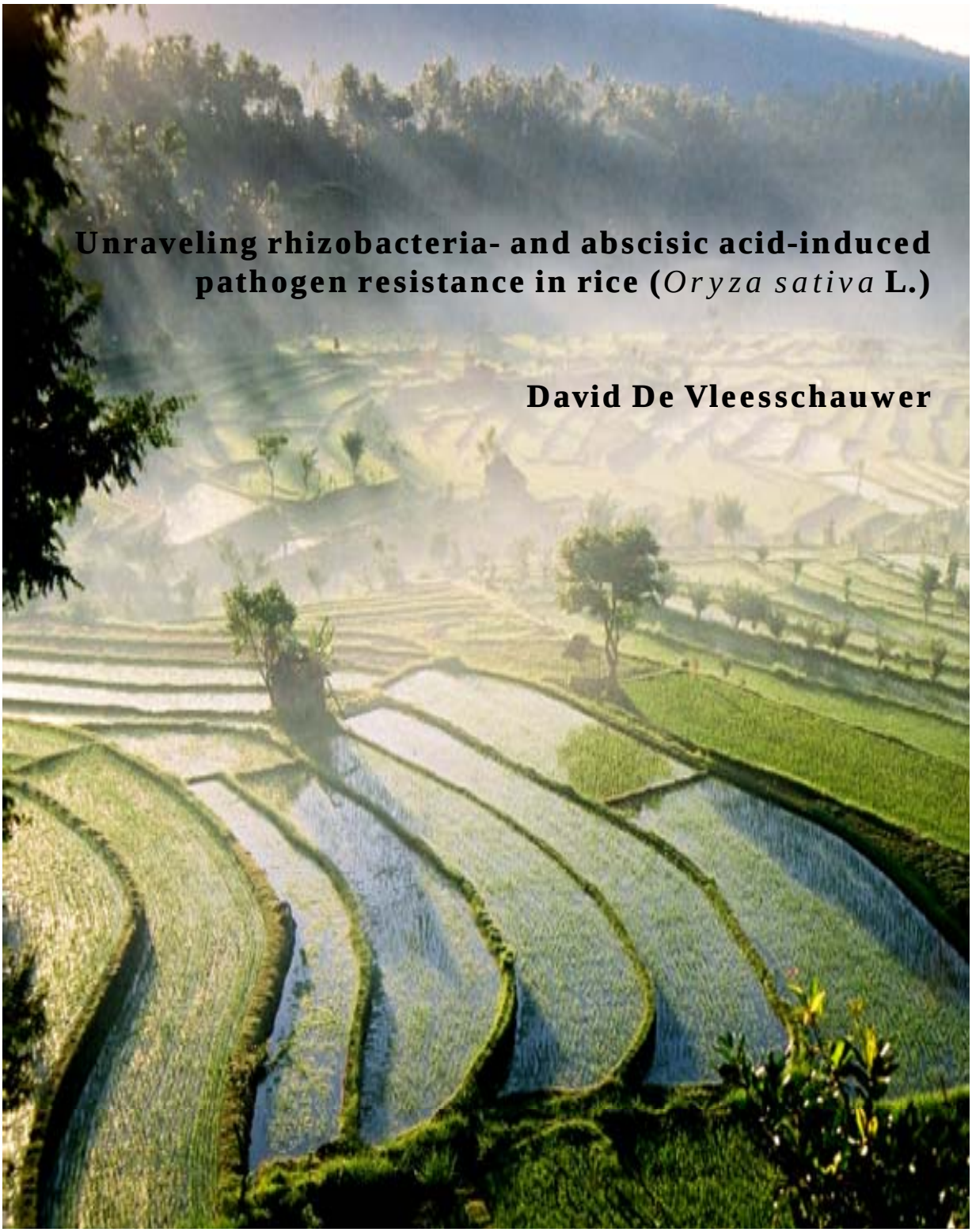

Unraveling rhizobacteria- and abscisic acid-induced pathogen resistance in rice (*Oryza sativa* L.)

David De Vleesschauwer

UNIVERSITEIT GENT

FACULTEIT BIO-INGENIEURSWETENSCHAPPEN

"Science is organized knowledge. Wisdom is organized life"

Immanuel Kant (1724-1804)

Promotor: Prof. Dr. ir. Monica Höfte
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Unraveling rhizobacteria- and abscisic acid-induced pathogen resistance in rice (*Oryza sativa* L.)

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

Dutch translation of the title:

Het ontrafelen van pathogeenresistentie in rijst (*Oryza sativa* L.) geïnduceerd door rhizobacteriën en abscisinezuur

Cover picture:

Illustration of a late afternoon sunset over an idyllic rice field in Inakadate, Japan

De Vleesschauwer D. (2008). Unraveling rhizobacteria- and abscisic acid-induced pathogen resistance in rice (*Oryza sativa* L.) *Phd Thesis*. Ghent University, Belgium.

ISBN-number: 978-90-5989-264-4

Een woordje van dank...

"de laatsten zullen de eersten zijn'...Ook dit cliché is van toepassing op het schrijven van een dankwoord, een moeilijke, niet-exacte wetenschap. Als minst technische, maar ongetwijfeld meest delicate onderdeel, steeds maar weer uitgesteld tot het allerlaatste moment, maar toch op een prominente plaats in dit boekje. Het schrijven van dit dankwoord is het eindpunt, het laatste wapenfeit van vijf lange jaren hard wetenschappelijk labeur. Terugblikkend op mijn bij momenten behoorlijk grillig en turbulent parcours, kan ik enkel maar beamen dat een doctoraat behalen niet zo evident is als het soms lijken kan. Wat aanvankelijk begon als een louter wetenschappelijk avontuur, bleek uiteindelijk een ware levensreis in al zijn facetten. Dat ik deze levensreis tot een min of meer goed einde heb weten te brengen, is in niet geringe mate te danken aan een groot aantal mensen die allen, elk op hun eigengereide manier, steeds bereid waren een handje toe te steken. Vandaar bij deze een woord van dank gericht aan allen die hebben bijgedragen tot dit werk.

In eerste instantie wil ik mijn promotor Monica Höfte bedanken. **Monica**, toen je me zo'n slordige zes jaar geleden polste voor een thesis en bijhorende stage in Vietnam, had ik nooit durven vermoeden dat dit het begin zou vormen van een jarenlange en bijwijlen uiterst harmonieuze en vruchtbare samenwerking die me zowel op wetenschappelijk als persoonlijk vlak veel heeft bijgebracht. Ik wil je dan ook oprecht bedanken voor de talloze discussies annex brainstormsessies die dit werk op een hoger niveau hebben gebracht, voor het minutieus nalezen en corrigeren van mijn schrijfsels, en, niet in het minst, voor de continue steun en talloze aanmoedigingen tijdens dit laatste 'annus horribilis'.

