planten, beschrijven we diverse mechanismen welke kunnen bijdragen aan breedspectrum ziekteresistentie in planten. Potentiële mechanismen betrokken bij het activeren van afweermechanismen Si zijn het op punt stellen van de immuunresponsen van de plant, wijzigingen van de hormonale balans van de plant, verhoogde fotorespiratie en de interactie met signaal componenten. Het verder ophelderen van de werking van Si kan leiden tot de ontwikkeling van planten die meer stressbestendig zijn.

In het tweede deel van deze thesis ontrafelen we verder de moleculaire mechanismen bij de interactie tussen rijst en *C. miyabeanus*. Infectie met *C. miyabeanus* is geassocieerd met de inductie van vroegtijdige senescentie en celdood, welke het infectie proces bevordert. *C. miyabeanus* produceert ethyleen (ET) via een traject dat verschilt van de plant ET biosynthese. Dit proces vereist 2-oxoglutaraat en arginine als cofactors en wordt gekatalyseerd door het Ethylene Forming Enzyme (EFE). ET geproduceerd door de schimmel, induceert tevens ET signalering in rijst, zowel direct als indirect via de activering van plant ET biosynthese. De door *C. miyabeanus* geactiveerde ET signalering zorgt voor de verzwakking van de afweermechanismen, onder andere door het induceren van senescentie en het onderdrukken van het fenylpropanoïde metabolisme. Bovendien tonen we aan dat de virulentie van *C. miyabeanus* isolaten gedeeltelijk gecorreleerd is met hun mogelijkheid om ET te produceren. Naast de goed beschreven rol van fytotoxines als een virulentiefactor van *C. miyabeanus*, wijzen deze resultaten op de belangrijke rol van ET in de virulentie van *C. miyabeanus*.

In het derde deel van dit manuscript, hebben we de positieve invloed van Si tijdens de interactie tussen rijst en *C. miyabeanus* bestudeerd. Microarray analyse toonde aan dat *C. miyabeanus* de fotosynthese cyclus inhibeert wat leidt tot foto-oxidatieve schade en senescentie in de bladeren. Toediending van Si anderzijds voorkwam senescentie, vermoedelijk door verhoogde fotorespiratie in Si-behandelde planten tijdens infectie. Deze bevindingen suggereren een scenario waarin het centraal metabolisme van de plant een essentiële rol speelt tijdens de interactie tussen rijst en *C. miyabeanus*. Enerzijds beïnvloedt C. miyabeanus *C. miyabeanus* de stofwisseling in rijstbladeren om senescentie te veroorzaken, wat het infectieproces bespoedigt. Anderzijds zorgt Si toediening voor een heroriëntatie van het centraal metabolisme door het induceren van fotorespiratie om zo te leiden tot verhoogde resistentie. In overeenkomst met eerdere microarray experimenten, tonen onze resultaten bovendien aan dat Si het negatieve effect van *C. miyabeanus* op rijstplanten te

niet doet, eerder dan afweermechansimen te induceren. Verder zagen we ook dat ET de belangrijste regulerende factor is tijdens basale en Si- geïnduceerde afweer tijdens infectie. Zowel het blokkeren van de ET signalering in rijst als het voorkomen van ET biosynthese door *C. miyabeanus* tijdens infectie bootsen Si-geïnduceerde resistentie na, wat leidt tot minder chlorose en een substantiÃ≪le reductie van de lesiegrootte. Dit resultaat is waarschijnlijk te wijten aan de massale accumulatie van phenolische componenten rond de plaats van infectie. Deze bevindingen lijken te herbevestigen dat ET productie een belangrijke rol speelt als virulentie factor en dat Si zorgt voor resistentie door het voorkomen van ET productie door *C. miyabeanus*.

In conclusie, de resultaten in deze thesis hebben gezorgd voor nieuwe inzichten in het moleculaire radarwerk dat zorgt voor zowel ziekte door *C. miyabeanus* als resistentie in Si-geïnduceerde planten. Hoewel we geen centraal mechanisme konden karakteriseren dat het effect van Si als initiator van breed spectrum ziekteresistentie kan verklaren, vormt dit manuscript een uitstekende uitgangsbasis voor verder onderzoek naar de positieve rol van Si. Meer inzicht in de mechanismen die verantwoordelijk zijn voor Si-geïnduceerde ziekteresistentie zal niet alleen een nieuw licht werpen op hoe breed spectrum resistentie in planten werkt, maar kan ook als leidraad dienen ter ontwikkeling van nieuwe strategieën die moeten zorgen voor een meer duurzame gewasbescherming in de toekomst.

Bibliography

Abed-Ashtiani, F, Kadir, JB, Selamat, AB, Hanif, AHBM and Nasehi, A. Effect of foliar and root application of silicon against rice blast fungus in MR219 rice variety. The plant pathology journal, **2012**: 28(2):164–171

Adie, B, Chico, JM, Rubio-Somoza, I and Solano, R. Modulation of plant defenses by ethylene. Journal of plant growth regulation, **2007**: 26(2):160–177

Agarie, S, Agata, W, Kubota, F and Kaufman, PB. Physiological roles of silicon in photosynthesis and dry matter production in rice plants. Japanese journal of crop science, **1992**: 64(2):200–206

Ahmad, S, Gordon-weeks, R, Pickett, J and Ton, J. Natural variation in priming of basal resistance: from evolutionary origin to agricultural exploitation. Molecular plant pathology, **2010**: 11:817–827

Ahn, IP, Kim, S, Kang, S, Suh, SC and Lee, YH. Rice defense mechanisms against *Cochliobolus miyabeanus* and *Magnaporthe grisea* are distinct. Phytopathology, **2005**: 95(11):1248–1255

Ahn, IP, Kim, S, Lee, YH and Suh, SC. Vitamin B1-induced priming is dependent on hydrogen peroxide and the *NPR1* gene in Arabidopsis. Plant physiology, **2007**: 143(2):838–848

Alexander, L and Grierson, D. Ethylene biosynthesis and action in tomato: a model for climacteric fruit ripening. Journal of experimental botany, **2002**: 53(377):2039–2055

Alfano, JR. Micro-Review Roadmap for future research on plant pathogen effectors. Molecular plant Pathology, **2009**: 10(6):805–813

Aravind, L and Koonin, EV. The DNA-repair protein AlkB, EGL-9, and leprecan define new families of 2-oxoglutarate- and iron-dependent dioxygenases. Genome biology, **2001**: 2(3): doi:10.1186/gb-2001-2-3-research0007

Arnon, DI and Stout, PR. The essentiality of certain elements in minute quantity for plants with special reference to copper. Plant physiology, **1939**: 14:371–375

Arshad, M and Frankenberger, WT. Microbial production of plant hormones. Plant and soil, **1991**: 133(1):1–8

Arshad, M and Frankenberger, WTJ. Microbial biosynthesis of ethylene and its influence on plant growth. Advances in microbial ecology, **1992**: 12:69–111

Arshad, M and Frankenberger, WJ. Ethylene: agricultural sources and applications, volume 90. Kluwer Academic/ Plenum Publishers, New York (USA), **2002**

Ashikari, M, Sakakibara, H, Lin, S, Yamamoto, T, Takashi, T, Nishimura, A, Angeles, ER, Qian, Q, Kitano, H and Matsuoka, M. Cytokinin oxidase regulates rice grain production. Science, **2005**: 309(5735):741–745

Asselbergh, B, De Vleesschauwer, D and Höfte, M. Global switches and fine-tuning-ABA modulates plant pathogen defense. Molecular plant-microbe interactions, **2008**: 21(6):709–719

Au, TK, Chick, WS and Leung, PC. The biology of ophiobolins. Life sciences, **2000**: 67(7):733–742

Aver'yanov, AA, Lapikova, VP, Nikolaev, ON and Stepanov, AI. Active oxygen-associated control of rice blast disease by riboflavin and roseoflavin. Biochemistry, **2000**: 65(11):1292–1298

Babu, RM, Sajeena, A, Smundeeswari, AV, Sreedhar, A, Vidhyasekaran, P, Seetharaman, K and Reddy, MS. Induction of systemic resistance to *Xanthomonas oryzae* pv. *oryzae* by salicylic acid in *Oryza sativa* (L.). Journal of plant diseases and protection, **2003**: 110:419–431

Bailey, TA, Zhou, X, Chen, J and Yang, Y. Role of ethylene, abscisic acid and MAP kinase pathways in rice blast resistance. In Advances in genetics, genomics and control of rice blast disease, Wang, G-L, and Valent, B, eds. (Springer Science, Dordrecht, NL). **2009**: pp. 185–190

Baltrus, Da, Nishimura, MT, Romanchuk, A, Chang, JH, Mukhtar, MS, Cherkis, K, Roach, J, Grant, SR, Jones, CD and Dangl, JL. Dynamic evolution of pathogenicity revealed by sequencing and comparative genomics of 19 *Pseudomonas syringae* isolates. PLoS pathogens, **2011**: 7(7): doi:10.1371/journal.ppat.1002132

Barhoom, S and Sharon, A. cAMP regulation of "pathogenic" and "saprophytic" fungal spore germination. Fungal genetics and biology, **2004**: 41(3):317–326

Bari, R and Jones, JDG. Role of plant hormones in plant defence responses. Plant molecular biology, **2009**: 69(4):473–488

Barnwal, MK, Kotasthane, a, Magculia, N, Mukherjee, PK, Savary, S, Sharma, aK, Singh, HB, Singh, US, Sparks, aH, Variar, M and Zaidi, N. A review on crop losses, epidemiology and disease management of rice brown spot to identify research priorities and knowledge gaps. European journal of plant pathology, **2013**: 136(3):443–457

Barry, CS, Llop-Tous, MI and Grierson, D. The regulation of *1-aminocyclopropane-1-carboxylic acid synthase* gene expression during the transition from system-1 to system-2 ethylene synthesis in tomato. Plant physiology, **2000**: 123(3):979–986

Bauer, P, Elbaum, R and Weiss, IM. Calcium and silicon mineralization in land plants: transport, structure and function. Plant science, **2011**: 180(6):746–756

Bauwe, H, Hagemann, M and Fernie, AR. Photorespiration: players, partners and origin. Trends in plant science, **2010**: 15(6):330–336

Bauwe, H, Hagemann, M, Kern, R and Timm, S. Photorespiration has a dual origin and manifold links to central metabolism. Current opinion in plant biology, **2012**: 15(3):269–275

Beckers, GJM, Jaskiewicz, M, Liu, Y, Underwood, WR, He, SY, Zhang, S and Conrath, U. Mitogen-activated protein kinases 3 and 6 are required for full priming of stress responses in *Arabidopsis thaliana*. The plant cell, **2009**: 21(3):944–953

Bélanger, RR, Benhamou, N and Menzies, JG. Cytological evidence of an active role of silicon in wheat resistance to powdery mildew (*Blumeria graminis* f. sp. *tritici*). Phytopathology, **2003**: 93(4):402–412

Bender, CL, Alarcón-Chaidez, F and Gross, DC. *Pseudomonas syringae* phytotoxins: mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. Microbiology and molecular biology reviews, **1999**: 63(2):266–292

Benhamou, N and Nicole, M. Review cell biology of plant immunization against microbial infection: the potential of induced resistance in controlling plant diseases. Plant physiology and biochemistry, **1999**: 37(10):703–719

Berger, S, Sinha, AK and Roitsch, T. Plant physiology meets phytopathology: plant primary metabolism and plant-pathogen interactions. Journal of experimental botany, **2007**: 58:4019–4026

Block, A and Alfano, JR. Plant targets for *Pseudomonas syringae* type III effectors: virulence targets or guarded decoys? Current opinion in microbiology, **2011**: 14(1):39–46

Bloom, AJ, Burger, M, Rubio Asensio, JS and Cousins, AB. Carbon dioxide enrichment inhibits nitrate assimilation in wheat and Arabidopsis. Science, **2010**: 328(5980):899–903

Bolton, MD. Primary metabolism and plant defense-fuel for the fire. Molecular plant-microbe interactions, **2009**: 22(5):487–497

Bonfig, KB, Schreiber, U, Gabler, A, Roitsch, T and Berger, S. Infection with virulent and avirulent *P. syringae* strains differentially affects photosynthesis and sink metabolism in Arabidopsis leaves. Planta, **2006**: 225(1):1–12

Borges-Walmsley, M and Walmsley, AR. cAMP signalling in pathogenic fungi: control of dimorphic switching and pathogenicity. Trends in microbiology, **2000**: 8(3):133–141

Boyd, La, Ridout, C, O'Sullivan, DM, Leach, JE and Leung, H. Plant-pathogen interactions: disease resistance in modern agriculture. Trends in genetics, **2013**: 29(4):233–240

Bradford, MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry, **1976**: 72:248–254

Brissot, P, Bardou-Jacquet, E, Jouanolle, AM and Loreal, O. Iron disorders of genetic origin: a changing world. Trends in molecular medicine, **2011**: 17:707–713

Broekaert, WF, Delauré, SL, De Bolle, MFC and Cammue, BPA. The role of ethylene in host-pathogen interactions. Annual review of phytopathology, **2006**: 44:393–416

Brooks, DM, Bender, CL and Kunkel, BN. The *Pseudomonas syringae* phytotoxin coronatine promotes virulence by overcoming salicylic acid-dependent defences in *Arabidopsis thaliana*. Molecular plant pathology, **2005**: 6(6):629–639

Brunings, A, Datnoff, L, Ma, J, Mitani, N, Nagamura, Y, Rathinasabapathi, B and Kirst, M. Differential gene expression of rice in response to silicon and rice blast fungus *Magnaporthe oryzae*. Annals of applied biology, **2009**: 155(2):161–170

Cai, K, Gao, D, Luo, S, Zeng, R, Yang, J and Zhu, X. Physiological and cytological mechanisms of silicon-induced resistance in rice against blast disease. Physiologia plantarum, **2008**: 134(2):324–333

Cai, K, Gao, D, Chen, J and Luo, S. Probing the mechanisms of silicon-mediated pathogen resistance. Plant signaling & behavior, **2009**: 4(1):1–3

Cai, Y, Cao, F, Wei, K, Zhang, G and Wu, F. Genotypic dependent effect of exogenous glutathione on Cd-induced changes in proteins, ultrastructure and antioxidant defense enzymes in rice seedlings. Journal of hazardous materials, **2011**: 192(3):1056–1066

Caldana, C, Scheible, WR, Mueller-Roeber, B and Ruzicic, S. A quantitative RT-PCR platform for high-throughput expression profiling of 2500 rice transcription factors. Plant methods, **2007**: 3:7

Carvalho, MP, Rodrigues, FA, Silveira, PR, Andrade, CCL, Baroni, JCP, Paye, HS and Loureiro Junior, JE. Rice resistance to brown spot mediated by nitrogen and potassium. Journal of phytopathology, **2010**: 158(3):160–166

Carver, T, Thomas, B, Robbins, M and Zeyen, R. Phenylalanine ammonia-lyase inhibition, autofluorescence, and localized accumulation of silicon, calcium and manganese in oat epidermis attacked by the powdery mildew fungus *Blumeria graminis*(DC) Speer. Physiological and molecular plant pathology, **1998**: 52(4):223–243

Castell-Miller, CV and Samac, D. Population genetic structure, gene flow and recombination of *Cochliobolus miyabeanus* on cultivated wildrice (*Zizania palustris*). Plant pathology, **2012**: 61(5):903–914