Geachte voorzitter en leden van de examencommissie, dank voor het kritisch nalezen van dit manuscript. Graag houd ik er ook aan **Peter Bakker** te bedanken voor de prettige en vlotte samenwerking. Zonder hem was er van Chapter 5 en het volledige pseudobactin-verhaal immers geen sprake geweest. Ook zonder al de transgene planten en bacteriële mutanten was voorliggend boekje waarschijnlijk een heel pak dunner geworden. Daarom, bedankt **Pierre Cornelis**, **Theresa Pattery**, **Mohammad Djavaheri**, **Leonid Chernin**, **Peter Nick**, **Gynheung An, Makoto Matsuoka** en **Yinong Yang**. Ook **Casiana Vera-Cruz** mag aan dit lijstje niet ontbreken. **Nollie**, thanks a lot for taking such good care of me when I was in the Philippines. My visit to IRRI and the many field trips I participated in were a truly breathtaking and highly inspiring experience. I'll hope to see you soon!

Tevens wil ik alle **collega's** en **ex-collega's** aan het labo Fytopathologie, jaargang 2008 en ver daarvoor, hartelijk bedanken; niet alleen voor de dagdagelijkse hulp, de continue interesse en vele wetenschappelijke discussies, maar bovenal voor de fantastische sfeer die er voor zorgt dat het labowerk nooit een sleur wordt. Merci, iedereen! In het bijzonder wil ik **Lieve** bedanken voor alle welgekomen hulp bij de administratieve rompslomp die inherent verbonden is met een door het IWT-gefinancierd doctoraat, alsook voor de talloze keren dat je goedlachs mijn verzoek tot een of andere obscure en doorgaans behoorlijk laattijdige bestelling hebt ingewilligd. Ook **Ilse** krijgt een speciale vermelding voor al die keren dat ze 'magic-finger gewijs' mijn verloren gewaande experimenten van de afgrond heeft gered, alsmede voor het nalezen van dit doctoraat, iets wat niet meteen tot haar normale takenpakket behoort. **Greet**, ook jij verdient een pluim voor die ontelbare momenten waarop je me geholpen hebt telkens ik weer eens te veel experimenten gepland had en het slachtoffer dreigde te worden van mijn eigen vakkundig georchestreerde chaos. **Soraya**, your scientific knowledge, wisdom in life, and ever present enthusiasm and happy spirit have been an enduring source of inspiration throughout my PhD. Thanks a lot! **Orelvis**, despite being a self-acclaimed Real adept, you turned out to be a great colleague. Good luck with

your PhD, and do never forget: Barça, Barça, hasta la muerte! **Kris**, 'honorary member of the most renowned rice team in Flanders and far beyond', jij was het die me met veel enthousiasme and kunde introduceerde in de wereld van de geïnduceerde resistentie. Naast mijn leermeester werd je echter al snel ook een echte vriend, iets wat zo mogelijk nog verrijkender bleek dan al je wetenschappelijke genialiteit. Ook 'Meniier' **Bob** en 'Mr Secretaris' **Bart** hebben na al die jaren als 'labo fytopath-buddies' een bevoorrecht plaatsje opgeëist in mijn vriendenbestand. Samen bonden we de strijd aan tegen het steeds verder oprukkende oestrogeenleger dat de ons zo geliefde labogangen onveilig maakte. Merci mannen, voor al het zalige 'gezwets en gezwans', de nu al legendarische en met het nodige Bush-bier opgeluisterde kerstmarkt avonturen, de voetbal-georiënteerde cafébezoekjes, en al die andere heerlijke momenten van dolle pret en intens plezier. En Bart, haal je agenda maar al boven, ik geef je afspraak komende zomer bovenop de Ventoux! Finally, I would like to express my deepest gratitude to **Joseph** and **Ninh Thuan**, the most compatible duo science has ever produced. They built the foundations upon which this work stands and taught me everything I needed to know about rice and *Magnaporthe*. Thanks a lot, I owe you big time!

Jeroen, **Bram**, **Daphnis**, **Frederic**, **Stephanie**, **Hanne**, en **Bert**: mijn thesistudenten van de afgelopen vijf jaar. Hoewel ieder van jullie aan den lijve ondervonden heeft dat zelfs het leven van een fytopatholoog niet altijd over rozen loopt, hoop ik toch dat jullie ten volle genoten hebben van jullie al dan niet kortstondig verblijf aan labo Fytopath. En hoewel het misschien niet meteen duidelijk moge zijn, is jullie bijdrage ontzettend constructief geweest voor dit werk. Het ga jullie goed!

Koen en **Tineke**, ik kan me voorstellen dat jullie meer dan eens de wenkbrauwen hebben gefronst bij het aanhoren van de vreemde capriolen van ondertekende wetenschappelijke brokkenpiloot. Desondanks hadden jullie toch steeds een kwinkslag en opbeurend woord klaar. Merci, het is een fantastisch gevoel zulke vrienden te hebben! Ook de rest van de vrienden en (schoon)familie, bedankt voor de vele gezellige en inspirerende momenten, de continue interesse en de talloze aanmoedigingen die ik steeds van jullie kreeg.

Uiteraard reserveer ik hier ook een bijzonder plaatsje voor het thuisfront. **Pa** en **ma**, bedankt voor de niet aflatende steun en de talrijke kansen die jullie me steeds geboden hebben. Ook al konden jullie bij momenten moeilijk vatten hoe iemand zo begeesterd (of is het 'geobsedeerd') kan zijn door een 'rijstplant en wat schimmels', toch waren jullie altijd bereid om bij te springen daar waar het kon. Ik prijs me gelukkig te weten dat ik steeds op jullie kan rekenen. Pa, jij verdient alvast een speciale vermelding voor al die keren dat je me bijstond in je rol als 'rijstboer-hobbyist', voor je doortastende en niet aflatende hulp in mijn zelfverklaarde kruistocht tegen een horde bloeddorstige mijten en, niet in het minst, om me in volle schrijfperiode ten gepaste tijde van achter m'n laptop te komen wegplukken voor een verkwikkende fietstocht, al dan niet gecombineerd met het nuttigen van een frisse Karmeliet. En geloof me vrij, diep vanbinnen ben ik stiekem misschien dan toch niet helemaal ontevreden over mijn 'boekske'.

Tenslotte is er **Hanne**, mijn persoonlijke engel, eeuwige steun en onverstoorbare rots in de branding. De ontelbare momenten die je hebt doorgebracht luisterend naar mijn grote en minder grote laboperikelen, mijn eindeloos geratel en geleuter over 7NSK2, WCS374r, IC1270, PTI en menig ander idioot vakjargon, het stereotiepe en vele nachtelijke tikwerk, mijn depressieve buien, de hulp bij het samplen van een of ander cruciaal experiment middenin een trouwfeest...Eigenlijk valt het niet te overzien wat jij de afgelopen vijf jaar voor me betekend hebt. Bedankt voor alles, zonder jouw steun en alles overwinnende liefde was het me niet gelukt!!!