Castilla, NP and Savary, S. Brown Spot. Rice fact sheets (IRRI), **2010**: pp. 1–3

Chagué, V, Danit, LV, Siewers, V, Schulze-Gronover, C, Tudzynski, P, Tudzynski, B and Sharon, A. Ethylene sensing and gene activation in *Botrytis cinerea*: a missing link in ethylene regulation of fungus-plant interactions? Molecular plant-microbe interactions, **2006**: 19(1):33–42

Chagué, V. Ethylene production by fungi: biological questions and future developments towards a sustainable polymers industry. In Handbook of hydrocarbon and lipid microbiology, Timmis, KN, ed. (Springer Berlin Heidelberg, Berlin, DE). **2010**: pp. 3012–3020

Chain, F, Côté-Beaulieu, C, Belzile, F, Menzies, JG and Bélanger, RR. A comprehensive transcriptomic analysis of the effect of silicon on wheat plants under control and pathogen stress conditions. Molecular plant-microbe interactions, **2009**: 22(11):1323–1330

Chalmardi, ZK, Abdolzadeh, A and Sadeghipour, HR. Silicon nutrition potentiates the antioxidant metabolism of rice plants under iron toxicity. Acta physiologiae plantarum, **2013**: doi:10.1007/s11738-013-1430-7

Chang, SJ, Tzeng, DS and Li, CC. Effect of silicon nutrient on bacterial blight resistance of rice (*Oryza sativa* L .). Second silicon in agriculture conference, **2002**: pp. 31–33

Chattopadhyay, SB and Dickson, JG. Relation of nitrogen to disease development in rice seedlings infected with *Helminthosporium oryzae*. Phytopathology, **1960**: 50:434–438

Chattopadhyay, AK and Samaddar, KR. Effects of *Helminthosporium oryzae* infection and ophiobolin on the cell membranes of host tissues. Physiological plant pathology, **1976**: 8:131–139

Chattopadhyay, SB and Bera, AK. Changes in nitrogen metabolism of rice leaves infected with *Helminthosporium oryzae*. Journal of phytopathology, **1978**: 91(1):52–59

Chattopadhyay, AK and Samaddar, KR. Effects of *Helminthosporium oryzae* infection and ophiobolin on the phenol metabolism of host tissues. Phytopathologische Zeitschrift, **1980**: 98(3):193–202

Chen, Z, Agnew, JL, Cohen, JD, He, P, Shan, L, Sheen, J and Kunkel, BN. *Pseudomonas syringae* type III effector AvrRpt2 alters *Arabidopsis thaliana* auxin physiology. Proceedings of the national academy of sciences of the United States of America, **2007**: 104(50):20131–20136

Chen, YY, Lin, YM, Chao, TC, Wang, JF, Liu, AC, Ho, FI and Cheng, CP. Virus-induced gene silencing reveals the involvement of ethylene-, salicylic acid- and mitogen-activated protein kinase-related defense pathways in the resistance of tomato to bacterial wilt. Physiologia plantarum, **2009**: 136(3):324–335

Chen, W, Yao, X, Cai, K and Chen, J. Silicon alleviates drought stress of rice plants by improving plant water status, photosynthesis and mineral nutrient absorption. Biological trace element research, **2011**: 142(1):67–76

Cheng, C, Yun, KY, Ressom, HW, Mohanty, B, Bajic, VB, Jia, Y, Yun, SJ and de los Reyes, BG. An early response regulatory cluster induced by low temperature and hydrogen peroxide in seedlings of chilling-tolerant *japonica* rice. BMC genomics, **2007**: 8(1): doi:10.1186/1471-2164-8-175

Chérif, M, Asselin, A and Bélanger, RR. Defense responses induced by soluble silicon in cucumber roots infected by *Pythium* spp. Phytopathology, **1994**: 84:236–242

Cheynier, V, Comte, G, Davies, KM, Lattanzio, V and Martens, S. Plant phenolics: recent advances on their biosynthesis, genetics, and ecophysiology. Plant physiology and biochemistry, **2013**: 72:1–20

Chiba, Y, Mitani, N, Yamaji, N and Ma, JF. HvLsi1 is a silicon influx transporter in barley. The plant journal, **2009**: 57(5):810–818

Choi, H, Hong, J, Ha, J, Kang, J and Kim, SY. ABFs, a family of ABA-responsive element binding factors. The journal of biological chemistry, **2000**: 275(3):1723–1730

Chowdappa, P, Mohan Kumar, S, Jyothi Lakshmi, M and Upreti, K. Growth stimulation and induction of systemic resistance in tomato against early and late blight by *Bacillus subtilis* OTPB1 or *Trichoderma harzianum* OTPB3. Biological control, **2013**: 65(1):109–117

Ciuffetti, LM, Manning, VA, Pandelova, I, Betts, MF and Martinez, JP. Host-selective toxins , Ptr ToxA and Ptr ToxB, as necrotrophic effectors in the *Pyrenophora tritici-repentis*-wheat interaction. New phytologist, **2010**: 187:911–919

Cocucci, M, Morgutti, S and Gianani, L. Effects of ophiobolin A on potassium permeability transmembrane electrical potential and proton extrusion in maize roots. Plant science letters, **1983**: 32:9–16

Cohn, JR and Martin, GB. *Pseudomonas syringae* pv. *tomato* type III effectors AvrPto and AvrPtoB promote ethylene-dependent cell death in tomato. The plant journal, **2005**: 44(1):139–154

Colebrook, EH, Creissen, G, McGrann, GRD, Dreos, R, Lamb, C and Boyd, La. Broad-spectrum acquired resistance in barley induced by the *Pseudomonas* pathosystem shares transcriptional components with Arabidopsis systemic acquired resistance. Molecular plant-microbe interactions, **2012**: 25(5):658–667

Colebrook, EH, Thomas, SG, Phillips, AL and Hedden, P. The role of gibberellin signalling in plant responses to abiotic stress. The journal of experimental biology, **2014**: 217:67–75

Condon, BJ, Leng, Y, Wu, D, Bushley, KE, Ohm, Ra, Otillar, R, Martin, J, Schackwitz, W, Grimwood, J, MohdZainudin, N, Xue, C, Wang, R, Manning, Va, Dhillon, B, Tu, ZJ, Steffenson, BJ, Salamov, A, Sun, H, Lowry, S, LaButti, K, Han, J, Copeland, A, Lindquist, E, Barry, K, Schmutz, J, Baker, SE, Ciuffetti, LM, Grigoriev, IV, Zhong, S and Turgeon, BG. Comparative genome structure, secondary metabolite, and effector coding capacity across *Cochliobolus* pathogens. PLoS genetics, **2013**: 9(1):1–29

Conrath, U, Beckers, GJM, Flors, V, García-Agustín, P, Jakab, G, Mauch, F, Newman, MA, Pieterse, CMJ, Poinssot, B, Pozo, MJ, Pugin, A, Schaffrath, U, Ton, J, Wendehenne, D, Zimmerli, L and Mauch-Mani, B. Priming: getting ready for battle. Molecular plant-microbe interactions, **2006**: 19(10):1062–1071

Conrath, U. Molecular aspects of defence priming. Trends in plant science, **2011**: 16(10):524–531

Cristescu, SM, Woltering, EJ and Harren, FJM. Real time monitoring of ethylene during fungal-plant interaction by laser-based photoacoustic spectroscopy. In Food mycology, a multifaceted approach to fungi and food, Dijksterhuis, J, and Samson, R, eds. (Traylor and Francis, New York, USA). **2006**: pp. 25–47

Cristescu, SM, Mandon, J, Arslanov, D, De Pessemier, J, Hermans, C and Harren, FJM. Current methods for detecting ethylene in plants. Annals of botany, **2013**: 111(3):347–360

Crownshaw, DK and Pegg, GF. Ethylene as a toxin synergist in *Verticillium* wilt of tomato. Physiological plant pathology, **1976**: 9:33–44

Cui, J, Bahrami, AK, Pringle, EG, Hernandez-Guzman, G, Bender, CL, Pierce, NE and Ausubel, FM. *Pseudomonas syringae* manipulates systemic plant defenses against pathogens and herbivores. Proceedings of the national academy of sciences of the United States of America, **2005**: 102(5):1791–1796

Currie, Ha and Perry, CC. Silica in plants: biological, biochemical and chemical studies. Annals of botany, **2007**: 100(7):1383–1389

Dallagnol, LJ, Rodrigues, Fa, Mielli, MVB, Ma, JF and Datnoff, LE. Defective active silicon uptake affects some components of rice resistance to brown spot. Phytopathology, **2009**: 99(1):116–121

Dallagnol, LJ, Rodrigues, Fa, DaMatta, FM, Mielli, MVB and Pereira, SC. Deficiency in silicon uptake affects cytological, physiological, and biochemical events in the rice-*Bipolaris oryzae* interaction. Phytopathology, **2011**a: 101(1):92–104

Dallagnol, LJ, Rodrigues, Fa, Martins, SCV, Cavatte, PC and DaMatta, FM. Alterations on rice leaf physiology during infection by *Bipolaris oryzae*. Australasian plant pathology, **2011**b: 40(4):360–365

Dallagnol, LJ, Rodrigues, Fa, Chaves, aRM, Vale, FXR and DaMatta, FM. Photosynthesis and sugar concentration are impaired by the defective active silicon uptake in rice plants infected with *Bipolaris oryzae*. Plant pathology, **2013**: 62(1):120–129

De Angeli, a, Monachello, D, Ephritikhine, G, Frachisse, JM, Thomine, S, Gambale, F and Barbier-Brygoo, H. The nitrate/proton antiporter AtCLCa mediates nitrate accumulation in plant vacuoles. Nature, **2006**: 442(7105):939–942

De Bruyne, L, Höfte, M and De Vleesschauwer, D. Connecting growth and defense: the emerging roles of brassinosteroids and gibberellins in plant innate immunity. Molecular plant, **2014**: 7(6):943–959

de Jong, A, Yakimova, E, Kapchina, V and Woltering, E. A critical role for ethylene in hydrogen peroxide release during programmed cell death in tomato suspension cells. Planta, **2002**: 214(4):537–545

De Meyer, G, Audenaert, K and Höfte, M. *Pseudomonas aeruginosa* 7NSK2-induced systemic resistance in tobacco depends on *in planta* salicylic acid accumulation but is not associated with *PR1a* expression. European journal of plant pathology, **1999**: 105(5):513–517

De Schutter, O. The transformative potential of the right to food. FAO, **2014**: 1-28

de Torres-Zabala, M, Truman, W, Bennett, MH, Lafforgue, G, Mansfield, JW, Rodriguez Egea, P, Bögre, L and Grant, M. *Pseudomonas syringae* pv. *tomato* hijacks the Arabidopsis abscisic acid signalling pathway to cause disease. The EMBO journal, **2007**: 26(5):1434–1443

de Torres Zabala, M, Bennett, MH, Truman, WH and Grant, MR. Antagonism between salicylic and abscisic acid reflects early host-pathogen conflict and moulds plant defence responses. The plant journal, **2009**: 59(3):375–386

De Vleesschauwer, D, Cornelis, P and Höfte, M. Redox-active pyocyanin secreted by *Pseudomonas aeruginosa* 7NSK2 triggers systemic resistance to *Magnaporthe grisea* but enhances *Rhizoctonia solani* susceptibility in rice. Molecular plant-microbe interactions, **2006**: 19(12):1406–1419

De Vleesschauwer, D, Djavaheri, M, Bakker, PaHM and Höfte, M. *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, **2008**: 148(4):1996–2012

De Vleesschauwer, D, Chernin, L and Höfte, MM. Differential effectiveness of *Serratia plymuthica* IC1270-induced systemic resistance against hemibiotrophic and necrotrophic leaf pathogens in rice. BMC plant biology, **2009**: 9(9): doi:10.1186/1471–2229–9–9

De Vleesschauwer, D and Höfte, M. Rhizobacteria-induced systemic resistance. Advances in botanical research, **2009**: 51:223–281

De Vleesschauwer, D, Yang, Y, Cruz, CV and Höfte, M. Abscisic acid-induced resistance against the brown spot pathogen *Cochliobolus miyabeanus* in rice involves MAP kinase-mediated repression of ethylene signaling. Plant physiology, **2010**: 152(4):2036–2052

De Vleesschauwer, D, Gheysen, G and Höfte, M. Hormone defense networking in rice: tales from a different world. Trends in plant science, **2013**: 18(10):555–565

De Vos, M, Van Oosten, VR, Van Poecke, RMP, Van Pelt, JA, Pozo, MJ, Mueller, MJ, Buchala, AJ, Métraux, JP, Van Loon, LC, Dicke, M and Pieterse, CMJ. Signal signature and transcriptome changes of Arabidopsis during pathogen and insect attack. Molecular plant-microbe interactions, **2005**: 18(9):923–937

Degani, O, Maor, R, Hadar, R, Sharon, A and Horwitz, BA. Host physiology and pathogenic variation of *Cochliobolus heterostrophus* strains with mutations in the *G protein alpha* subunit, *CGA1*. Applied and environmental microbiology, **2004**: 70(8):5005–5009

Dela Paz, MAG, Goodwin, PH, Raymundo, AK, Ardales, EY and Cruz, CMV. Phylogenetic analysis based on *ITS* sequences and conditions affecting the type of conidial germination of *Bipolaris oryzae*. Plant pathology, **2006**: 55(6):756–765

Denancé, N, Sánchez-Vallet, A, Goffner, D and Molina, A. Disease resistance or growth: the role of plant hormones in balancing immune responses and fitness costs. Frontiers in plant science, **2013**: 4:155

D'Enfert, C. Fungal spore germination: insights from the molecular genetics of *Aspergillus nidulans* and *Neurospora crassa*. Fungal genetics and biology, **1997**: 21:163–172

Detmann, KC, Araújo, WL, Martins, SCV, Sanglard, LMVP, Reis, JV, Detmann, E, Rodrigues, FA, Nunes-Nesi, A, Fernie, AR and DaMatta, FM. Silicon nutrition increases grain yield, which, in turn, exerts a feed-forward stimulation of photosynthetic rates via enhanced mesophyll conductance and alters primary metabolism in rice. New phytologist, **2012**: 196(3):752–762

Ding, X, Cao, Y, Huang, L, Zhao, J, Xu, C, Li, X and Wang, S. Activation of the *indole-3-acetic acid-amido synthetase GH3-8* suppresses *expansin* expression and promotes salicylate- and jasmonate-independent basal immunity in rice. The plant cell, **2008**: 20(1):228–240

Dixon, Ra, Achnine, L, Kota, P, Liu, CJ, Reddy, MSS and Wang, L. The phenylpropanoid pathway and plant defence-a genomics perspective. Molecular plant pathology, **2002**: 3(5):371–390

Domenech, J, Ramos, SB, Probanza, A, Lucas, GJA and Gutierrez, MFJ. Elicitation of systemic resistance and growth promotion of *Arabidopsis thaliana* by PGPRs from *Nicotiana glauca*: a study of the putative induction pathway. Plant and soil, **2006**: 290(1-2):43–50

Domingo, C, Andrés, F, Tharreau, D, Iglesias, DJ and Talón, M. Constitutive expression of *OsGH3.1* reduces auxin content and enhances defense response and resistance to a fungal pathogen in rice. Molecular plant-microbe interactions, **2009**: 22(2):201–210

dos Santos, LS and de Oliveira, AC. Rice iron metabolism: from source to solution. Journal of crop science and biotechnology, **2007**: 10:64–72

Dou, D and Zhou, JM. Phytopathogen effectors subverting host immunity: different foes, similar battleground. Cell host & microbe, **2012**: 12(4):484–495

Dubois, F, Tercé-Laforgue, T, Gonzalez-Moro, MBn, Estavillo, JM, Sangwan, R, Gallais, A and Hirel, B. Glutamate dehydrogenase in plants: is there a new story for an old enzyme? Plant physiology and biochemistry, **2003**: 41(6-7):565–576

Durrant, WE and Dong, X. Systemic acquired resistance. Annual review of phytopathology, **2004**: 42:185–209

Eckert, C, Xu, W, Xiong, W, Lynch, S, Ungerer, J, Tao, L, Gill, R, Maness, PC and Yu, J. Ethylene-forming enzyme and bioethylene production. Biotechnology for biofuels, **2014**: 7(1):33: doi:10.1186/1754-6834-7-33

Ehleringer, JR and Cerling, TE. C3 and C4 photosynthesis. In The earth system: biological and ecological dimensions of global environmental change, Mooney, HA, and Canadell, JG, eds. (John Wiley and sons, Chichester, UK). **2002**: pp. 186–190

Epstein, E. The anomaly of silicon in plant biology. Proceedings of the national academy of sciences of the United States of America, **1994**: 91:11–17

Epstein, E. Silicon. Annual review of plant physiology and plant molecular biology, **1999**: 50:641–664

Epstein, E and Bloom, AJ. Mineral nutrition of plants: principles and perspectives, (Sinauer Associates, Sunderland, USA). **2005**: p. 380

Epstein, E. Silicon: its manifold roles in plants. Annals of applied biology, **2009**: 155(2):155–160

Fang, CX, Wang, QS, Yu, Y, Li, QM, Zhang, HL, Wu, XC, Chen, T and Lin, WX. Suppression and overexpression of *Lsi1* induce differential gene expression in rice under ultraviolet radiation. Plant growth regulation, **2011**: 65(1):1–10

FAO. How to feed the world in 2050. FAO, **2009**

Farooq, MA, Ali, S, Hameed, A, Ishaque, W, Mahmood, K and Iqbal, Z. Alleviation of cadmium toxicity by silicon is related to elevated photosynthesis, antioxidant enzymes; suppressed cadmium uptake and oxidative stress in cotton. Ecotoxicology and environmental safety, **2013**: 96:242–249

Fauteux, F, Rémus-Borel, W, Menzies, JG and Bélanger, RR. Silicon and plant disease resistance against pathogenic fungi. FEMS microbiology letters, **2005**: 249(1):1–6

Fauteux, F, Chain, F, Belzile, F, Menzies, JG and Bélanger, RR. The protective role of silicon in the Arabidopsis-powdery mildew pathosystem. Proceedings of the national academy of sciences of the United States of America, **2006**: 103(46):17554–17559

Fawe, a, Abou-Zaid, M, Menzies, JG and Bélanger, RR. Silicon-mediated accumulation of flavonoid phytoalexins in cucumber. Phytopathology, **1998**: 88(5):396–401

Flaishman, MA and Kolattukudy, PE. Timing of fungal invasion using host's ripening hormone as a signal. Proceedings of the national academy of sciences of the United States of America, **1994**: 91:6579–6583

Flaishman, MA, Hwang, CS and Kolattukudy, PE. Involvement of protein phosphorylation in the induction of appressorium formation in *Colletotrichum gloeosporioides* by its host surface wax and ethylene. Physiological and molecular plant pathology, **1995**: 47:103–117

Fleck, AT, Nye, T, Repenning, C, Stahl, F, Zahn, M and Schenk, MK. Silicon enhances suberization and lignification in roots of rice (*Oryza sativa*). Journal of experimental botany, **2011**: 62(6):2001–2011

Flood, J. The importance of plant health to food security. Food security, **2010**: 2(3):215–231

Fonteyne, S. De rol van ethyleen , auxine en abscisinezuur in de interactie tussen rijst en de brown spot pathogeen *Cochliobolus miyabeanus*. Master thesis, Ghent University, Ghent, Belgium (in Dutch), **2011**:

Foyer, CH, Bloom, AJ, Queval, G and Noctor, G. Photorespiratory metabolism: genes, mutants, energetics, and redox signaling. Annual review of plant biology, **2009**: 60:455–484

Friesen, TL, Faris, JD, Solomon, PS and Oliver, RP. Host-specific toxins: effectors of necrotrophic pathogenicity. Cellular microbiology, **2008**: 10(7):1421–1428

Fu, J, Liu, H, Li, Y, Yu, H, Li, X, Xiao, J and Wang, S. Manipulating broad-spectrum disease resistance by suppressing pathogen-induced auxin accumulation in rice. Plant physiology, **2011**: 155(1):589–602

Fu, YQ, Shen, H, Wu, DM and Cai, KZ. Silicon-mediated amelioration of Fe^{2+} toxicity in rice (*Oryza sativa* L.) roots. Pedosphere, **2012**: 22(6):795–802

Fu, ZQ and Dong, X. Systemic acquired resistance: turning local infection into global defense. Annual review of plant biology, **2013**: 64:839–863

Fukuda, H, Fuji, T and Ogawa, T. Preparation of a cell-free ethylene-forming system from *Penicillium digitatum*. Agricultural and biological chemistry, **1986**: 50:977–981

Fukuda, H, Fuji, T and Ogawa, T. Production of ethylene by a growth-suppressed mutant of *Penicillium digitatum*. Biotechnology and bioengineering, **1988**: 31:620–623

Fukuda, H, Takahashi, M, Fujii, T, Tazaki, M and Ogawa, T. An NADH:Fe(III)EDTA oxidoreductase from *Cryptococcus albidus*: an enzyme involved in ethylene production *in vivo*? FEMS microbiology letters, **1989**: 51(1):107–111

Fukuda, H, Ogawa, T, Masato, T, Nagahama, K, Fujii, T, Tanase, S and Morino, Y. Two reactions are simultaneously catalyzed by a single enzyme: the arginine-dependent simultaneous formation of two products, ethylene and succinate, from 2-oxoglutarate by and enzyme from *Pseudomonas syringae*. Biochemical and biophysical research communications, **1992**: 188(2):483–489

Gan, S and Amasino, RM. Inhibition of leaf senescence by autoregulated production of cytokinin. Science, **1995**: 270(5244):1986–1988

Gao, D, Cai, K, Chen, J, Luo, S, Zeng, R, Yang, J and Zhu, X. Silicon enhances photochemical efficiency and adjusts mineral nutrient absorption in *Magnaporthe oryzae* infected rice plants. Acta physiologiae plantarum, **2011**: 33(3):675–682

Garg, R, Tyagi, AK and Jain, M. Microarray analysis reveals overlapping and specific transcriptional responses to different plant hormones in rice. Plant signaling & behavior, **2012**: 7(8):951–956

Gaur, VS, Singh, US, Gupta, AK and Kumar, A. Understanding the differential nitrogen sensing mechanism in rice genotypes through expression analysis of *high* and *low affinity ammonium transporter* genes. Molecular biology reports, **2012**: 39(3):2233–2241

Geelen, D, Lurin, C, Bouchez, D, Frachisse, JM, Lelièvre, F, Courtial, B, Barbier-Brygoo, H and Maurel, C. Disruption of putative anion channel gene *AtCLC-a* in Arabidopsis suggests a role in the regulation of nitrate content. The plant journal, **2000**: 21(3):259–267

Genin, S and Boucher, C. Pathogen profile *Ralstonia solanacearum*: secrets of a major pathogen unveiled by analysis of its genome. Molecular plant pathology, **2002**: 3:111–118

Ghanmi, D, McNally, DJ, Benhamou, N, Menzies, JG and Bélanger, RR. Powdery mildew of *Arabidopsis thaliana*: a pathosystem for exploring the role of silicon in plant-microbe interactions. Physiological and molecular plant pathology, **2004**: 64(4):189–199

Ghareeb, H, Bozsó, Z, Ott, PG, Repenning, C, Stahl, F and Wydra, K. Transcriptome of silicon-induced resistance against *Ralstonia solanacearum* in the silicon non-accumulator tomato implicates priming effect. Physiological and molecular plant pathology, **2011**: 75(3):83–89

Gianani, L, Cocucci, S, Pardi, D and Randazzo, G. Effects of ophiobolin B on cell enlargement and H^+/K^+ exchange in maize coleoptile tissues. Planta, **1979**: 146(3):271–274

Glazebrook, J. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annual review of phytopathology, **2005**: 43:205–227

Glick, BR. Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase. FEMS microbiology letters, **2005**: 251(1):1–7

Glick, BR, Todorovic, B, Czarny, J, Cheng, Z, Duan, J and McConkey, B. Promotion of plant growth by bacterial ACC deaminase. Critical reviews in plant sciences, **2007**: 26(5-6):227–242

Glick, BR. Bacteria with ACC deaminase can promote plant growth and help to feed the world. Microbiological research, **2014**: 169(1):30–39

Gocal, GF, Sheldon, CC, Gubler, F, Moritz, T, Bagnall, DJ, MacMillan, CP, Li, SF, Parish, RW, Dennis, ES, Weigel, D and King, RW. *GAMYB*-like genes, flowering, and gibberellin signaling in Arabidopsis. Plant physiology, **2001**: 127(4):1682–1693

Gojon, A, Krouk, G, Perrine-Walker, F and Laugier, E. Nitrate transceptor(s) in plants. Journal of experimental botany, **2011**: 62(7):2299–2308

Gomes, D, Agasse, A, Thi'ebaud, P, Delrot, S, Gerós, H and Chaumont, F. Aquaporins are multifunctional water and solute transporters highly divergent in living organisms. Biophysica acta, **2009**: 1788:1213–1228

Gómez-Ariza, J, Campo, S, Rufat, M, Estopà, M, Messeguer, J, San Segundo, B and Coca, M. Sucrose-mediated priming of plant defense responses and broad-spectrum disease resistance by overexpression of the maize pathogenesis-related PRms protein in rice plants. Molecular plant-microbe interactions, **2007**: 20(7):832–842

Goto, M, Yaguchi, Y and Hyodo, H. Ethylene production in citrus leaves infected with *Xanthomonas citri* and its relation to defoliation. Physiological plant pathology, **1980**: 16(3):343–350

Govrin, EM and Levine, A. The hypersensitive response facilitates plant infection by the necrotrophic pathogen Botrytis cinerea. Current biology, **2000**: 10(13):751–757

Grant, M and Lamb, C. Systemic immunity. Current opinion in plant biology, **2006**: 9(4):414–420

Grant, MR and Jones, JDG. Hormone (dis)harmony moulds plant health and disease. Science, **2009**: 324(5928):750–752

Grant, MR, Kazan, K and Manners, JM. Exploiting pathogens' tricks of the trade for engineering of plant disease resistance: challenges and opportunities. Microbial biotechnology, **2013**: 6(3):212–222

Grigoriev, IV, Nikitin, R, Haridas, S, Kuo, A, Ohm, R, Otillar, R, Riley, R, Salamov, A, Zhao, X, Korzeniewski, F, Smirnova, T, Nordberg, H, Dubchak, I and Shabalov, I. MycoCosm portal: gearing up for 1000 fungal genomes. Nucleic acids research, **2014**: 42:699–704

Gross, J, Stein, RJ, Fett-neto, AG and Fett, JP. Iron homeostasis related genes in rice. Genetics and molecular biology, **2003**: 26(4):477–497

Gu, HH, Qiu, H, Tian, T, Zhan, SS, Deng, THB, Chaney, RL, Wang, SZ, Tang, YT, Morel, JL and Qiu, RL. Mitigation effects of silicon rich amendments on heavy metal accumulation in rice (Oryza sativa L.) planted on multi-metal contaminated acidic soil. Chemosphere, **2011**: 83(9):1234–1240

Guan, X and Gu, S. Photorespiration and photoprotection of grapevine (*Vitis vinifera* L. cv. Cabernet Sauvignon) under water stress. Photosynthetica, **2009**: 47(3):437–444

Guinel, FC and Geil, RD. A model for the development of the rhizobial and arbuscular mycorrhizal symbioses in legumes and its use to understand the roles of ethylene in the establishment of these two symbioses. Canadian journal of botany, **2002**: 80(7):695–720

Guntzer, F, Keller, C and Meunier, JD. Benefits of plant silicon for crops: a review. Agronomy for sustainable development, **2011**: 32(1):201–213

Guo, AY, Chen, X, Gao, G, Zhang, H, Zhu, QH, Liu, XC, Zhong, YF, Gu, X, He, K and Luo, J. PlantTFDB: a comprehensive plant transcription factor database. Nucleic acids research, **2008**: 36:966–999

Guo, M, Tian, F, Wamboldt, Y and Alfano, JR. The majority of the type III effector inventory of *Pseudomonas syringae* pv. *tomato* DC3000 can suppress plant immunity. Molecular plant-microbe interactions, **2009**: 22(9):1069–1080

Hagel, JM and Facchini, PJ. Dioxygenases catalyze the O-demethylation steps of morphine biosynthesis in opium poppy. Nature chemical biology, **2010**: 6(4):273–275

Hamayun, M, Sohn, Ey, Khan, SA, Shinwari, ZK, Khan, AL and Lee, IJ. Silicon alleviates the adverse effects of salinity and drought stress on growth and endogenous plant growth hormones of soybean (*Glycine max* L.). Pakistan journal of botany, **2010**: 42(3):1713–1722

Hanson, MR, Gray, BN and Ahner, BA. Chloroplast transformation for engineering of photosynthesis. Journal of experimental botany, **2013**: 64(3):731–742

Hao, D, Yamasaki, K, Sarai, A and Ohme-takagi, M. Determinants in the sequence specific binding of two plant transcription factors, CBF1 and NtERF2, to the DRE and GCC motifs. Biochemistry, **2002**: 11:4202–4208

Hase, S, Van Pelt, Ja, Van Loon, L and Pieterse, CM. Colonization of Arabidopsis roots by *Pseudomonas fluorescens* primes the plant to produce higher levels of ethylene upon pathogen infection. Physiological and molecular plant pathology, **2003**: 62(4):219–226

Hausinger, RP. Fe(II)/α-ketoglutarate-dependent hydroxylases and related enzymes. Critical reviews in biochemistry and molecular biology, **2004**: 39(1):21–68

Heinai, H, Saigusa, M, Yoshida, K and Okazaki, H. Effect of application of acidified porous hydrate calcium silicate and porous hydrated calcium silicate on the growth of rice plants (*Oryza sativa* L .). Soil science and plant nutrition, **2005**: 1(7):961–966

Helliwell, EE, Wang, Q and Yang, Y. Transgenic rice with inducible ethylene production exhibits broad-spectrum disease resistance to the fungal pathogens *Magnaporthe oryzae*. and *Rhizoctonia solani* Plant biotechnology journal, **2013**: 11(1):33–42

Hewitt, EJ and Smith, TA. Plant mineral nutrition. In Experimental methods for the investigation of plant nutrient requirements, Hewitt, EJ, and Smith, TA, eds. (English Universities Press Ltd., London, UK). **1975**: pp. 176–222

Higo, K, Ugawa, Y, Iwamoto, M, and Korenaga, T. Plant cis-acting regulatory DNA elements (PLACE) database. Nucleic acids research, **1999**: 27:297–300