David

Contents

List of Abbreviations

3-AT: 3-aminotriazole
2-DDG: 2-deoxy-D-glucose
ABA : abscisic acid
ABRE: ABA-responsive elements
ACC : amino cyclopropane carboxylate
AM : arbuscular mycorrhiza
Avr : avirulence
BABA: ß-amino butyric acid
BTH: benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester
CFU: colony-forming units
Cm: *Cochliobolus miyabeanus*
d1: rice mutant defective in the alpha subunit of heterotrimeric G-proteins
DAB: 3,3'-diaminobenzidine
dpi: days post-inoculation
DPI: diphenilene iodonium
EREBP: ethylene-response element binding protein
ERF: ethylene-response factor
ET: ethylene
ETI: effector-triggered immunity
G/GO: glucose/glucose oxidase
hai: hours after infection
HR: hypersensitive response
INA: dichloroisonicotinic acid
IR: induced resistance
ISR: induced systemic resistance
JA: jasmonic acid
KB : King's medium B
LB : Luria Bertiana medium
LOX : lipoxygenase
LPS: lipopolysaccharides
MAMP: microbial-associated molecular pattern
M(A)PK: mitogen-activated protein kinase
MeJA: methyl jasmonate
NADPH: nicotinamide adenine dinucleotide phosphate
NahG: salicylate hydroxylase
NH1: NPR1 homolog 1
NIL: near-isogenic line
NO: nitric oxide
NPR1: non-expresser of PR proteins 1
PAL: phenylalanine ammonia lyase
PAMP: pathogen-associated molecular pattern
PBZ: probenazole
PCA: phenazine-1-carboxylic acid
PCD: programmed cell death
Pch: pyochelin
PDF1.2: plant defensin 1.2
PGPR: plant growth-promoting rhizobacteria

PR: pathogenesis-related
PRR: pattern recognition receptor
Psb: pseudobactin
PTI: PAMP-triggered immunity
Pvd: pyoverdine
Pyo: pyocyanin
R: resistance
ROS: reactive oxygen species
SA: salicylic acid
SAR: systemic acquired resistance
SNARE: specific N-ethyl-malmeimide-sensitive fusion protein attachment protein receptors
T3SE : type III-secreted effectors
TF : transcription factor
WPSR : whole plant-specific resistance
WT: wild-type
X/XO: xanthine/xanthine oxidase
Xoo: *Xanthomonas oryzae* pv. *oryzae*

Glossary

Avirulent
A pathogen strain that carries an avirulence (*Avr*) gene and cannot multiply in a resistant host plant cultivar expressing a complementary resistance (*R*) gene.

Induced Systemic Resistance (ISR)
The phenomenon that plants acquire an enhanced level of resistance against subsequent pathogen attack following root colonization by selected strains of non-pathogenic rhizobacteria.

Systemic Acquired Resistance (SAR)
The phenomenon that plants acquire an enhanced defensive capacity against future pathogen attack as a result of a primary, limited infection with a necrotizing pathogen.

Hypersensitive response (HR)
Rapid collapse (programmed cell death) of cells after attack by an avirulent pathogen.

Oxidative burst
Rapid accumulation of reactive oxygen species (e.g. O_2^-, H_2O_2) with direct antimicrobial activity, but also implicated in plant signaling.

Elicitor
Compound inducing defense responses in plants.

Potentiated
Augmented induction of pathogen- or elicitor-induced plant defense responses.

Primed
State of enhanced ability to mobilize pathogen- or elicitor-induced cellular defense responses.

Biotroph
A pathogen that establishes a long-term feeding relationship with the living cells of the host, rather than killing the host cells as part of the infection process. Typically, biotrophic pathogens grow between the host cells, invading only a few of the cells to produce nutrient-absorbing structures termed **haustoria**. By their feeding activities, they create a nutrient sink to the infection site, so that the host is disadvantaged but is not killed.

Necrotroph
A pathogen that kills living host tissues and feeds on the remains. Necrotrophic pathogens are typically characterized by having a broad host range and are considered insensitive to *R* gene-triggered plant defense responses.

Hemibiotroph
Pathogens that are characterized by an initial period of biotrophy before switching to a necrotrophic growth stage.

Chapter 1

1.1 Problem Statement

Rice is the world's most important staple food grain, providing the bulk of the caloric intake of no less than two billion people living in the rural and urban areas of tropical and subtropical Asia (Leung et al., 2003). During the last few decades, major progress has been made in increasing rice productivity. As a result, world rice production has more than doubled, from a mere 257 million tons in 1996 to over 600 million tons in 2006 (FAO, 2007). This stunning yet much needed rise in production has primarily been achieved through the adoption of modern high-yielding varieties, adequate irrigation, use of fertilizers and other complementary inputs. However, in recent years, population growth has outpaced rice production (Hossain, 1999). Whereas the annual population growth in rice-producing and rice-consuming nations continues to swell, rice yields have stuck fast at approximately 6 tons per hectare in the countries accounting for 75% of the global rice output. If these trends continue, demand for rice in many parts of Asia will outstrip supply within a few years. Such lag in production will disproportionally affect the low-income countries where people consume more rice and the population grows faster.

Diseases caused by pathogenic microbes have always had a significant impact on rice supply. Historically, severe epidemics have led to serious food shortages, claiming the lives of millions. For instance, the great Bengal famine in 1942 was, in part, attributed to an outbreak of brown spot disease (*Cochliobolus miyabeanus*), while rice blast (*Magnaporthe oryzae*) epidemics caused a major food crisis in Korea in the 1970s (Ou, 1985). Nowadays, diseases are still among the major constraints on high rice productivity. Considering the staggering 150 millions of global rice

plantings, even a conservative estimate of 1 to 5% annual yield loss translates into thousands of tons of rice and billions of dollars lost (Mew et al., 2004). For decades, rice disease management systems have relied primarily on the release of new resistant varieties and the application of pesticides. However, whilst the use of hazardous chemicals is environmentally undesirable as well as economically costly, particularly in less-affluent regions of the world, resistant rice cultivars often do not withstand more than one or two years of cultivation before succumbing to diseases, due to either breakdown or gradual erosion of the resistance in face of the high variability of the pathogen population (Mew et al., 2004). Hence, there is considerable incentive to develop new disease control strategies providing durable, environmentally sound, and broad-spectrum pathogen protection. Among such strategies, approaches capitalizing on the plant's own defensive repertoire seem very promising for sustainable rice production in the future (Song and Goodman, 2001).

To resist their potential colonization by microbial pathogens and parasites, plants have evolved a plethora of sophisticated mechanisms to perceive attack by these deleterious microorganisms and to respond adequately by activating an appropriate set of defense responses (Koornneef and Pieterse, 2008). Apart from reacting locally, plants can also establish immunity in systemic tissues, thereby augmenting their defensive capacity against future attack (Van der Ent et al., 2008a). Depending on the organism interacting with the plant, plants are able to activate several types of this so-called induced resistance, including systemic acquired resistance (SAR), which is triggered upon a localized infection with a necrotizing pathogen (Durrant and Dong, 2004), and induced systemic resistance (ISR), which is activated following colonization of the plant roots by selected strains of nonpathogenic rhizobacteria (Van Loon et al., 1998). Contrary to the attacker-specific primary immune response, induced resistance is typically effective against a broad spectrum of otherwise virulent pathogens. Moreover, induced resistance often spreads systemically throughout the plant, thereby protecting the entire plant against subsequent invaders. Unfortunately, compared to the wealth of information on inducible defense responses in dicot plant species, in the class of the *Monocotyledoneae,* including the most important agronomic cereals, molecular information on induced defense mechanisms is still largely missing, this knowledge being key to optimal deployment and commercial acceptance of induced resistance in an agricultural setting. In view of aforementioned knowledge gap, the primary objective of this work was to expand our knowledge on the mechanistic basis of microbially and chemically induced pathogen resistance in rice, a central monocot plant model. In particular, we sought to:

- assess whether rhizobacteria known to elicit resistance in dicots are also capable of triggering resistance in rice against various pathogens exhibiting different modes of infection
- elucidate the bacterial determinants and host effector responses governing the induced resistance phenotype
- explore the tapestry of signaling networks underlying rhizobacteria-mediated systemic resistance in rice
- gain insight into the specificity and compatibility of hormonal signal transduction systems leading to the induced defense state in rice and elucidate if, and how, crosstalk among these signaling conduits affects the interaction of rice with the brown spot pathogen *Cochliobolus miyabeanus*.