Hildebrand, M, Dahlin, K and Volcani, BE. Characterization of a silicon transporter gene family in *Cylindrotheca fusiformis*: sequences , expression analysis , and identification of homologs in other diatoms. Molecular genetics and genomics, **1998**: 260:480–486

Hodges, CF. Endogenous ethylene response and chlorophyll loss in sequentially older leaves of *Poa pratensis* infected by *Bipolaris sorokiniana*. Journal of plant physiology, **1990**: 136(6):670–674

Horst, WJ, Wang, Y, and Eticha, D. The role of the root apoplast in aluminium-induced inhibition of root elongation and in aluminium resistance of plants: a review. Annals of botany, **2010**: 106:185–197

Hoshida, H, Tanaka, Y, Hibino, T, Hayashi, Y, Tanaka, a and Takabe, T. Enhanced tolerance to salt stress in transgenic rice that overexpresses chloroplast *glutamine synthetase*. Plant molecular biology, **2000**: 43(1):103–111

Hossain, M, Khalequzzaman, KM, Mollah, MRA, Abid Hussain, M and Abdur Rahim, M. Reaction of breeding cultivars of rice against brown spot and blast under field conidtion. Asian journal of plant sciences, **2004**: 3(5):641–617

Hottiger, T and Boller, T. Ethylene biosynthesis in *Fusarium oxysporum* f.sp. *tulipae* proceeds from glutamate/2-oxoglutarate and requires oxygen and ferrous ions *in vivo*. Archives of microbiology, **1991**: 157(1):18–22

Hwang, S, Gou, Z and Kuznetsov, IB. DP-Bind: a web server for sequence-based prediction of DNA-binding residues in DNA-binding proteins. Bioinformatics, **2007**: 23(5):634–636

Ichinose, Y, Taguchi, F and Mukaihara, T. Pathogenicity and virulence factors of *Pseudomonas syringae*. Journal of general plant pathology, **2013**: 79(5):285–296

Iqbal, N, Nazar, R, Syeed, S, Masood, A and Khan, NA. Exogenously-sourced ethylene increases stomatal conductance , photosynthesis, and growth under optimal and deficient nitrogen fertilization in mustard. Journal of experimental botany, **2011**: 62(14):1–9

Ishihara, A, Nakao, T, Mashimo, Y, Murai, M, Ichimaru, N, Tanaka, C, Nakajima, H, Wakasa, K and Miyagawa, H. Probing the role of tryptophan-derived secondary metabolism in defense responses against *Bipolaris oryzae* infection in rice leaves by a suicide substrate of tryptophan decarboxylase. Phytochemistry, **2011**: 72(1):7–13

Islam, A and Saha, R. Effects of silicon on the chemical composition of rice plants. Plant soil, **1969**: 30:446–458

Iwai, T, Miyasaka, A, Seo, S and Ohashi, Y. Contribution of ethylene biosynthesis for resistance to blast fungus infection in young rice plants. Plant physiology, **2006**: 142(3):1202–1215

Jia, C, Zhang, L, Liu, L, Wang, J, Li, C and Wang, Q. Multiple phytohormone signalling pathways modulate susceptibility of tomato plants to *Alternaria alternata* f. sp. *lycopersici*. Journal of experimental botany, **2013**: 64(2):637–650

Jiang, CJ, Shimono, M, Sugano, S, Kojima, M, Yazawa, K, Yoshida, R, Inoue, H, Hayashi, N, Sakakibara, H and Takatsuji, H. Abscisic acid Interacts antagonistically with salicylic acid signaling pathway in rice-*Magnaporthe grisea* interaction. Molecular plant-microbe interactions, **2010**: 23:791–798

Johnson, L. Iron and siderophores in fungal-host interactions. Mycological research, **2008**: 112:170–183

Jones, LHP and Handreck, KA. Silica in soils, plants and animals. Advances in agronomy, **1967**: 19:107–149

Jones, JDG and Dangl, JL. The plant immune system. Nature, **2006**: 444(7117):323–329

Jugdaohsingh, R. Silicon and bone health. The journal of nutrition, health & aging, **2007**: 11(2):99–110

Jun, SH, Han, MJ, Lee, S, Seo, YS, Kim, WT and An, G. OsEIN2 is a positive component in ethylene signaling in rice. Plant & cell physiology, **2004**: 45(3):281–289

. Kaneko, M, Inukai, Y, Ueguchi-tanaka, M, Itoh, H, Izawa, T and Kobayashi, Y. Loss-of-function mutations of the rice *GAMYB* gene impair *alpha-amylase* expression in aleurone and flower development the plant cell. The plant cell, **2004**: 16:33–44

Kangasjärvi, S, Neukermans, J, Li, S, Aro, EM and Noctor, G. Photosynthesis, photorespiration, and light signalling in defence responses. Journal of experimental botany, **2012**: 63(4):1619–1636

Katagiri, F and Tsuda, K. Understanding the plant immune system. Molecular plant-microbe interactions, **2010**: 23(12):1531–1536

Kauss, H, Seehaus, K, Franke, R, Gilbert, S, Dietrich, Ra and Kröger, N. Silica deposition by a strongly cationic proline-rich protein from systemically resistant cucumber plants. The plant journal, **2003**: 33(1):87–95

Kawahara, Y, de la Bastide, M, Hamilton, JP, Kanamori, H, McCombie, WR, Ouyang, S, Schwartz, DC, Tanaka, T, Wu, J, Zhou, S, Childs, KL, Davidson, RM, Lin, H, Quesada-Ocampo, L, Vaillancourt, B, Sakai, H, Lee, SS, Kim, J, Numa, H, Itoh, T, Buell, CR and Matsumoto, T. Improvement of the *Oryza sativa* Nipponbare reference genome using next generation sequence and optical map data. Rice, **2013**: 6(1):4: doi:10.1186/1939-8433-6-4

Kawamura, Y, Hase, S, Takenaka, S, Kanayama, Y, Yoshioka, H, Kamoun, S and Takahashi, H. INF1 elicitin activates jasmonic acid and ethylene-mediated signalling pathways and induces resistance to bacterial wilt disease in tomato. Journal of phytopathology, **2009**: 157:287–297

Kenigsbuch, D and Cohen, Y. Inheritance of resistance to downy mildew in *Cucumis melo* Pi-124112 and commonality of resistance genes with Pi-124111F. Plant disease, **1992**: 76(6):615–617

Kesarwani, M, Yoo, J and Dong, X. Genetic interactions of TGA transcription factors in the regulation of pathogenesis-related genes and disease. **2007**: 144:336–346

Khatabi, B and Schäfer, P. Ethylene in mutualistic symbioses. Plant signaling & behavior, **2012**: 7(12):1634–1638

Kim, Jm and Hyeon, Sb. Isolation of ophiobolin A and its analogs as inhibitors to photosynthesis. Agricultural and biological chemistry, **1984**: 48(3):803–805

Kim, Hj, Kim, Jc, Kim, BS, Kim, HG and Cho, KY. Antibiotic and phytotoxic activities of ophiobolins from *Helminthosporium* species. The plant pathology journal, **1999**: 15(1):14–20

Kim, Yk, Liu, Zm, Li, D and Pappachan, E. Two novel genes induced by hard-surface contact of *Colletotrichum gloeosporioides* conidia. Journal of bacteriology, **2000**: 182(17):4688–4695

Kim, SG, Kim, KW, Park, EW and Choi, D. Silicon-induced cell wall fortification of rice leaves: a possible cellular mechanism of enhanced host resistance to blast. Phytopathology, **2002**: 92(10):1095–1103

Kim, YH, Khan, AL, Kim, DH, Lee, SY, Kim, KM, Waqas, M, Jung, HY, Shin, JH, Kim, JG and Lee, IJ. Silicon mitigates heavy metal stress by regulating P-type heavy metal ATPases, *Oryza sativa low silicon* genes, and endogenous phytohormones. BMC plant biology, **2014**: 14: doi:10.1186/1471-2229-14-13

Kimura, M, Anzai, H and Yamaguchi, I. Microbial toxins in plant-pathogen interactions: biosynthesis, resistance mechanisms, and significance. The journal of general and applied microbiology, **2001**: 47(4):149–160

Kinrade, SD, Gillson, AME and Knight, CTG. Silicon-29 NMR evidence of a transient hexavalent silicon complex in the diatom *Navicula pelliculosa*. Journal of the chemical society, **2002**: pp. 307–309

Kobayashi, T, Suzuki, M, Inoue, H, Itai, RN, Takahashi, M, Nakanishi, H, Mori, S and Nishizawa, NK. Expression of iron-acquisition-related genes in iron-deficient rice is co-ordinately induced by partially conserved iron-deficiency-responsive elements. Journal of experimental botany, **2005**: 56(415):1305–1316

Koga, H, Dohi, K and Mori, M. Abscisic 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, **2004**: 65(1):3–9

Kováčik, J and Bačkor, M. Phenylalanine ammonia-lyase and phenolic compounds in chamomile tolerance to cadmium and copper excess. Water, air, and soil pollution, **2007**: 185(1-4):185–193

Krizsán, K, Bencsik, O, Nyilasi, I, Galgóczy, L, Vágvölgyi, C and Papp, T. Effect of the sesterterpene-type metabolites, ophiobolins A and B, on zygomycetes fungi. FEMS microbiology letters, **2010**: 313(2):135–140

Kropff, M, Bouma, J and Jones, J. Systems approaches for the design of sustainable agro-ecosystems. Agricultural systems, **2001**: 70:369–393

Kubo, Y, Tsuda, M, Furusawa, I and Shishiyama, J. Genetic analysis of genes involved in melanin biosynthesis of *Cochliobolus miyabeanus*. Experimental mycology, **1989**: 13(1):77–84

Kubo, Y and Takano, Y. Dynamics of infection-related morphogenesis and pathogenesis in *Colletotrichum orbiculare*. Journal of general plant pathology, **2013**: 79(4):233–242

Kulkarni, RD, Thon, MR, Pan, H and Dean, RA. Novel G-protein-coupled receptor-like proteins in the plant pathogenic fungus *Magnaporthe grisea*. Genome biology, **2005**: 6(3):R24

Kumagai, E, Araki, T, Hamaoka, N and Ueno, O. Ammonia emission from rice leaves in relation to photorespiration and genotypic differences in glutamine synthetase activity. Annals of botany, **2011**: 108(7):1381–1386

Lakshmanan, M, Zhang, Z, Mohanty, B, Kwon, JY, Choi, HY, Nam, HJ, Kim, DI and Lee, DY. Elucidating rice cell metabolism under flooding and drought stresses using flux-based modeling and analysis. Plant physiology, **2013**: 162(4):2140–2150

Lee, RH, Wang, CH, Huang, LT and Chen, SC. Leaf senescence in rice plants: cloning and characterization of senescence up-regulated genes. Journal of experimental botany, **2001**: 52(358):1117–1121

Lee, SK, Sohn, EY, Hamayun, M, Yoon, JY and Lee, IJ. Effect of silicon on growth and salinity stress of soybean plant grown under hydroponic system. Agroforestry Systems, **2010**: 80(3):333–340

Lenné, J. Pests and poverty: the continuing need for crop protection research. Outlook on agriculture, **2000**: 29(4):235–250

Leung, PC, Taylor, Wa, Wang, JH and Tipton, CL. Role of calmodulin inhibition in the mode of action of ophiobolin A. Plant physiology, **1985**: 77(2):303–308

Leung, H, Zhu, Y, Revilla-Molina, I, Fan, JX, Chen, H, Pangga, I, Cruz, CV and Mew, TW. Using genetic diversity to achieve sustainable rice disease management. Plant disease, **2003**: 87(10):1156–1169

Li, G, Meng, X, Wang, R, Mao, G, Han, L, Liu, Y and Zhang, S. Dual-level regulation of ACC synthase activity by MPK3/MPK6 cascade and its downstream WRKY transcription factor during ethylene induction in Arabidopsis. PLoS genetics, **2012**: 8(6): doi:10.1371/journal.pgen.1002767

Liang, Y, Si, J and Römheld, V. Silicon uptake and transport is an active process in *Cucumis sativus*. New phytologist, **2005**a: 167(3):797–804

Liang, YC, Sun, WC, Si, J and Romheld, V. Effects of foliar- and root-applied silicon on the enhancement of induced resistance to powdery mildew in *Cucumis sativus*. Plant pathology, **2005**b: 54(5):678–685

Liang, Y, Sun, W, Zhu, YG and Christie, P. Mechanisms of silicon-mediated alleviation of abiotic stresses in higher plants: a review. Environmental pollution, **2007**: 147(2):422–428

Lin, Z, Alexander, L, Hackett, R and Grierson, D. LeCTR2, a CTR1-like protein kinase from tomato, plays a role in ethylene signalling, development and defence. The plant journal, **2008**: 54(6):1083–1093

Linka, M and Weber, APM. Shuffling ammonia between mitochondria and plastids during photorespiration. Trends in plant science, **2005**: 10(10):461–465

Liu, C and Mehdy, MC. A nonclassical arabinogalactan protein gene highly expressed in vascular tissues, *AGP31*, is transcriptionally repressed by methyl jasmonic acid in Arabidopsis. Plant physiology, **2007**: 145(3):863–874

López-Ráez, JA, Verhage, A, Fernández, I, García, JM, Azcón-Aguilar, C, Flors, V and Pozo, MJ. Hormonal and transcriptional profiles highlight common and differential host responses to arbuscular mycorrhizal fungi and the regulation of the oxylipin pathway. Journal of experimental botany, **2010**: 61(10):2589–25601

Lu, SF, Tzeng, DD and Hsu, ST. *In vitro* and *in vivo* ethylene production in relation to pathogenesis of *Pseudomonas solanacearum* (Smith) on *Lycopersicon esculentum* M.. Plant protection bulletin, **1989**: 31:60–76

Lu, BR. Taxonomy of the genus *Oryza* (poaceae): historical perspective and current status. IRRN, **1999**: 24:4–8

Lund, ST, Stall, RE and Klee, HJ. Ethylene regulates the susceptible response to pathogen infection in tomato The plant cell, **1998**: 10(3):371–382

Ma, JF and Takahashi, E. Effect of silicon on the growth and phosphorus uptake of rice. Plant and soil, **1990**: 126:115–119

Ma, JF, Mitani, N, Nagao, S, Konishi, S, Tamai, K, Iwashia, T and Yano, M. Characterization of the silicon uptake system and molecular mapping of the *silicon transporter* gene in rice. Plant physiology, **2004**: 136:3284–3289

Ma, JF and Yamaji, N. Silicon uptake and accumulation in higher plants. Trends in plant science, **2006**: 11(8):392–397

Ma, JF, Tamai, K, Yamaji, N, Mitani, N, Konishi, S, Katsuhara, M, Ishiguro, M, Murata, Y and Yano, M. A silicon transporter in rice. Nature, **2006**: 440(7084):688–691

Ma, JF, Yamaji, N, Mitani, N, Tamai, K, Konishi, S, Fujiwara, T, Katsuhara, M and Yano, M. An efflux transporter of silicon in rice. Nature, **2007**: 448(7150):209–212

Ma, JF and Yamaji, N. Functions and transport of silicon in plants. Cellular and molecular life sciences, **2008**: 65(19):3049–3057

Ma, JF, Yamaji, N and Mitani-Ueno, N. Transport of silicon from roots to panicles in plants. Proceedings of the Japan academy, **2011**: 87(7):377–385

Macho, AP, Guidot, A, Barberis, P, Beuzón, CR and Genin, S. *Ralstonia solancearum* type III effector mutant strains with reduced fitness in host plants. Molecular plant-microbe interactions, **2010**: 23(9):1197–1205

Marchler-Bauer, A, Lu, S, Anderson, JB, Chitsaz, F, Derbyshire, MK, DeWeese-Scott, C, Fong, JH, Geer, LY, Geer, RC, Gonzales, NR, Gwadz, M, Hurwitz, DI, Jackson, JD, Ke, Z, Lanczycki, CJ, Lu, F, Marchler, GH, Mullokandov, M, Omelchenko, MV, Robertson, CL, Song, JS, Thanki, N, Yamashita, RA, Zhang, D, Zhang, N, Zheng, C and Bryant, SH. CDD: a conserved domain database for the functional annotation of proteins. Nucleic acids research, **2011**: 39(Database issue):225–229

Martin-Jézéquel, V. Coupling of silicon, carbon and nitrogen metabolisms in marine diatoms. In integrated marine system analysis, Dehairs, FE, Elskens, M, and Goeyens, L, eds. (FWO Vlaanderen, Brussels, Belgium). **1998**: pp. 65–83

Martin-jézéquel, V, Hildebrand, M and Brezinski, MA. Silicon metabolism in diatoms: Implications for growth. Journal of phycology, **2000**: 36:821–840

Martinoia, E, Maeshima, M and Neuhaus, HE. Vacuolar transporters and their essential role in plant metabolism. Journal of experimental botany, **2007**: 58(1):83–102

Masclaux, BC, Quilleré, I, Gallais, A and Hirel, B. The challenge of remobilisation in plant nitrogen economy. A survey of physio-agronomic and molecular approaches. Annals of applied biology, **2001**: 138:69–81

Masclaux-Daubresse, C, Carrayol, E and Valadier, MH. The two nitrogen mobilisation- and senescence-associated *GS1* and *GDH* genes are controlled by C and N metabolites. Planta, **2005**: 221(4):580–588

Masclaux-Daubresse, C, Daniel-Vedele, F, Dechorgnat, J, Chardon, F, Gaufichon, L and Suzuki, A. Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. Annals of botany, **2010**: 105(7):1141–1157

Mase, K, Ishihama, N, Mori, H, Takahashi, H, Kaminaka, H, Kodama, M and Yoshioka, H. Transcription factor MACD1 participates in phytotoxin-triggered programmed cell death. Molecular plant-microbe interactions, **2013**: 26(8):868–879

. Mase, K, Mizuno, T, Ishihama, N, Fujii, T, Mori, H, Kodama, M and Yoshioka, H. Ethylene signaling pathway and MAPK cascades are required for AAL toxin-induced programmed cell death. Molecular plant-microbe interactions, **2012**: 25(8):1015–1025

Matsubara, M and Kuroda, H. Physiological and biochemical studies on germinating fungal spores. IV. Accumulation of glutamine and its origin in germinating conidia of *Cochliobolus miyabeanus*. Chemical & pharmaceutical bulletin, **1980**: 28(5):1365–1373

Matsumura, Ki, Hijmans, RJ, Chemin, Y, Elvidge, CD, Sugimoto, K, Wu, W, Lee, Yw and Shibasaki, R. Mapping the global supply and demand structure of rice. Sustainability science, **2009**: 4(2):301–313

Matys, V, Kel-Margoulis, OV, Fricke, E, Liebich, I, Land, S, Barre-Dirrie, A, Reuter, I, Chekmenev, D, Krull, M, Hornischer, K, Voss, N, Stegmaier, P, Lewicki-Potapov, B, Saxel, H, Kel, AE and Wingender, E. TRANSFAC and its module TRANSCompel: transcriptional gene regulation in eukaryotes. Nucleic acids research, **2006**: 34:108–110

Maxwell, K and Johnson, GN. Chlorophyll fluorescence-a practical guide. Journal of experimental botany, **2000**: 51(345):659–668

McKeague, JA and Cline, MG. Silica in soils. Advances in agronomy, **1963**: 15:339–396

McMichael, P. A food regime analysis of the world food crisis. Agriculture and human values, **2009**: 26(4):281–295

Mei, C, Qi, M, Sheng, G and Yang, Y. 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, **2006**: 19(10):1127–1137

Melotto, M, Underwood, W, Koczan, J, Nomura, K and He, SY. Plant stomata function in innate immunity against bacterial invasion. Cell, **2006**: 126(5):969–980

Mengiste, T. Plant immunity to necrotrophs. Annual review of phytopathology, **2012**: 50:267–294

Mercado Vergnes, D, Renard, ME, Duveiller, E and Maraite, H. Effect of growth stage on host sensitivity to helminthosporol toxin and susceptibility to *Cochliobolus sativus* causing spot blotch on wheat. Physiological and molecular plant pathology, **2006**: 68(1-3):14–21

Merchante, C, Alonso, JM and Stepanova, AN. Ethylene signaling: simple ligand, complex regulation. Current opinion in plant biology, **2013**: 16(5):554–560

Mergemann, H and Sauter, M. Ethylene induces epidermal cell death at the site of adventitious root emergence in rice. Plant physiology, **2000**: 124(2):609–614

Mew, TW and Gonzales, P. A handbook of rice seedborne fungi. **2002**

Mew, TW, Leung, H, Savary, S, Vera Cruz, CM and Leach, JE. Looking ahead in rice disease research and management. Critical reviews in plant sciences, **2004**: 23(2):103–127

Ming, DF, Pei, ZF, Naeem, MS, Gong, HJ and Zhou, WJ. Silicon alleviates PEG-induced aater-deficit stress in upland rice seedlings by enhancing osmotic adjustment. Journal of agronomy and crop science, **2012**: 198(1):14–26

Mitani, N, Chiba, Y, Yamaji, N and Ma, JF. Identification and characterization of maize and barley Lsi2-like silicon efflux transporters reveals a distinct silicon uptake system from that in rice. The plant cell, **2009**a: 21(7):2133–2142

Mitani, N, Yamaji, N and Ma, JF. Identification of maize silicon influx transporters. Plant & cell physiology, **2009**b: 50(1):5–12

Mitani, N, Yamaji, N, Ago, Y, Iwasaki, K and Ma, JF. Isolation and functional characterization of an influx silicon transporter in two pumpkin cultivars contrasting in silicon accumulation. The plant journal, **2011**: 66(2):231–240

Miyashita, Y and Good, AG. NAD(H)-dependent glutamate dehydrogenase is essential for the survival of *Arabidopsis thaliana* during dark-induced carbon starvation. Journal of experimental botany, **2008**: 59(3):667–680

Montpetit, J, Vivancos, J, Mitani-Ueno, N, Yamaji, N, Rémus-Borel, W, Belzile, F, Ma, JF and Bélanger, RR. Cloning, functional characterization and heterologous expression of *TaLsi1*, a wheat silicon transporter gene. Plant molecular biology, **2012**: 79(1-2):35–46

Moore, T, Martineau, B, Bostock, RM, Lincoln, JE and Gilchrist, DG. Molecular and genetic characterization of ethylene involvement in mycotoxin-induced plant cell death. Physiological and molecular plant pathology, **1999**: 54:73–85

Moreno, JI, Martín, R and Castresana, C. Arabidopsis SHMT1, a serine hydroxymethyltransferase that functions in the photorespiratory pathway influences resistance to biotic and abiotic stress. The plant journal, **2005**: 41(3):451–463

Müller, C, Scheible, W, Stitt, M and Krapp, A. Influence of malate and 2-oxoglutarate on the *NIA* transcript level and nitrate reductase activity in tobacco leaves. Plant cell, **2001**: 204:191–203

Mundle, SOC, Johnson, T, Lacrampe-Couloume, G, Pérez-de Mora, A, Duhamel, M, Edwards, EA, McMaster, ML, Cox, E, Révész, K and Sherwood Lollar, B. Monitoring biodegradation of ethene and bioremediation of chlorinated ethenes at a contaminated site using compound-specific isotope analysis (CSIA). Environmental science & technology, **2012**: 46(3):1731–1738

Mur, LAJ, Kenton, P, Atzorn, R, Miersch, O and Wasternack, C. The outcomes of concentration-specific interactions between salicylate and jasmonate signaling include synergy, antagonism, and oxidative stress leading to cell death. Plant physiology, **2006**: 140(1):249–262

Mur, LaJ, Laarhoven, LJJ, Harren, FJM, Hall, Ma and Smith, AR. Nitric oxide interacts with salicylate to regulate biphasic ethylene production during the hypersensitive response. Plant physiology, **2008**: 148:1537–1546

Mur, LaJ, Simpson, C, Gay, a, Smith, Ja, Paveley, N, Sánchez-Martin, J and Prats, E. Stomatal lock-up following pathogenic challenge: source or symptom of costs of resistance in crops? Plant pathology, **2013**: 62:72–82

Mutasa-Göttgens, E and Hedden, P. Gibberellin as a factor in floral regulatory networks. Journal of experimental botany, **2009**: 60(7):1979–1989

Mutka, AM, Fawley, S, Tsao, T and Kunkel, BN. Auxin promotes susceptibility to *Pseudomonas syringae* via a mechanism independent of suppression of salicylic acid-mediated defenses. The plant journal, **2013**: 74(5):746–754

Nagahama, K, Ogawa, T, Fujii, T, Masato, T, Goto, M and Fukuda, H. L-arginine is essential for the formation *in vitro* of ethylene by an extract of *Pseudomonas syringae*. Journal of general microbiology, **1991**a: 137:1641–1646

Nagahama, K, Ogawa, T, Fujii, T, Tazaki, M, Tanase, S, Morino, Y and Fukuda, H. Purification and properties of an ethylene-forming enzyme from *Pseudomonas syringae* pv. *phaseolicola* PK2. Journal of general microbiology, **1991**b: 137:2281–2286

Naik, RM. Dependence of *in vivo* nitrate reduction on photorespiration and mitochondrial respiration in leaves of *Nicotiana sylvestris*. Journal of plant biochemistry & biotechnology, **2006**: 15:59–61

Navarre, Da and Wolpert, TJ. Inhibition of the glycine decarboxylase multienzyme complex by the host-selective toxin victorin. The plant cell, **1995**: 7(4):463–471

Navarre, Da and Wolpert, TJ. Victorin induction of an apoptotic/senescence-like response in oats. The plant cell, **1999**: 11(2):237–249

Nisler, J, Zatloukal, M, Popa, I, Dolezal, K, Strnad, M and Spíchal, L. Cytokinin receptor antagonists derived from 6-benzylaminopurine. Phytochemistry, **2010**: 71(7):823–830

Novák, O, Hauserová, E, Amakorová, P, Dolezal, K and Strnad, M. Cytokinin profiling in plant tissues using ultra-performance liquid chromatography-electrospray tandem mass spectrometry. Phytochemistry, **2008**: 69(11):2214–2224

Nunes-Nesi, A, Fernie, AR and Stitt, M. Metabolic and signaling aspects underpinning the regulation of plant carbon nitrogen interactions. Molecular plant, **2010**: 3(6):973–96

Nwugo, CC and Huerta, AJ. Silicon-induced cadmium resistance in rice (*Oryza sativa*). Journal of plant nutrition and soil science, **2008**: 171(6):841–848

Nwugo, CC and Huerta, AJ. The effect of silicon on the leaf proteome of rice (*Oryza sativa* L.) plants under cadmium-stress. Journal of proteome research, **2011**: 10(2):518–528

Oide, S, Moeder, W, Krasnoff, S, Gibson, D, Haas, H, Yoshioka, K and Turgeon, BG. NPS6, encoding a nonribosomal peptide synthetase involved in siderophore-mediated iron metabolism, is a conserved virulence determinant of plant pathogenic ascomycetes. The plant cell, **2006**: 18(10):2836–2853

Okita, TW and Volcani, BE. Role of silicon in diatom metabolism: differential synthesis of DNA polymerases and DNA-binding proteins during silicate starvation and recovery in *Cylindrotheca fusiformis*. Biochimica et biophysica acta, **1978**: 519:76–86

Okuda, A and Takahashi, E. The role of silicon. In The mineral nutrition of the rice plant, Chandler, RF, ed. (Johns Hopkins University Press, Baltimore, USA). **1964**: pp. 132–146

Oliveros, J. VENNY. An interactive tool for comparing lists with Venn diagrams. BioinfoGP **2007**: http://bioinfogp.cnb.csic.es/tools/venny/index.html

Ou, SH. Rice Diseases. Commonwealth Mycological Institute, Kew, UK, **1985**

Pageau, K, Reisdorf-Cren, M, Morot-Gaudry, JF and Masclaux-Daubresse, C. The two senescence-related markers, *GS1* (cytosolic glutamine synthetase) and *GDH* (glutamate dehydrogenase), involved in nitrogen mobilization, are differentially regulated during pathogen attack and by stress hormones and reactive oxygen species in *Nicotiana tabacum* L. leaves. Journal of experimental botany, **2006**: 57(3):547–557

Pall, ML and Robertson, CK. Fatty acid control of cyclic AMP levels in *Neurospora crassa*. Current microbiology, **1987**: 15(1):25–27

Pantelides, IS, Tjamos, SE and Paplomatas, EJ. Ethylene perception via ETR1 is required in Arabidopsis infection by *Verticillium dahliae*. Molecular plant pathology, **2010**: 11:191–202

Pantelides, IS, Tjamos, SE, Pappa, S, Kargakis, M and Paplomatas, EJ. The ethylene receptor ETR1 is required for *Fusarium oxysporum* pathogenicity. Plant pathology, **2013**: 62(6):1302–1309

Park, CJ, Bart, R, Chern, M, Canlas, PE, Bai, W and Ronald, PC. Overexpression of the endoplasmic reticulum chaperone *BiP3* regulates XA21-mediated innate immunity in rice. PloS one, **2010**: 5(2: doi:10.1371/journal.pone.0009262.g001

Pastor, V, Luna, E, Mauch-Mani, B, Ton, J and Flors, V. Primed plants do not forget. Environmental and experimental botany, **2013**: 94:46–56

Pazout, J and Pazoutova, S. Ethylene is synthesized by vegetative mycelium in surface cultures of *Penicillium cyclopium* Westling. Canadian journal of microbiology, **1989**: 35:384–387

Pegg, G. Changes in host glycosidase activity following infection of the tomato plant with *Verticillium albo-atrum*. Agronomie, **1981**: v. 1(9) p.