1.2. Thesis Outline

This dissertation starts with a comprehensive literature review summarizing our current knowledge on the basic mechanisms and regulation of rice innate immunity (**Chapter 2**). In addition to providing an overview of the various effector mechanisms associated with the establishment and/or maintenance of the induced defense state, we survey recent advances in our understanding of the signaling circuitry orchestrating basal, *R*-gene-mediated and induced pathogen resistance in rice, thereby focusing on the connections and crosstalk between the different phytohormonal networks involved and the role played by such pathway crosstalk in shaping the outcome of rice-pathogen interactions.

Chapters 3, **4** and **5** are dedicated to the phenomenon of rhizobacteria-mediated ISR. In **Chapter 3**, we investigate the bacterial traits and host immune responses associated with induction of ISR by the well-characterized rhizobacterium *Pseudomonas aeruginosa* 7NSK2. Using a set of bacterial mutants defective in the production of several biocontrol-associated metabolites, we pinpoint the redox-active pigment pyocyanin as a two-faced ISR elicitor capable of inducing resistance against the hemibiotrophic rice blast pathogen *Magnaporthe oryzae* while promoting infection by the necrotrophic pathogen *Rhizoctonia solani,* causal agent of rice sheath blight. In addition, evidence is brought forward demonstrating that the differential effectiveness of pyocyanin with respect to 7NSK2-mediated ISR is due to its ability to modulate the plant's oxidative machinery.

In **Chapter 4,** we discuss the resistance mechanisms underlying ISR triggered by the biocontrol bacterium *Serratia plymuthica* IC1270. Pursuing a combined histochemical and pharmacological approach, we show that, like 7NSK2, root colonization by IC1270 locks plant into a pathogen-inducible program of boosted ROS generation, culminating in the prompt expression of hypersensitive response-like cell death at sites of attempted pathogen entry. Highly effective against *M. oryzae*, blocking the pathogen in its hemibiotrophic stage, this H_2O_2-fueled resistance response appears to act as a double-edged sword in the rice induced resistance program as IC1270-colonized plants are rendered hypersusceptible to the necrotrophic pathogens *R. solani* and *Cochliobolus miyabeanus*. As an **addendum** to this chapter, a review paper dealing with the use or potential use of *S. plymuthica* strains as low-input practical agents of plant protection against fungal pathogens is included as well.

Aiming to further dissect the rhizobacteria-induced ISR response, we progress through **Chapter 5** exploring the bacterial determinants and host effector mechanisms underpinning ISR elicited by the *Pseudomonas fluorescens* strain WCS374r. The cumulative results presented in this chapter favor a model in which WCS374r bacteria trigger ISR against *Magnaporthe oryzae* through the secretion of pseudobactin-type siderophores, thereby sensitizing naïve leaves for potentiated expression of a salicylic acid-repressible yet jasmonate/ethylene-dependent multifaceted defense response. In addition to unraveling the signaling circuitry governing WCS374r-induced resistance, this chapter addresses the differences and similarities between WCS374r-mediated ISR and blast resistance induced by the salicylic acid analog benzothiadiazole (BTH).

In the second part of this work, covering **Chapters 6** and **7**, the focus is shifted to the role of the phytohormone abscisic acid (ABA) in modulating plant pathogen defense. Most comprehensively studied as a key endogenous signal functioning in abiotic stress adaptation, ABA has only recently been implicated in the response to biotic challenges. Introducing this topic in **Chapter 6** with a literature review focusing on the principles and mechanisms of ABA's broad and divergent impact on plant disease resistance, we show in **Chapter 7** that pretreatment of rice with ABA confers enhanced resistance against the fungal pathogen *Cochliobolus miyabeanus*, causal agent of the devastating rice brown spot disease. Using a multidisciplinary approach, evidence is provided supporting ABA-mediated repression of pathogen-induced ethylene (ET) signaling as a core resistance mechanism. In addition, we present a novel role for the ABA-inducible mitogen-activated protein kinase gene *OsMPK5* as a critical modulator of this ABA/ET crosstalk, and describe how ABA might interfere with the postulated fungal manipulation of the plant.

Finally, in **Chapter 8**, we briefly recapitulate the main findings and discuss the practical implications and future prospects of the research conducted.

Chapter

2

Defense signaling and induced disease resistance in rice: putting the pieces together

David De Vleesschauwer and Monica Höfte

In preparation

2.1. Introduction

Plant innate immunity is based on a surprisingly complex response that is highly flexible in its capacity to recognize and counteract different invaders. To effectively combat invasion by microbial pathogens, plants have evolved a plethora of sophisticated mechanisms providing several strategic layers of coordinated defenses. Pre-formed structural and physical barriers, as well as inducible plant responses triggered by invariant pathogen- or microbe-associated molecular patterns (MAMPs/PAMPs) constitute the first line of defense and result in a basal level of resistance (Schwessinger and Zipfel, 2008). To achieve their full virulent potential, successful pathogens need to suppress this basal resistance by injecting effector proteins into the host cells that intercept MAMP-triggered defense signals. However, if a plant possesses cognate resistance (R) proteins competent to directly or indirectly recognize these pathogen-secreted effectors, an alternative suite of signaling pathways is activated, culminating in the programmed execution of challenged host cells and rapid containment of the pathogen (Dangl and Jones, 2006).

Triggering of local responses can also mount systemic immunity that primes naïve tissues against subsequent attack. Once initiated, this so-called induced resistance is generally durable and broad-spectrum. Effective induced resistance requires amplification of the primary inducing signal through the generation of secondary messengers such as reactive oxygen and nitrogen species and a range of phytohormones, including salicylic acid, jasmonic acid and ethylene. Rapidly accumulating evidence indicates that the signaling conduits modulated by these endogenous signal molecules do not constitute simple, linear cascades but rather consist of elaborate regulatory networks with frequent crosstalk, allowing the plant to activate an appropriate spectrum of responses depending on the type of intruder encountered (Koornneef et al., 2008).

Historically, research aimed at elucidating the molecular mechanisms underpinning plant immune responses has been polarized towards the use of experimentally tractable dicotyledons, such as Arabidopsis and tobacco. However, the use of rice as an alternative system for studying innate plant immunity is now gaining momentum (Zhou et al., 2006). Primarily fueling this keen interest is the emergence of rice as a pivotal model for cereal crops, many of which are among the world's most important staple foods. This new-born status of rice as a central plant model arises from several key features, including its relatively small genome (430 Mb approximately; Bennetzen, 2002; Goff et al., 2002; Yu et al., 2002), ease of transformation (Kathuria et al., 2007), full genome sequences for both *indica* and *japonica* cultivars, and the availability of myriad tools for reverse genetics such as transposon- and T-DNA-tagged populations (Miyao et al.,

2007; Piffanelli et al., 2007). Furthermore, rice shares extensive synteny and collinearity with other cereal species, further increasing the utility of this system (Devos and Gale, 2000). The use of rice as a model plant therefore offers an unprecedented opportunity to identify and characterize the signaling circuitry and biochemical defenses underpinning biotic stress adaptation in a staple food of world significance, while providing a foundation for comparison with other commercially important crops, such as maize, barley, and wheat. Furthermore, profound knowledge of the mechanistic basis and regulation of inducible immune responses in rice not only promises to offer fundamental insights into the genetic architecture and crosstalk of disease resistance pathways in crop species, but also may guide novel strategies to translate the value of information emerging from such fundamental research for effective utilization of basal and induced resistance phenomena in an agricultural context.