Perez, CEA, Rodrigues, FA, Moreira, WR and Damatta, FM. Leaf gas exchange and chlorophyll a fluorescence in wheat plants supplied with silicon and infected with *Pyricularia oryzae*. Phytopathology, **2014**: 104(2):143–149

Perry, CC, Moss, J and Williams, RJP. A staining agent for biological silica. Proceedings of the royal society, **1990**: 241(1300):47–50

Perry, CC and Keeling-Tucker, T. Aspects of the bioinorganic chemistry of silicon in conjunction with the biometals calcium, iron and aluminium. Journal of inorganic biochemistry, **1998**: 69(3):181–191

Peterhansel, C, Blume, C and Offer. Photorespiratory bypasses: how can they work? Journal of experimental botany, **2013**: 64(3):709–715

Petti, C, Reiber, K, Ali, SS, Berney, M and Doohan, FM. Auxin as a player in the biocontrol of *Fusarium* head blight disease of barley and its potential as a disease control agent. BMC plant biology, **2012**: 12:224

Pieterse, C, van Wees, S, Hoffland, E, van Pelt, JA and van Loon, LC. Systemic resistance in Arabidopsis induced by biocontrol bacteria is independent of salicylic acid accumulation and *pathogenesis-related* gene expression. Plant cell, **1996**: 8(8):1225–1237

Pieterse, CM, van Wees, SC, van Pelt, JA, Knoester, M, Laan, R, Gerrits, H, Weisbeek, PJ and van Loon, LC. A novel signaling pathway controlling induced systemic resistance in Arabidopsis. The plant cell, **1998**: 10(9):1571–1580

Pieterse, CM, Van Pelt, Ja, Ton, J, Parchmann, S, Mueller, MJ, Buchala, AJ, Métraux, JP and Van Loon, LC. Rhizobacteria-mediated induced systemic resistance (ISR) in Arabidopsis requires sensitivity to jasmonate and ethylene but is not accompanied by an increase in their production. Physiological and molecular plant pathology, **2000**: 57(3):123–134

Pieterse, CMJ, Leon-Reyes, A, Van der Ent, S and Van Wees, SCM. Networking by small-molecule hormones in plant immunity. Nature chemical biology, **2009**: 5(5):308–316

Pieterse, CMJ, Van der Does, D, Zamioudis, C, Leon-Reyes, A and Van Wees, SCM. Hormonal modulation of plant immunity. Annual review of cell and developmental biology, **2012**: 28:489–521

Pondaven, P, Gallinari, M, Chollet, S, Bucciarelli, E, Sarthou, G, Schultes, S and Jean, F. Grazing-induced changes in cell wall silicification in a marine diatom. Protist, **2007**: 158:21–28

Pozo, MJ, Van Der Ent, S, Van Loon, LC and Pieterse, CMJ. Transcription factor MYC2 is involved in priming for enhanced defense during rhizobacteria-induced systemic resistance in *Arabidopsis thaliana*. New phytologist, **2008**: 180(2):511–523

Priyatno, TP, Abu Bakar, FD, Kamaruddin, N, Mahadi, NM and Abdul Murad, AM. Inactivation of the catalytic subunit of cAMP-dependent protein kinase A causes delayed appressorium formation and reduced pathogenicity of *Colletotrichum gloeosporioides*. The scientific world journal, **2012**: 2012: doi:10.1100/2012/545784

Qiu, X, Xie, W, Lian, X and Zhang, Q. Molecular analyses of the rice *glutamate dehydrogenase* gene family and their response to nitrogen and phosphorous deprivation. Plant cell reports, **2009**: 28(7):1115–1126

Rachmilevitch, S, Cousins, AB and Bloom, AJ. Nitrate assimilation in plant shoots depends on photorespiration. Proceedings of the national academy of sciences of the United States of America, **2004**: 101(31):11506–11510

Raven, JA. Cycling silicon - the role of accumulation in plants - commentary. New Phytologist, **2003**: 158:419–421

Ray, DK, Mueller, ND, West, PC and Foley, Ja. Yield trends are insufficient to double global crop production by 2050. PloS one, **2013**: 8(6: doi:10.1371/journal.pone.0066428

Reeves, CD and Volcani, BE. Role of silicon in diatom metabolism: patterns of protein phosphorylation in *Cylindrotheca fusiformis* during recovery from silicon starvation. Archives of microbiology, **1984**: 137:291–294

Rémus-Borel, W, Menzies, JG and Bélanger, RR. Silicon induces antifungal compounds in powdery mildew-infected wheat. Physiological and molecular plant pathology, **2005**: 66(3):108–115

Rémus-Borel, W, Menzies, JG and Bélanger, RR. Aconitate and methyl aconitate are modulated by silicon in powdery mildew-infected wheat plants. Journal of plant physiology, **2009**: 166(13):1413–1422

Resende, RS, Rodrigues, FA, Cavatte, PC, Martins, SCV, Moreira, WR, Chaves, ARM and Damatta, FM. Leaf gas exchange and oxidative stress in sorghum plants supplied with silicon and infected by *Colletotrichum sublineolum*. Phytopathology, **2012**: 102(9):892–898

Reynolds, O, Keeping, M and Meyer, J. Silicon-augmented resistance of plants to herbivorous insects: a review. Annals of applied biology, **2009**: 155(2):171–186

Rezende, DC, Rodrigues, FA, Carré-Missio, V, Schurt, Da, Kawamura, IK and Korndörfer, GH. Effect of root and foliar applications of silicon on brown spot development in rice. Australasian plant pathology, **2009**: 38(1):67–73

Richmond, KE and Sussman, M. Got silicon? The non-essential beneficial plant nutrient. Current opinion in plant biology, **2003**: 6(3):268–272

Rico, A, Mccraw, SL and Preston, GM. The metabolic interface between *Pseudomonas syringae* and plant cells. Current opinion in microbiology, **2011**: 14(1):31–38

Riechmann, JL, Krizek, BA and Meyerowitz, EM. Dimerization specificity of Arabidopsis MADS domain homeotic proteins APETALA1, APETALA3, PISTILLATA, and AGAMOUS. Proceedings of the national academy of sciences of the United States of America, **1996**: 93(10):4793–4798

Riedel, T, Groten, K and Baldwin, IT. Symbiosis between *Nicotiana attenuata* and *Glomus intraradices*: ethylene plays a role, jasmonic acid does not. Plant, cell & environment, **2008**: 31(9):1203–1213

Riemann, M, Mu, A, Korte, A, Furuya, M, Weiler, EW, Nick, P and Ii, BI. Impaired Induction of the jasmonate pathway in the rice mutant *hebiba*. Plant physiology, **2003**: 133:1820–1830

Riemann, M, Haga, K, Shimizu, T, Okada, K, Ando, S, Mochizuki, S, Nishizawa, Y, Yamanouchi, U, Nick, P, Yano, M, Minami, E, Takano, M, Yamane, H and Iino, M. Identification of rice *allene oxide cyclase* mutants and the function of jasmonate for defence against *Magnaporthe oryzae*. The plant journal, **2013**: 74(2):226–238

Rivero, RM, Shulaev, V and Blumwald, E. Cytokinin-dependent photorespiration and the protection of photosynthesis during water deficit. Plant physiology, **2009**: 150(3):1530–1540

Robert-Seilaniantz, A, Grant, M and Jones, JDG. Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. Annual review of phytopathology, **2011**: 49:317–343

Rodrigues, FA. Effect of silicon and host resistance on sheath blight development in rice. Plant disease, **2001**:85:827–832

Rodrigues, F, Vale, F, Korndörfer, G, a.S. Prabhu, Datnoff, L, a.M.a. Oliveira and Zambolim, L. Influence of silicon on sheath blight of rice in Brazil. Crop protection, **2003**: 22(1):23–29

Rodrigues, Fa, McNally, DJ, Datnoff, LE, Jones, JB, Labbé, C, Benhamou, N, Menzies, JG and Bélanger, RR. Silicon enhances the accumulation of diterpenoid phytoalexins in rice: a potential mechanism for blast resistance. Phytopathology, **2004**: 94(2):177–183

Rodrigues, FA, Jurick, WM, Datnoff, LE, Jones, JB and Rollins, Ja. Silicon influences cytological and molecular events in compatible and incompatible rice-*Magnaporthe grisea* interactions. Physiological and molecular plant pathology, **2005**: 66(4):144–159

Rogalla, H and Römheld, V. Role of leaf apoplast in silicon-mediated manganese tolerance of *Cucumis sativus* L. Plant, Cell and Environment, **2002**: 25(4):549–555

Rojas, CM, Senthil-Kumar, M, Tzin, V and Mysore, KS. Regulation of primary plant metabolism during plant-pathogen interactions and its contribution to plant defense. Frontiers in plant science, **2014**: 5: doi:10.3389/fpls.2014.00017

Rojas, CM, Senthil-Kumar, M, Wang, K, Ryu, CM, Kaundal, A and Mysore, KS. Glycolate oxidase modulates reactive oxygen species-mediated signal transduction during nonhost Resistance in *Nicotiana benthamiana* and Arabidopsis. Plant cell, **2012**: 24(1):336–352

Rojo, E, Solano, R and Sánchez-Serrano, JJ. Interactions between signaling compounds involved in plant defense. Journal of plant growth regulation, **2003**: 22(1):82–98

Romero-Puertas, MC, Corpas, FJ, Rodríguez-Serrano, M, Gómez, M, Del Río, LA and Sandalio, LM. Differential expression and regulation of antioxidative enzymes by cadmium in pea plants. Journal of plant physiology, **2007**: 164(10):1346–1357

Rontein, D, Dieuaide-Noubhani, M, Dufourc, EJ, Raymond, P and Rolin, D. The metabolic architecture of plant cells. Stability of central metabolism and flexibility of anabolic pathways during the growth cycle of tomato cells. The Journal of biological chemistry, **2002**: 277(46):43948–43960

Rouault, TA. The role of iron regulatory proteins in mammalian iron homeostasis and disease. Nature chemical biology, **2006**: 2:406–414

Roychoudhury, A, Gupta, B and Sengupta, DN. Trans-acting factor designated OSBZ8 interacts with both typical abscisic acid responsive elements as well as abscisic acid responsive element-like sequences in the vegetative tissues of *indica* rice cultivars. Plant cell reports, **2008**: 27(4):779–794

Sacristan, S and Garcia-Arenal, F. The evolution of virulence and pathogenicity in plant pathogen populations. Molecular plant pathology, **2008**: 9:369–384

Saeed, AI, Sharov, V, White, J, Li, J, Liang, W, Bhagabati, N, Braisted, J, Klapa, M, Currier, T, Thiagarajan, M, Sturn, A, Snuffin, M, Rezantsev, A, Popov, D, Ryltsov, A, Kostukovich, E, Borisovsky, I, Liu, Z, Vinsavich, A, Trush, V and Quackenbush, J. TM4: a free, open-source system for microarray data management and analysis. BioTechniques, **2003**: 34(2):374–378

Saeki, K. Adsorption of Fe^{2+} and Mn^{2+} on silica, gibbsite, and humic acids. Soil science, **2004**: 169:832–840

Samuels, AL, Glass, ADM, Ehret, DL and Menzies, JG. Mobility and deposition of silicon in cucumber plants. Plant cell and environment, **1991**: 14:485–492

Sangster, AG and Hodson, MJ. Silicon and aluminium codeposition in the cell wall phytoliths of gymnosperm leaves. In Phytoliths - Applications in earth sciences and human history, Colin, F, ed. (Taylor and Francis, Oxon, UK). **2001**: pp. 343–355

Sarry, JE, Kuhn, L, Ducruix, C, Lafaye, A, Junot, C, Hugouvieux, V, Jourdain, A, Bastien, O, Fievet, JB, Vailhen, D, Amekraz, B, Moulin, C, Ezan, E, Garin, J and Bourguignon, J. The early responses of *Arabidopsis thaliana* cells to cadmium exposure explored by protein and metabolite profiling analyses. Proteomics, **2006**: 6(7):2180–2198

Sato, M, Watanabe, K, Yazawa, M, Takikawa, Y and Nishiyama, K. Detection of new ethylene-producing bacteria, *Pseudomonas syringae* pvs. *cannabina* and *sesami*, by PCR amplification of genes for the ethylene-forming enzyme. Phytopathology, **1997**: 87(12):1192–1196

Sato, H, Ando, I, Hirabayashi, H, Takeuchi, Y and Arase, S. QTL analysis of brown spot resistance in rice (*Oryza sativa* L .). Breeding science, **2008**: 96:93–96

Satoh, K, Kondoh, H, Sasaya, T, Shimizu, T, Choi, IR, Omura, T and Kikuchi, S. Selective modification of rice (*Oryza sativa*) gene expression by rice stripe virus infection. The journal of general virology, **2010**: 91(Pt 1):294–305

Savant, NK, Snyder, GH and Datnoff, LE. Silicon management and sustainable rice production. Advances in agronomy, **1997**: 58:151–199

Savary, S, Willocquet, L, Elazegui, FA, Teng, PS, Van Du, P, Zhu, DF, Tang, QY, Huang, SW, Lin, XQ, Singh, HM and Srivastava, RK. Rice pest constraints in tropical Asia: characterization of injury profiles in relation to production situations. Plant disease, **2000**: 84:341–356

Savary, S, Castilla, NP, Elazegui, F and Teng, PS. Multiple effects of two drivers of agricultural change, labour shortage and water scarcity, on rice pest profiles in tropical Asia. Field crops research, **2005**: 91(2-3):263–271

Savary, S, Nelson, A, Sparks, AH, Willocquet, L, Duveiller, E, Mahuku, G, Hodson, D, Yuen, J, Djurle, A, Forbes, G and Garrett, KA. International agricultural research tackling the effects of global and climate changes on plant diseases in the developing world. Plant disease, **2011**: 95(10):1204–1216

Savary, S, Horgan, F, Willocquet, L and Heong, K. A review of principles for sustainable pest management in rice. Crop protection, **2012**: 32:54–63

Schmiederer, T, Rausch, S, Valdebenito, M, Mantri, Y, Mosker, E, Baramov, T, Stelmaszyk, K, Schmieder, P, Butz, D, Muller, S, Schneider, K, Baik, M, Hantke, K and Sussmuth, R. The *E. coli* siderophores enterobactin and salmochelin form six-coordinate silicon complexes at physiological pH. Angewandte chemie-international Edition, **2011**: 50:4230–4233

Schut, M, Rodenburg, J, Klerkx, L, van Ast, A and Bastiaans, L. Systems approaches to innovation in crop protection. A systematic literature review. Crop protection, **2014**: 56:98–108

Schweizer, P, Buchala, A, Dudler, R and Metraux, JP. Induced systemic resistance in wounded rice plants. The plant journal, **1998**: 14(4):475–481

Seth, CS. A review on mechanisms of plant tolerance and role of transgenic plants in environmental clean-up. The botanical review, **2012**: 78(1):32–62

Seck, PA, Diagne, A, Mohanty, S and Wopereis, MCS. Crops that feed the world 7: Rice. Food security, **2012**: 4:7–24

Seifi, HS, Van Bockhaven, J, Angenon, G and Höfte, M. Glutamate metabolism in plant disease and defense: friend or foe? Molecular plant-microbe interactions, **2013**: 26(5):475–485

Seo, S, Mitsuhara, I, Feng, J, Iwai, T, Hasegawa, M and Ohashi, Y. Cyanide, a coproduct of plant hormone ethylene biosynthesis, contributes to the resistance of rice to blast fungus. Plant physiology, **2011**: 155(1):502–514

Shabana, YM, Ismail, AE, Rashad, YM and Survey, D. Control of brown spot pathogen of rice (Bipolaris oryzae) using some phenolic components. Brazilian journal of microbiology, **2008**: 39:438–444

Shain, L and Wheeler, H. Production of ethylene by oats resistant and susceptible to victorin. Phytopathology, **1975**: 65:88–89

Shamir, R, Maron-Katz, A, Tanay, A, Linhart, C, Steinfeld, I, Sharan, R, Shiloh, Y and Elkon, R. EXPANDER-an integrative program suite for microarray data analysis. BMC bioinformatics, **2005**: 6(1): doi:10.1186/1471-2105-6-232