Here, we survey recent progress in our understanding of pathogen-induced defense signaling in rice, with a focus on the main hormonal signaling conduits operative in the rice signaling infrastructure. We also aim to highlight the molecular players that orchestrate the regulatory crosstalk between these conduits and pay special attention to the role of pathway crosstalk in shaping the outcome of rice-pathogen interactions. A thorough overview of the various effector responses associated with the establishment and/or maintenance of the induced defense state is provided as well. For additional background, the reader is referred to some excellent recent reviews (Bostock, 2005; Koornneef and Pieterse, 2008, Angel-Lopez et al., 2008; Van Wees et al., 2008) and book chapters (Van der Ent et al., 2008a), and the concepts and references herein that deal with induced disease resistance and pathogen defense signaling in plants.

2.2. Hormonal signaling cascades involved in the rice defense response

Not more than 15 years ago, it was generally accepted that pathogen-inducible defense mechanisms were steered through a central signaling conduit controlling a multicomponent defense response. Much progress has since been made in understanding plant defense signaling and it is now firmly established that both basal and induced disease resistance are regulated by multiple signal transduction pathways in which phytohormones, such as salicylic acid (SA), jasmonic acid (JA) or ethylene (ET), function as key signaling molecules. In this section, we outline the latest discoveries dealing with hormonal regulation of rice pathogen defense and highlight interactions between hormone signaling, plant defense and microbial virulence.

2.2.1. SA-dependent defense signaling

Salicylic acid is a natural phenolic compound present in many plant species at various levels. In dicots, the role of SA as a key defense signal controlling a variety of inducible immune responses is widely documented (Durrant and Dong, 2004). Following pathogen infection, endogenous levels of SA and its conjugates increase dramatically, immediately preceding the induction of pathogenesis-related (PR) proteins and the onset of local and systemic acquired resistance (Malamy et al., 1990; Metraux et al., 1990; Rasmussen et al., 1991). In rice, however, the role of SA in the signaling network regulating disease resistance is still poorly understood, and even a matter of debate.

Rice plants normally accumulate high levels of free endogenous SA. Under noninducing conditions, SA concentrations in rice leaves (up to 37 μg g^{-1} fresh weight) are at least two orders of magnitude higher than those found in healthy tobacco or *Arabidopsis* (< 0.1 μg g^{-1} fresh weight) (Silverman et al., 1995; Chen et al., 1997). Notwithstanding such high basal SA content, rice plants are not insensitive to exogenously administered SA. For instance, SA is capable of quickly inducing activation of SA-glucosyl transferase, an enzyme that converts free SA into ß-O-D-glucosyl SA (Silverman et al., 1995). In addition, exogenous SA treatment can also induce H_2O_2 accumulation in the veins and interveinal regions of rice leaves, suggesting that SA may promote oxidative stress through the production of reactive oxygen species (ROS; Ganesan and Thomas, 2001).

Another layer of complexity in developing a coherent view of the role of SA in innate rice immunity is added by a series of recent studies demonstrating that rice is endowed with an SA signaling pathway that shares downstream components with the systemic acquired resistance (SAR) pathway in *Arabidopsis*. SAR refers to a pathogen-inducible plant defense response that involves a cascade of transcriptional events induced by SA through the master regulatory protein NPR1 (Durrant and Dong, 2004). To date, five *NPR1*-like genes have been identified in the rice genome, among which *OsNH1* is the closest rice *NPR1* homolog, sharing 60% similarity with Arabidopsis *NPR1* (Chern et al., 2005a; Yuan et al., 2007). As is the case with *AtNPR1* (Chern et al., 2001), ectopic expression of *OsNH1* in transgenic rice conditions resistance to the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), suggesting that rice shares a resistance route similar to the *NPR1*-mediated pathway (Chern et al., 2005b; Yuan et al., 2007b). However, interpretation of the experiments utilizing NPR1/NH1-modified transgenics is not without complication, particularly in light of peculiar phenotypes in certain backgrounds. Although most of the evidence indicates exquisite sensitivity in the plant to respond rapidly to changes in the cellular NPR1/NH1 levels, the data also suggest that there are impacts on yet-ill defined

response pathways when NPR1/NH1 levels are severely altered as in the overexpression transgenics. For example, ectopic expression of *OsNH1* not only confers high levels of resistance to *Xoo*, but also leads to constitutive accumulation of *PR* transcripts and hypersensitivity to light (Chern et al., 2005b). In contrast, defense genes are significantly expressed in rice overexpressing *AtNPR1* only when lesion-mimic spots are visible, a phenotype potentiated by SA and low-light intensities (Fitzgerald et al., 2004). Moreover, in Arabidopsis overexpressing *AtNPR1*, defense genes are not activated until induction by chemical or pathogen treatment (Cao et al., 1998). These conflicting observations suggest a marked difference between rice and Arabidopsis in the regulation of NPR1/NH1-controlled defense gene expression, possibly due to the fact that the endogenous SA levels in rice are several hundred-fold higher than those in Arabidopsis (Silverman et al., 1995; Kogel and Langen, 2005).

Notwithstanding these apparent discrepancies at the level of defense gene induction, several lines of evidence suggest that the core molecular mechanisms underlying NPR1-dependent SA signaling are conserved in rice. For instance, using yeast-two-hybrid assays, Chern et al. (2005b) showed that, analogous to the situation in Arabidopsis, wild-type OsNH1, but not a point mutant corresponding to npr1-1, interacts strongly with rTGA2.2, a rice bZIP transcription factor (TF) homolog. In addition, rTGA2.2 was shown to bind to a *cis*-element required sequence-specifically for SA responsiveness of *PR* gene promoters, implicating a role for this TF in activation of SA-regulated gene expression in rice (Chern et al., 2001; Fitzgerald et al., 2004).