Sharma, R, De Vleesschauwer, D, Sharma, MK and Ronald, PC. Recent advances in dissecting stress-regulatory crosstalk in rice. Molecular plant, **2013**: 6(2):250–560

Shen, X, Li, X, Li, Z, Li, J, Duan, L and Eneji, aE. Growth, physiological attributes and antioxidant enzyme activities in soybean seedlings treated with or without silicon under UV-b radiation stress. Journal of agronomy and crop science, **2010**: 196(6):431–439

Shen, X, Liu, H, Yuan, B, Li, X, Xu, C and Wang, S. OsEDR1 negatively regulates rice bacterial resistance via activation of ethylene biosynthesis. Plant, cell & environment, **2011**: 34(2):179–191

Shennan, JL. Utilisation of C_2-C_4 gaseous hydrocarbons and isoprene by microorganisms. Journal of chemical technology & biotechnology, **2006**: 81(3):237–256

Shetty, R, Fretté, X, Jensen, B, Shetty, NP, Jensen, JD, Jø rgensen, HJrL, Newman, MA and Christensen, LPr. Silicon-Induced changes in antifungal phenolic acids, flavonoids, and key phenylpropanoid pathway genes during the interaction between miniature roses and the biotrophic pathogen *Podosphaera pannosa*. Plant physiology, **2011**: 157(4):2194–2205

Shetty, R, Jensen, B, Shetty, NP, Hansen, M, Hansen, CW, Starkey, KR and Jø rgensen, HJL. Silicon induced resistance against powdery mildew of roses caused by *Podosphaera pannosa*. Plant pathology, **2012**: 61(1):120–131

Shi, XH, Zhang, CC, Wang, H, and Zhang, FS. Effect of Si on the distribution of Cd in rice seedlings. Plant soil, **2005**: 272:53–60

Shi, J, An, HL, Zhang, L, Gao, Z and Guo, XQ. *GhMPK7*, a novel multiple stress-responsive cotton group C *MAPK* gene, has a role in broad spectrum disease resistance and plant development. Plant molecular biology, **2010**: 74:1–17

Shi, Y, Wang, Y, Flowers, TJ and Gong, H. Silicon decreases chloride transport in rice (*Oryza sativa* L.) in saline conditions. Journal of plant physiology, **2013**: 170(9):847–853

Shoresh, M, Yedidia, I and Chet, I. Involvement of jasmonic acid/ethylene signaling pathway in the systemic resistance induced in cucumber by *Trichoderma asperellum* T203. Phytopathology, **2005**: 95(1):76–84

Shoresh, M, Harman, GE and Mastouri, F. Induced systemic resistance and plant responses to fungal biocontrol agents. Annual review of phytopathology, **2010**: 48:21–43

Siedow, J and Day, D. Respiration and photorespiration. In Biochemistry and molecular biology of plants, Buchanan, BB, Gruissem, W, and Jones, RL, eds. (American society of plant physiologists, Rockville, USA). **2000**: pp. 692–728

Silva, MRJ, Pereira, SC, Rodrigues, FA, Antônio, L, Júnior, Za, Fontes, LF and Oliveira, MGA. Silicon and manganese on the activity of enzymes involved in rice resistance against brown spot. Tropical plant pathology, **2012**: 37(5):339–345

Singh, RK, Singh, CV and Shukla, VD. Phosphorus nutrition reduces brown spot incidence in rainfed upland rice. IRRI notes, **2005**: 30(2):31–32

Sørhagen, K, Laxa, M, Peterhänsel, C and Reumann, S. The emerging role of photorespiration and non-photorespiratory peroxisomal metabolism in pathogen defence. Plant biology, **2013**: 15(4):723–736

Song, FM and Goodman, RM. Molecular biology of disease resistance in rice. Physiological and molecular plant pathology, **2001**: 59:1–11

Song, A, Li, P, Li, Z, Fan, F, Nikolic, M and Liang, Y. The alleviation of zinc toxicity by silicon is related to zinc transport and antioxidative reactions in rice. Plant and Soil, **2011**: 344(1-2):319–333

Spíchal, L, Werner, T, Popa, I, Riefler, M, Schmülling, T and Strnad, M. The purine derivative PI-55 blocks cytokinin action via receptor inhibition. The FEBS journal, **2009**: 276(1):244–253

Spoel, SH, Johnson, JS and Dong, X. Regulation of tradeoffs between plant defenses against pathogens with different lifestyles. Proceedings of the national academy of sciences of the United States of America, **2007**: 104(47):18842–18847

Spoel, SH and Dong, X. Making sense of hormone crosstalk during plant immune responses. Cell host & microbe, **2008**: 3(6):348–351

Srivasta, A, Mehta, S, Lindlof, A and Bhargava, S. Over-represented promoter motifs in abiotic stress-induced *DREB* genes of rice and sorghum and their probable role in regulation of gene expression. Plant signaling & behavior, **2010**: 5(7):775–784

Stone, JM, Heard, JE, Asai, T and Ausubel, FM. Simulation of fungal-mediated cell death by fumonisin B1 and selection of *fumonisin B1-resistant* (*fbr*) Arabidopsis mutants. The plant cell, **2000**: 12(10):1811–1822

Suenaga, A, Moriya, K, Sonoda, Y, Ikeda, A, Wirén, NV, Yamaguchi, J and Yamaya, T. Constitutive expression of a novel-type ammonium transporter *OsAMT2* in rice plants. Plant & cell physiology, **2003**: 44(2):206–211

Sullivan, CW and Volcani, BE. Role of silicon in diatom metabolism III. the effects of silicic acid on *DNA polymerase*, *TMP kinase* and DNA synthesis in *Cylindrotheca fusiformis*. Biochimica et biophysica acta, **1973**: 308:212–229

Sun, W, Zhang, J, Fan, Q, Xue, G, Li, Z and Liang, Y. Silicon-enhanced resistance to rice blast is attributed to silicon-mediated defence resistance and its role as physical barrier. European journal of plant pathology, **2010**: 128(1):39–49

Tabuchi, M, Abiko, T and Yamaya, T. Assimilation of ammonium ions and reutilization of nitrogen in rice (*Oryza sativa* L.). Journal of experimental botany, **2007**: 58(9):2319–2327

Tada, Y, Kusaka, K, Betsuyaku, S, Shinogi, T, Sakamoto, M, Ohura, Y, Hata, S, Mori, T, Tosa, Y and Mayama, S. Victorin triggers programmed cell death and the defense response via interaction with a cell surface mediator. Plant & cell physiology, **2005**: 46(11):1787–1798

Taheri, P and Tarighi, S. Riboflavin induces resistance in rice against *Rhizoctonia solani* via jasmonate-mediated priming of phenylpropanoid pathway. Journal of plant physiology, **2010**: 167(3):201–208

Takatsuji, H, Jiang, Cj and Sugano, S. Salicylic acid signaling pathway in rice and the potential applications of its regulators. Japan agricultural research quarterly, **2010**: 44:217–223

Taler, D, Galperin, M, Benjamin, I, Cohen, Y and Kenigsbuch, D. Plant *R* genes that encode photorespiratory enzymes confer resistance against disease. Plant cell, **2004**: 16(1):172–184

Tamura, K, Peterson, D, Peterson, N, Stecher, G, Nei, M and Kumar, S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular biology and evolution, **2011**: 28(10):2731–2739

Tanaka, N, Fujita, M, Handa, H, Murayama, S, Uemura, M, Kawamura, Y, Mitsui, T, Mikami, S, Tozawa, Y, Yoshinaga, T and Komatsu, S. Proteomics of the rice cell: systematic identification of the protein populations in subcellular compartments. Molecular genetics and genomics, **2004**: 271(5):566–576

Tao, Z, Liu, H, Qiu, D, Zhou, Y, Li, X, Xu, C and Wang, S. A pair of allelic *WRKY* genes play opposite roles in rice-bacteria interactions. Plant physiology, **2009**: 151(2):936–948

Teng, TS, Heong, KL and Moody, K. Rice pest science and managament. (IRRI, Los Baños, PH). **1994**: p. 289

Thamatrakoln, K, Alverson, AJ and Hildebrand, M. Comparative sequence analysis of diatom silicon transporters: toward a mechanistic model of silicon transport. Journal of phycology, **2006**: 42(4):822–834

Thines, E, Anke, H and Weber, RW. Fungal secondary metabolites as inhibitors of infection-related morphogenesis in phytopathogenic fungi. Mycological research, **2004**: 108(1):14–25

Thordal-Christensen, H. Fresh insights into processes of nonhost resistance. Current opinion in plant biology, **2003**: 6(4):351–357

Thuan, NTN, Bigirimana, J, Roumen, E, Straeten, D and Höfte, M. Molecular and pathotype analysis of the rice blast fungus in North Vietnam. European journal of plant pathology, **2006**: 114(4):381–396

Timmer, CP, Block, S and Dawe, D. Long-run dynamics of rice consumption, 1960-2050. In Rice in the global economy: strategic research and policy issues for food security, Pandey, S, Byerlee, D, Dawe, D, Dobermann, A, Mohanty, S, and Hardy, B, eds. (IRRI, Los Baños, PH). **2011**: pp. 139–174

Tipton, CL, Paulsen, PV and Betts, RE. Effects of ophiobolin A on ion leakage and hexose uptake by maize roots. Plant physiology, **1977**: 59(5):907–910

Toledo, MZ, Castro, GSa, Crusciol, CAC, Soratto, RP, Cavariani, C, Ishizuka, MS and Picoli, LB. Silicon leaf application and physiological quality of white oat and wheat seeds. Semina: Ciências Agrárias, **2012**: 33(5):1693–1702

Ton, J, De Vos, M, Robben, C, Buchala, A, Métraux, JP, Van Loon, LC and Pieterse, CMJ. Characterization of Arabidopsis enhanced disease susceptibility mutants that are affected in systemically induced resistance. The plant journal, **2002**: 29(1):11–21

Tripathi, DK, Singh, VP, Kumar, D and Chauhan, DK. Impact of exogenous silicon addition on chromium uptake, growth, mineral elements, oxidative stress, antioxidant capacity, and leaf and root structures in rice seedlings exposed to hexavalent chromium. Acta physiologiae plantarum, **2011**: 34(1):279–289

Tripathi, P, Deo, R, Pratap, R, Dwivedi, S and Goutam, D. Silicon mediates arsenic tolerance in rice (*Oryza sativa* L .) through lowering of arsenic uptake and improved antioxidant defence system. Ecological engineering, **2013**: 52:96–103

Tripathi, DK, Singh, VP, Gangwar, S, Prasad, SM, Maurya, JN and Chauhan, DK. Role of silicon in enrichment of plant nutrients and protection from biotic and abiotic stresses. In Improvement of crops in the era of climatic changes, Ahmad, P, Wani, MR, Azooz, MM, and Tran, LSP, eds. (Springer, New York, USA). **2014**: pp. 39–56

Tsuji, H, Aya, K, Ueguchi-Tanaka, M, Shimada, Y, Nakazono, M, Watanabe, R, Nishizawa, NK, Gomi, K, Shimada, A, Kitano, H, Ashikari, M and Matsuoka, M. GAMYB controls different sets of genes and is differentially regulated by microRNA in aleurone cells and anthers. The plant journal, **2006**: 47(3):427–444

Tzima, A, Paplomatas, EJ, Rauyaree, P and Kang, S. Roles of the catalytic subunit of cAMP-dependent protein kinase A in virulence and development of the soilborne plant pathogen *Verticillium dahliae*. Fungal genetics and biology, **2010**: 47(5):406–415

Tzima, AK, Paplomatas, EJ, Tsitsigiannis, DI and Kang, S. The G protein β subunit controls virulence and multiple growth- and development-related traits in *Verticillium dahliae*. Fungal genetics and biology, **2012**: 49(4):271–283

Usadel, B, Poree, F, Nagel, A, Lohse, M, Czedik-Eysenberg, A and Stitt, M. A guide to using MapMan to visualize and compare omics data in plants: a case study in the crop species, maize. Plant, cell & environment, **2009**: 32(9):1211–1229

Valentini, R, EPRON, D, Angelis, P, Matteucci, G and Dreyer, E. *In situ* estimation of net CO_2 assimilation, photosynthetic electron flow and photorespiration in Turkey oak (*Q. cerris* L.) leaves: diurnal cycles under different levels of water supply. Plant, cell & environment, **1995**: 18(6):631–640

Valls, M, Genin, S and Boucher, C. Integrated regulation of the type III secretion system and other virulence determinants in *Ralstonia solancearum*. PLoS pathogens, **2006**: 2(8): doi:10.1371/journal.ppat.0020082

Van der Ent, S, Verhagen, BWM, Van Doorn, R, Bakker, D, Verlaan, MG, Pel, MJC, Joosten, RG, Proveniers, MCG, Van Loon, LC, Ton, J and Pieterse, CMJ. MYB72 is required in early signaling steps of rhizobacteria-induced systemic resistance in Arabidopsis. Plant physiology, **2008**: 146(3):1293–1304

Van der Ent, S, Van Wees, SCM and Pieterse, CMJ. Jasmonate signaling in plant interactions with resistance-inducing beneficial microbes. Phytochemistry, **2009**: 70(13-14):1581–1588

Van der Ent, S and Pieterse, CM. Ethylene: multi-tasker in plant-attacker interactions. Annual plant reviews, **2012**: 44:343–377

van Hulten, M, Pelser, M, van Loon, LC, Pieterse, CMJ and Ton, J. Costs and benefits of priming for defense in Arabidopsis. Proceedings of the national academy of sciences of the United States of America, **2006**: 103(14):5602–5607

van Loon, LC, Geraats, BPJ and Linthorst, HJM. Ethylene as a modulator of disease resistance in plants. Trends in plant science, **2006**: 11(4):184–191

Vaughan, Da, Lu, BR and Tomooka, N. The evolving story of rice evolution. Plant science, **2008**: 174(4):394–408

Vidhyasekaran, P, Borromeo, ES and Mew, TW. *Helminthosporium oryzae* toxin suppresses phenol metabolism in rice plants and aids pathogen. Physiological and molecular plant pathology, **1992**: 41:307–315

Vidhyasekaran, P, Ponmalar, TR, Samiyappan, R, Velazhahan, R, Vimala, R, Ramanathan, a, Paranidharan, V and Muthukrishnan, S. Host-specific toxin production by *Rhizoctonia solani*, the rice sheath blight pathogen. Phytopathology, **1997**: 87(12):1258–1263

Vleeshouwers, VGaa and Oliver, RP. Effectors as tools in disease resistance breeding against biotrophic, hemibiotrophic, and necrotrophic plant pathogens. Molecular plant-microbe interactions, **2014**: 27(3):196–206

Vlot, aC, Dempsey, DA and Klessig, DF. Salicylic acid, a multifaceted hormone to combat disease. Annual review of phytopathology, **2009**: 47:177–206

Vos, IA, Pieterse, CMJ and van Wees, SCM. Costs and benefits of hormone-regulated plant defences. Plant pathology, **2013**: 62:43–55

Walker, EL and Connolly, EL. Time to pump iron: iron-deficiency-signaling mechanisms of higher plants. Current opinion in plant biology, **2008**: 11(5):530–535

Wally, O, Jayaraj, J and Punja, ZK. Broad-spectrum disease resistance to necrotrophic and biotrophic pathogens in transgenic carrots (*Daucus carota* L.) expressing an Arabidopsis *NPR1* gene. Planta, **2009**: 231(1):131–141