The fact that NPR1/NH1 function in rice is fairly similar to that in Arabidopsis begs the question of whether SA has a central role in the induced resistance program of rice. In support of such a role, some studies claim that exogenous SA application can induce partial resistance against the rice blast pathogen *Magnaporthe oryzae*, albeit to a much lesser extent than its functional analogs BTH, dichloroisonicotinic acid (DCINA) and probenazole (Sakamoto et al., 1999; Manandhar et al., 2000; Kogel and Langen, 2005; Iwai et al., 2007). On the other hand, SA levels do not change significantly after infection with either compatible or incompatible rice pathogens, which suggests that SA is not a limiting factor in the signaling circuitry leading to disease resistance in rice (Silverman et al., 1995). This view is corroborated by the finding that depletion of high levels of endogenous SA in transgenic NahG rice expressing the SA-degrading salicylate hydroxylase gene does not measurably affect *PR* transcript accumulation (Yang et al., 2004). Interestingly, SA-deficient NahG rice contains elevated levels of reactive oxygen species (ROS) and exhibits spontaneous lesion formation in an age- and light-dependent manner. Moreover, SA-deficient rice is hypersusceptible to avirulent but not virulent blast isolates, an effect that the investigators attribute to the reduced capacity of the NahG transgenics to cope

with the strong oxidative burst elicited by avirulent pathogens (Yang et al., 2004). Therefore, rather than being an effective signal for activation of defense genes and induced resistance, the high-level endogenous SA in rice may act as a preformed antioxidant, regulating cellular redox balance and protecting rice plants from oxidative stress (Yang et al., 2004).

2.2.2. JA-dependent defense signaling

Jasmonic acid and its metabolites, collectively known as jasmonates (JAs), are important lipid-derived regulators that modulate a number of vital physiological processes, including wound responses, secondary metabolite biosynthesis, and defense against herbivorous insects (Cheong and Choi, 2003; Dombrecht et al., 2007). Although the role of JA in disease resistance has been comprehensively studied in dicotyledoneous plants (Pozo et al., 2005; Kazan and Manners, 2008), surprisingly little is known about its function in the defense response of rice and other economically important monocot plants. To date, most of the information comes from studies investigating the effect of exogenous JA or methyl jasmonate (MeJA) treatments on the induction of defense-related host effector responses. Early experiments showed that addition of JA or MeJA to rice cell suspension cultures induced multiple stress- or defense-associated transcripts and elicited the accumulation of the phytoalexins momilactone A and sakuranetin (Nojiri et al., 1996; Tamogami et al., 1997, 2000; Jwa et al., 2006). Moreover, the activity of lipoxygenase, a key enzyme in the octadecanoid pathway leading to the synthesis of JA, was activated earlier and to a higher extent in incompatible interactions between rice and *M. oryzae* than in compatible interactions (Ohta et al., 1991; Peng et al., 1994), while transcripts of *RCI-1*, a chloroplastic lipoxygenase, were found to be specifically upregulated upon plant treatments with chemical inducers of acquired resistance, such as BTH, INA and probenazole (Schaffrath et al., 2000). Corroborating these findings, Mei et al. (2006) recently demonstrated that pathogen-inducible overexpression of a rice allene oxide synthase gene, encoding a key JA biosynthetic enzyme, results in elevated JA levels, increased *PR* gene transcript accumulation, and significantly enhanced resistance to *M. oryzae* infection. Increased levels of JA, followed by induction of defense-related genes and enhanced blast resistance was also observed in rice leaves exogenously treated with INA or wounding (Schweizer et al., 1997a,b; Schweizer et al., 1998). Together these observations constitute a large body of circumstantial evidence supporting a role for the octadecanoid signaling pathway and, hence, JA action, in defense gene activation and disease resistance responses in rice.

Yet, a major challenge in finding coherence among models for JA-responsive defense signaling is that individual investigators may obtain seemingly different results with the same or similar system. The example of JA-induced resistance to *M. oryzae* is a case in point. Whereas Schweizer et al. (1998) found that exogenous application of JA was able to induce resistance to *M. oryzae* in systemic, but not in local, treated leaves, Mei et al. (2006) observed the opposite phenomenon, with clear induction of *PR1* and induced resistance in local, JA-treated tissues, rather than in systemic, naïve plant parts. Moreover, another recent study found little evidence of JA-conditioned blast resistance in either local or systemic leaves (Ahn et al., 2005), indicating that conditions for expression of JA-inducible resistance are critical. Further complexity in the signaling network controlling JA-responsive defenses in rice is evident from the observation that pathogen-induced activation of jasmonate-responsive *PR* genes is not associated with an increase in endogenous JA levels, whereas reduction of endogenous JA levels with tetcyclacis, an inhibitor of JA biosynthesis, significantly reduces infection-triggered *PR* transcription (Schweizer et al., 1997b; Mei et al., 2006). However, these apparently contradictory results may be reconciled by assuming that JA is embedded in an interactive regulatory signaling network, resulting in the cooperative induction of *PR* genes upon pathogen attack. In this model, JA may not need to accumulate but be required at a certain minimal level for activation of a specific subset of JA-responsive *PR* genes. In other words, it is not unlikely that the octadecanoid pathway may function as an enhancer of pathogen-induced defense reactions in rice. This view is consistent with other findings reporting a role for JA in priming defense gene expression and plant protection induced by physiologically relevant, non-toxic doses of the synthetic blast resistance inducer dichloroisonicotinic acid (INA) (Schweizer et al., 1997a). Nevertheless, owing to the inherent complexity of biotic stress-response signaling, assays using mutant or transgenic rice lines impaired in JA biosynthesis or signal perception are imperative to unequivocally delineate the role of JA and its position within the signal transduction paths leading to the expression of rice disease resistance in various contexts.

2.2.3. Ethylene-dependent signaling

Rice farmers and researchers have long observed the phenomenon of partial resistance to blast in rice growing in anaerobic conditions such as moisture-saturated soils or flooded paddies. Drought stress and upland culture conditions increase severity of rice blast in disease-susceptible cultivars (Singh et al., 2004), whereas flood conditions reduce the number of blast lesions and flatten disease gradients (Kim et al., 1985; Lai et al., 1999). However, until recently, the mechanism behind this phenomenon remained elusive.

Biotic and abiotic stresses, including pathogen infection and anaerobic conditions caused by water submergence, induce the biosynthesis of the simple gaseous hormone, ethylene (ET). For instance, submergence leads to activation of *OsACS1* and *OsACS5*, two rice ET biosynthetic genes, culminating in rapid ET accumulation (Zarembinski et al., 1997; Van der Straeten et al., 2001). In addition, topical application of Ethephon (2-chloroethylphosphonic acid), an ethylene-releasing chemical, was shown to increase resistance in blast-susceptible rice cultivars (Singh et al., 2004), indicating a circumstantial association between ET accumulation and disease resistance. Further evidence for the involvement of ET biosynthesis in rice resistance to *M. oryzae* comes from Iwai and associates (2006), who demonstrated that accumulation of ET and its coproduct, cyanide, are indispensable for *R*-gene-mediated resistance to blast in young rice plants.

During the past decade, several key components of the ET signal transduction pathway have been successfully identified and characterized in *Arabidopsis* and tomato using various genetic approaches (Broekaert et al., 2006). Orthologs have also been found in other plants species, including rice (Chen et al., 2005). Furthermore, a dominant negative mutant of the *Arabidopsis* ET receptor gene *ETR1* confers ethylene insensitivity in heterologous plants, including tobacco (Knoester et al., 1998), tomato and petunia (Wilkinson et al., 1997), suggesting the universal existence of the ET signaling pathway throughout the plant kingdom (van Loon et al., 2006a). In addition to the *ETR1* receptor gene, *EIN2* encodes an integral membrane protein that plays a central regulatory role in ET signaling. Recessive loss-of-function mutations in the *Arabidopsis EIN2* gene block ethylene responses completely (Alonso and Stepanova, 2004; van Loon et al., 2006a). Based primarily on sequence similarity, two *EIN2* orthologs, which share, respectively, 57 and 32% sequence identity with AtEIN2, were isolated from rice (Jun et al., 2004; Zhou et al., 2004). *OsEIN2* antisense lines and *OsEIN2-2* RNAi lines exhibit ethylene insensitivity as reflected by reduced shoot elongation and a decreased expression of ethylene-responsive genes, suggesting that both genes are integral to ethylene signaling in rice. Interestingly, silencing of *OsEIN2-2* results in increased susceptibility of the RNAi lines to *M. oryzae* and *Burkholderia glumae* (Zhou et al., 2006), whereas antisense suppression of *OsEIN2* was recently shown to have a positive effect on basal resistance of rice to the fungal brown spot pathogen *Cochliobolus miyabeanus* (Chapter 7). Taken together with the opposite effects of Ethephon application on blast and brown spot development (Singh et al., 2004; Chapter 7), these data support a model in which ET acts as a two-faced defense regulator that alleviates stress caused by *M. oryzae* and *B. glumae* while suppressing basal resistance to *C. miyabeanus*.

2.2.4. Abscisic acid signaling pathway

Abscisic acid not only governs a variety of growth and developmental processes, including seed development and regulation of stomatal aperture, but also plays a crucial role in the initiation of adaptive responses to salt, drought, osmotic and cold stress (Fujita et al., 2006). In addition to this well-documented function in the response to abiotic stress, a fast-growing number of reports indicate that ABA is also prominently involved in regulating and integrating biotic stress-response signaling networks. Although both positive and negative effects of ABA on plant immune responses have been reported, ABA predominantly behaves as a negative regulator of disease resistance, with ABA deficiency resulting in enhanced resistance to an array of pathogens in several plant species (Chapter 6; Mauch-Mani and Mauch, 2005). Moreover, elegant research by de Torres-Zabala et al. (2007) recently revealed that *Pseudomonas syringae* specifically targets the ABA signaling pathway to cause disease in Arabidopsis, suggesting that ABA is a susceptibility factor for this bacterium. Curiously, this finding echoes a previous report in rice where foliar application of ABA or abiotic stress treatment was shown to enhance susceptibility to *M. oryzae* through suppression of the so-called 'whole-plant-specific resistance', an age-related resistance phenomenon that is observed exclusively in intact rice seedlings (Koga et al., 2004). Consonant with this is the finding that exogenous ABA, when applied to young rice seedlings, antagonizes transcription of the defense-related *Rir1b* gene and attenuates probenazole-induced resistance to *M. oryzae* (Schaffrath et al., 2000; Cooper et al., 2003). Additional data supporting the notion that ABA plays a negative role in the rice defense signaling network comes from some intriguing work by the Yang lab, demonstrating that RNAi suppression of *OsMPK5*, an ABA-inducible mitogen-activated protein kinase, leads to constitutive *PR* gene expression and enhancement of resistance to *M. oryzae* and the bacterial pathogen *Burkholderia glumae* (Xiong and Yang, 2003; Zhou et al., 2006). However, ABA does not appear to condition susceptibility against all rice pathogens, as the same RNAi lines were found to be compromised in the expression of ABA-inducible resistance to *C. miyabeanus* (Chapter 7). Like ethylene, ABA thus seems to play an ambivalent and widespread role in modulating rice defenses, acting as either a positive or negative regulator of disease resistance by interfering at multiple levels with biotic and abiotic stress signaling cascades.

2.2.5. Gibberellin signaling pathway

Gibberellins (GAs) are diterpenoid plant hormones that act at all stages in the plant life cycle by promoting germination, hypocotyl elongation, root, leaf, stem, and fruit growth, greening of leaves, flowering, and flower and seed development. The origin of research into GAs can be

traced back to Japanese plant pathologists in the late 1800's who were studying a devastating rice disease referred to as 'bakanae' (foolish seedling). Symptoms of the disease included exceptionally tall seedlings that toppled over before they had a chance to mature and flower, slender leaves, and stunted roots. In 1926, Eicchi Kurosawa tied the elongation of bakanae-infected rice seedlings to a stimulus derived from the fungus *Gibberella fujikuroi* (=*Fusarium moniliforme*). Later on, the stimulus was crystallized and named 'gibberellin' after the fungus it was isolated from. However, it was not until the mid 1950s that researchers became aware of gibberellins as naturally occurring substances in higher plants.

Research over the past few years has uncovered the principal steps associated with GA perception and signal transduction in rice and *Arabidopsis* (Fig. 2.1.). Current concepts suggest that GA promotes plant growth by inducing the degradation of DELLAs, a group of nuclear growth-repressing proteins belonging to the plant-specific GRAS superfamily (Harberd, 2003). In rice, binding of bioactive GA to the soluble GA receptor GID1 induces interaction with the only DELLA protein present, SLR1 (Hartweck and Olszewski, 2006; Ueguchi-Tanaka et al., 2007). The stabilized trio-complex consisting of GA, GID1, and SLR1 is then targeted for ubiquitination by the F-box protein GID2, resulting in rapid degradation of SLR1 by the 26S proteasome, thereby relieving the DELLA-mediated growth restraint (Eckardt, 2007). The same pathway is operative in *Arabidopsis* with three GA receptors (GIDa, GIDb, and GIDc), five DELLA proteins (RGA, GAI, RGL1, RLG2, and RLG3), and the F-box protein SLY1 (for review see Jiang and Fu, 2007).

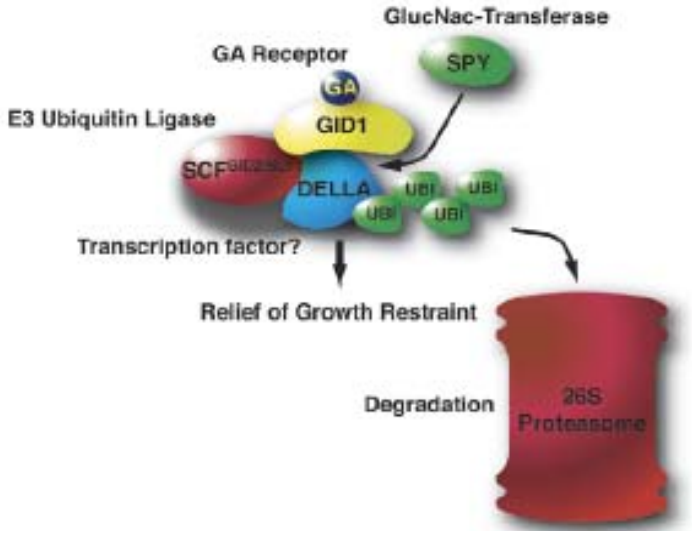

Figure 2.1. Model depicting the various interactions among the established GA signaling components (Schwechheimer, 2007). GA, when bound to the GID1 GA receptor, induces the interaction of the GA receptor with the DELLA proteins. This is followed by DELLA ubiquitylation via $SCF^{DELLA/GID2}$ and DELLA degradation by the 26S proteasome (UBI, ubiquitin). How $SCF^{DELLA/GID2}$ gains its DELLA protein specificity in response to GA binding is not yet understood.