Walters, D and Heil, M. Costs and trade-offs associated with induced resistance. Physiological and molecular plant pathology, **2007**: 71(1-3):3–17

Wang, KL, Li, H and Ecker, JR. Ethylene biosynthesis and signaling networks. The plant cell, **2002**: pp. S131–S152

Wang, YY, Hsu, PK and Tsay, YF. Uptake, allocation and signaling of nitrate. Trends in plant science, **2012**: 17(8):458–467

Watanabe, T. Studies on rice blast controlling agent of benzisothiazole analogs. Journal of pesticide science, **1977**: 2:395–404

Watanabe, K, Nagahama, K and Sato, M. A conjugative plasmid carrying the *EFE* gene for the ethylene-forming enzyme isolated from *Pseudomonas syringae* pv. *glycinea*. Phytopathology, **1998**: 88(11):1205–1209

Watanabe, S, Shimoi, E, Ohkama, N, Hayashi, H, Yoneyama, T, Yazaki, J, Fuji, F, Shinbo, K, Yamamoto, K, Sakata, K, Sasaki, T, Kishimoto, N, Kikuchi, S and Fujiwara, T. Identification of several rice genes regulated by Si nutrition. Soil science and plant nutrition, **2004**: 50:1273–1276

Waterhouse, AM, Procter, JB, Martin, DMA, Clamp, M and Barton, GJ. Jalview Version 2-a multiple sequence alignment editor and analysis workbench. Bioinformatics, **2009**: 25(9):1189–1191

Weber, A. Interaction of cytosolic and plastidic nitrogen metabolism in plants. Journal of experimental botany, **2002**: 53(370):865–874

Webster, RK and Gunnell, PS. Compendium of rice diseases. American phytopathological society, St. Paul (MN), USA, **1992**

Weingart, H and Volksch, B. Ethylene production by *Pseudomonas syringae* pathovars *in vitro* and *in planta*. Applied and environmental microbiology, **1997**: 63(1):156–161

Weingart, H, Völksch, B and Ullrich, MS. Comparison of ethylene production by *Pseudomonas syringae* and *Ralstonia solanacearum*. Phytopathology, **1999**: 89(5):360–365

Weingart, H, Ullrich, H, Geider, K and Völksch, B. The role of ethylene production in virulence of *Pseudomonas syringae* pvs. *glycinea* and *phaseolicola*. Phytopathology, **2001**: 91(5):511–518

Wickramasinghe, DB and Rowell, DL. The release of silicon from amorphous silica and rice straw in Sri Lankan soils. Biology and fertility of soils, **2005**: 42(3):231–240

Wingler, a, Lea, PJ, Quick, WP and Leegood, RC. Photorespiration: metabolic pathways and their role in stress protection. Philosophical transactions of the Royal society of London. Series B, biological sciences, **2000**: 355(1402):1517–1529

Wolpert, TJ, Dunkle, LD and Ciuffetti, LM. Host-selective toxins and avirulence determinants: what's in a name? Annual review of phytopathology, **2002**: 40:251–285

Woltering, EJ, Jong, AJ, Yakimova, ET and Hoeberichts, FA. Ethylene: mediator of oxidative stress and programmed cell death in plants. In Biology and biotechnology of the plant hormone ethylene, Vendrell, M., ed. (IOS Press, Amsterdam, NL). **2003**: pp. 315–323

Wood, NT. Nodulation by numbers: the role of ethylene in symbiotic nitrogen fixation. Trends in plant science, **2001**: 6(11):501–502

Wu, JW, Shi, Y, Zhu, YX, Wang,YC, and Gong, HJ. Mechanisms of enhanced heavy metal tolerance in plants by silicon: a review. Pedosphere, **2013**: 23(6):815–825

Wu, Y, Yi, G, Peng, X, Huang, B, Liu, E and Zhang, J. Systemic acquired resistance in Cavendish banana induced by infection with an incompatible strain of *Fusarium oxysporum* f. sp. *cubense*. Journal of plant physiology, **2013**: 170(11):1039–1046

Wubben, MJ, Su, H, Rodermel, SR and Baum, TJ. Susceptibility to the sugar beet cyst nematode is modulated by ethylene signal transduction in *Arabidopsis thaliana*. Molecular plant-microbe interactions, **2001**: 14(10):1206–1212

Xiao, JZ, Tsuda, M, Doke, N and Nishimura, S. Phytotoxins produced by germinating spores of *Bipolaris oryzae*. Phytopathology, **1991**: 81(1):58–64

Xiong, L and Yang, Y. Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid-inducible mitogen-activated protein kinase. The plant cell, **2003**: 15:745–759

Xu, Jr, Urban, M, Sweigard, JA and Hamer, JE. The *CPKA* gene of *Magnaporthe grisea* is essential for appressorial penetration. Molecular plant-microbe interactions, **1997**: 10(2):187–194

Xu, J, Audenaert, K, Hofte, M and De Vleesschauwer, D. abscisic acid promotes susceptibility to the rice leaf blight pathogen *Xanthomonas oryzae* pv *oryzae* by suppressing salicylic acid-mediated defenses. PloS one, **2013**: 8(6): doi:10.1371/journal.pone.0067413

Yamanaka, S, Takeda, H, Komatsubara, S, Ito, F, Usami, H, Togawa, E and Yoshino, K. Structures and physiological functions of silica bodies in the epidermis of rice plants. Applied physics letters, **2009**: 95(12): doi:10.1063/1.3232204

Yang, S, Sweetman, JP, Amirsadeghi, S, Barghchi, M, Huttly, AK, Chung, WI and Twell, D. Novel anther-specific *myb* genes from tobacco as putative regulators of *phenylalanine ammonia-lyase* expression. Plant physiology, **2001**: 126(4):1738–1753

Yang, Y, Qi, M and Mei, C. Endogenous salicylic acid protects rice plants from oxidative damage caused by aging as well as biotic and abiotic stress. The plant journal, **2004**: 40(6):909–919

Yaqoob, M, Mann, RA, Iqbal, SM, Anwar, M and Program, R. Reaction of rice genotypes to brown spot disease pathogen *Cochliobolus miyabeanus* under drought conditions. Mycopath, **2011**: 9(1):9–11

Ye, M, Song, Y, Long, J, Wang, R, Baerson, SR, Pan, Z, Zhu-Salzman, K, Xie, J, Cai, K, Luo, S and Zeng, R. Priming of jasmonate-mediated antiherbivore defense responses in rice by silicon. Proceedings of the national academy of sciences of the United States of America, **2013**: 110(38): doi:10.1073/pnas.1305848110

Yuan, Y, Ou, J, Wang, Z, Zhang, C, Zhou, Z and Lin, Q. Regulation of carbon and nitrogen metabolisms in rice roots by 2-oxoglutarate at the level of hexokinase. Physiologia plantarum, **2007**a: 129(2):296–306

Yuan, B, Shen, X, Li, X, Xu, C and Wang, S. Mitogen-activated protein kinase OsMPK6 negatively regulates rice disease resistance to bacterial pathogens. Planta, **2007**b: 226(4):953–960

Yuan, Y, Zhong, S, Li, Q, Zhu, Z, Lou, Y, Wang, L, Wang, J, Wang, M, Li, Q, Yang, D and He, Z. Functional analysis of rice *NPR1*-like genes reveals that *OsNPR1/NH1* is the rice orthologue conferring disease resistance with enhanced herbivore susceptibility. Plant biotechnology journal, **2007**c: 5(2):313–324

Yun, CH, Sugawara, F and Strobel, GA. The phytotoxic ophiobolins produced by *Drechslera oryzae*, their sturctures and biological activity on rice. Plant science, **1988**: 54:237–243

Zadoks, J. Fifty years of crop protection, 1950-2000. Wageningen journal of life sciences, **2003**: 50(2):181–193

Zargar, SM, Nazir, M, Agrawal, GK, Kim, DW and Rakwal, R. Silicon in plant tolerance against environmental stressors: towards crop improvement using omics approaches. Current proteomics, **2010**: 7:135–143

Zechmeister-Boltenstern, S and Smith, KA. Ethylene production and decomposition in soils. Biology and fertility of soils, **1998**: 26(4):354–361

Zhang, HB, Zhang, DB, Chen, J, Yang, YD, Huang, ZJ, Huang, DF, Wang, XC and Huang, RF. Tomato stress-responsive factor TSRF1 interacts with ethylene responsive element GCC box and regulates pathogen resistance to *Ralstonia solanacearum*. Plant molecular biology, **2004**: 55:825–834

Zhang, CC, Wang, LJ, Nie, Q, Zhang, WX, and Zhang, F. Long-term effects of exogenous silicon on cadmium translocation and toxicity in rice (*Oryza sativa* L.). Environmental and experimental botany, **2008**: 62:300–307

Zhang, Y, Shi, J, Liu, JY, Zhang, JD and Guo, XQ. Identification of a novel *NPR1*-like gene from *Nicotiana glutinosa* and its role in resistance to fungal, bacterial and viral pathogens. Plant biology, **2010**: 12(1):23–34

Zhang, G, Cui, Y, Ding, X and Dai, Q. Stimulation of phenolic metabolism by silicon contributes to rice resistance to sheath blight. Journal of plant nutrition and soil science, **2013**: 176(1):118–124

Zhao, Xc, Qu, X, Mathews, DE and Schaller, GE. Effect of ethylene pathway mutations upon expression of the ethylene receptor ETR1 from Arabidopsis. Plant physiology, **2002**: 130:1983–1991

Zhu, Y, Chen, H, Fan, J, Wang, Y, Li, Y, Chen, J, Yang, S, Hu, L, Leung, H, Mew, TW, Teng, PS, Wang, Z and Mundt, CC. Genetic diversity and disease control in rice. Nature, **2000**: 406(6797):718–722

Zwack, PJ, Robinson, BR, Risley, MG and Rashotte, AM. Cytokinin response factor 6 negatively regulates leaf senescence and is induced in response to cytokinin and numerous abiotic stresses. Plant & cell physiology, **2013**: 54(6):971–981

Curriculum vitae

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Master thesis: "Deciphering silicon-induced resistance against necrotrophic pathogens in rice (*Oryza sativa* L.)"
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2007: Bachelor in Bioscience Engineering: Agricultural Sciences, University of Gent (UGent)

2004: High school diploma (Science and Mathematics), Berkenboom Humaniora, Sint-Niklaas, Belgium

Additional training

Protein Chemistry (1 semester) 2010
Effective Scientific Communication (certified; 20 contact hours) 2010
Introduction to Gene Regulation (1 day) 2013

Professional Record

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PhD thesis: "Silicon-induced brown spot resistance in rice (*Oryza sativa* L.)"
Promotor: Prof. Dr. Monica Höfte

Publications

Peer reviewed:

Van Bockhaven J, De Vleesschauwer D, Höfte M (2013) Towards establishing broad-spectrum disease resistance in plants: silicon leads the way. Journal of experimental botany 64, 1281-93.

Seifi HS, Van Bockhaven J, Angenon G, Höfte M (2013) Glutamate metabolism in plant disease and defense: friend or foe? Molecular plant-microbe interactions. MPMI 26, 475-85.

Conference Proceedings

Van Bockhaven J, De Vleesschauwer D, Höfte M (2011) Silicon-induced brown spot resistance in rice (*Oryza sativa* L.). Communications in agricultural and applied biological sciences 76, 137-40.

Participation to conferences and symposia

Van Bockhaven J, De Vleesschauwer D and Höfte M. Silicon-induced brown spot resistance in rice (*Oryza sativa* L.). V simposio Brasileiro sobre silicio na agricultura,

December 12, 2009, Viçosa, Brazil. Poster presentation

Van Bockhaven J, De Vleesschauwer D and Höfte M. Silicon-induced brown spot resistance in rice (*Oryza sativa* L.). 62nd international symposium on crop protection, May 18, 2010, Gent, Belgium. Poster presentation

Van Bockhaven J, De Vleesschauwer D and Höfte M. Silicon Silicium-geinduceerde resistentie in monocotylen. Studiedag in het kader van AOG primaire plantaardige productie, June 2, 2010, Bottelare, Belgium. Oral presentation by Van Bockhaven J.

Van Bockhaven J, De Vleesschauwer D and Höfte M. Silicon-induced brown spot resistance in rice (*Oryza sativa* L.). Plant stress biotechnology (Belgian plant biotechnology association), December 3, 2010, Leuven, Belgium. Oral presentation by Van Bockhaven J.

Van Bockhaven J, De Vleesschauwer D and Höfte M. Silicon-induced brown spot resistance in rice (*Oryza sativa* L.). 63rd international symposium on crop protection, May 24, 2011, Gent, Belgium. Oral presentation by Van Bockhaven J.

Van Bockhaven J, De Vleesschauwer D and Höfte M. Silicon-induced brown spot resistance in rice (*Oryza sativa* L.). 5th international conference on silicon in agriculture, September 13-18, 2011, Beijing, China. Oral presentation by Van Bockhaven J.

Van Bockhaven J, Brockmans D, De Vleesschauwer D and Höfte M. The influence of nitrogen on brown spot resistance in rice. Plant research @ FBW, May 4, 2012, Gent, Belgium. Oral presentation by Van Bockhaven J.

Van Bockhaven J, De Vleesschauwer D and Höfte M. Silicon-induced brown spot resistance in rice (*Oryza sativa* L.). Silicon-soil-plant-pathogen interaction seminar, February 15, 2012, Louvain-la-Neuve, Belgium. Oral presentation by Van Bockhaven J.

Van Bockhaven J, De Vleesschauwer D, Balzadeh S, Mueller-Roeber B and Höfte M. Silicon-mediated priming results in broad spectrum resistance in rice (*Oryza sativa* L.). 64th international symposium on crop protection, May 22, 2012, Gent, Belgium. Oral presentation by Van Bockhaven J.

Van Bockhaven J, Kikuchi S, Asano T, Höfte M and De Vleesschauwer D. Silicon-induced resistance to the rice brown spot pathogen *Cochliobolus miyabeanus* involves repression of pathogen-induced ethylene action. 6^{th} silicon in agriculture conference, August 26-30, 2014, Stockholm, Sweden. Oral presentation by De Vleesschauwer D.

Van Bockhaven J, Steppe K, Bauweraerts I, Kikuchi S, Asano T, Höfte M and De Vleesschauwer D. Transcriptome analysis of Si-induced resistance in rice against the brown spot pathogen *Cochliobolus miyabeanus*. 6^{th} silicon in agriculture conference, August 26-30, 2014, Stockholm, Sweden. Oral presentation by Van Bockhaven J.

Attended conferences and symposia without participation

61st International Symposium on Crop Protection, May 19, 2009, Ghent, Belgium

Plant hormones: new insights for biotechnology (Belgian plant biotechnology association), November 13, 2009, Gembloux, Belgium

Living together: Plant - Microorganism Biotechnology (Belgian plant biotechnology association), November 18, 2011, Louvain-la-Neuve, Belgium

65th International Symposium on Crop Protection, May 21, 2013, Ghent, Belgium

65th International Symposium on Crop Protection, May 20, 2014, Ghent, Belgium

Awards

Green footsteps award, June 17, 2011
Van Bockhaven J, De Vleesschauwer D and Höfte M. Silicon and broad spectrum disease resistance: one step closer to the holy grail of plant pathology.