In contrast to its well-documented role as a plant growth regulator, GA has only recently been implicated in biotic stress-response signaling (Smirnoff and Grant, 2008). In some elegant work, Navarro et al. (2008) demonstrated that loss-of-function mutations in DELLA proteins render *Arabidopsis* more resistant to the bacterial speck pathogen *P. syringae* pv. *tomato* (Pst) through priming of the SA-dependent defense pathway. By contrast, the same set of mutants proved to be enhanced susceptible to the necrotrophic pathogen *Alternaria brassicicola*, a phenomenon which correlated with attenuated induction of the JA-reporter gene *PDF1.2*. On the basis of these and other findings, the authors contemplated that DELLAs promote resistance to necrotrophs and susceptibility to biotrophs, partly by modulating the SA/JA balance (Navarro et al., 2008). In rice, there are currently no published reports exploring the involvement of GAs or DELLA in the context of plant-pathogen interactions. However, a series of studies by Day and colleagues (2003, 2004) marks the first identification of two other members of the GRAS gene family, i.e. *CIGR1* and *CIGR2*, as having a possible involvement in attacker-induced defense responses. Both genes, which are rapidly inducible by bio-active GA, were shown to be dramatically upregulated upon exposure of rice cells to a *M. oryzae*-derived chitin elicitor, but not following challenge with bacterial pathogens, implicating the necessity for a fungal-specific factor in the signaling pathway leading to induction of *CIGR1* and *CIGR2* expression. Interestingly, localization experiments using GFP-fusions of both CIGR1 and CIGR2 in a transient onion assay confirmed the nuclear localization of both proteins, supporting a possible role of each as transcriptional regulators in elicitor-induced defense responses. As GA-induced activation of *CIGR1/2* is impaired in *slr1-1* mutant plants (Day et al., 2004), it will be interesting to assess whether SLR1 in turn acts as a transcriptional activator of *CIGR1* and *CIGR2* prior to its degradation in response to GA.

2.2.6. Auxin signaling pathway

Like gibberellins, auxin regulates almost every aspect of plant development. At the molecular level, auxin induces gene expression through direct physical interaction with TIR1-like F-box receptor proteins (Quint and Gray, 2006). These interactions catalyze the destruction of members of the AUX/IAA family of transcriptional repressor proteins via the SCF (Skp1-Cullin-F-box) E3-ubiquitin ligase proteasome pathway (Gray et al., 2001; Dharmasiri et al., 2005; Kepinski and Leyser, 2005). The degradation of AUX/IAA proteins in turn allows activation of Auxin Response Factors (ARFs), leading to expression of auxin-responsive genes (Hagen and Guilfoyle, 2002). Interestingly, a growing body of evidence indicates that some pathogens either produce auxin themselves or increase plant auxin biosynthesis upon infection to manipulate the

host's defensive and developmental machinery (Glickmann et al., 1998; Maor et al., 2004; Valls et al., 2006). Deregulation of auxin-responsive genes also occurs upon treatment with the PAMP surrogate flg22 (Navarro et al., 2006). Flg22 recognition by the plant triggers the upregulation of a canonical microRNA (miR393) that hampers the formation of F-box proteins, thereby contributing to the downregulation of auxin signaling (Navarro et al., 2006). Moreover, augmenting auxin signaling through overexpression of a TIR1 paralog that is partially refractory to miR393 renders Arabidopsis more susceptible to *Pseudomonas syringae*, while attenuation of auxin signaling through miR393 overexpression increases resistance to the latter pathogen. These findings indicate that repression of auxin signaling is an integral component of the bacterial-induced plant immune response (Navarro et al., 2006). Notably, in some interesting work using whole-genome transcription profiling, Wang et al. (2007) recently reported that plant treatment with SA causes a stabilization of AUX/IAA repressor proteins and inhibition of auxin responses, suggesting that SA antagonizes the auxin signaling pathway as part of the plant defense mechanism.

Consistent with auxin promoting disease susceptibility in dicots, Ding et al. (2008) recently uncovered auxin as an important virulence factor in *Xoo*-induced disease on rice. *Xoo* infection was found to induce expression of several auxin biosynthesis-related genes, leading to local accumulation of the main auxin indole-3-acetic acid (IAA). Curiously, *Xoo*-induced IAA accumulation triggered the induction of several expansins, a highly conserved multigene family of cell wall proteins that mediate pH-dependent wall loosening (Humphrey et al., 2007). Although loosening the cell wall is a vital process during auxin-regulated plant growth and development, it may also render the plant more vulnerable to biotic intruders by facilitating pathogen entry or allowing enhanced nutrient leakage. Indeed, disease tests with expansin-overexpressing rice plants revealed enhanced susceptibility, suggesting that *Xoo*-induced auxin-stimulated expansin production may be one of the mechanisms used by this pathogen to weaken the rice cell wall and, hence, inflict disease. In line with this assumption, comparative analysis of the defense mechanisms in compatible and incompatible rice-*Xoo* interactions demonstrated that resistant rice plants counteract *Xoo*-induced cell wall disturbance by suppressing attacker-induced auxin signaling through hyperactivation of the IAA-conjugating amido synthetase GH3-8 (Ding et al., 2008). Whether the auxin response pathway also impacts rice resistance to necrotrophic pathogen attack, as was recently shown in *Arabidopsis* (Llorente et al., 2008), remains to be explored.

2.2.7. Brassinosteroid signaling pathway

About a decade ago, the discovery of brassinosteroid-deficient Arabidopsis mutants uncovered brassinosteroids (BRs) as a novel class of polyhydroxylated phytohormones with important roles in regulating various cellular and developmental processes (Clouse and Sasse, 1998). Since then, molecular genetic studies in both *Arabidopsis* and rice have established a detailed model of the BR signaling pathway leading from BR perception at the cell surface to regulation of transcription in the nucleus (Fig. 2.2). According to current concepts, BRs directly bind to the extracellular domain of the receptor kinase BRI1 to activate its kinase activity and promote heterodimerization with, and phosphorylation of, another receptor kinase, BAK1 (Kinoshita et al., 2005; Nakamura et al., 2006). Downstream of these receptor kinases, the BIN2 kinase and BSU1 phosphatase regulate the phosphorylation status of the homologous transcription factors BZR1 and BZR2. BRs activate BZR1 and BZR2 by inducing their dephosphorylation, possibly by inhibiting BIN2 or activating BSU1. Finally, activated BZR1 and BZR2 directly bind BR-responsive promoters, causing transcriptional changes that ultimately increase plant growth and reduce BR biosynthesis (Gendron and Wang, 2007). Recently, 14-3-3 proteins joined the list of signaling components with a role in BR signaling (Gampala et al., 2007). 14-3-3s are highly conserved phosphopeptide-binding proteins that interact with a vast array of cellular proteins in a sequence-specific and phosphorylation-dependent manner. By using yeast-two-hybrid screens and a range of protein-protein interaction assays, Bai et al. (2007) recently demonstrated that all eight rice 14-3-3 proteins modulate BR signaling by specifically inhibiting the function of phosphorylated BZR1 through cytoplasmic retention. Interestingly, evidence is accumulating that the same set of 14-3-3 proteins is also prominently involved in the response to pathogens, thereby providing a potential functional interface between BR signaling and rice defense. For example, at least four 14-3-3 proteins were reported to be differentially regulated in interactions of rice with *Xoo* and *M. oryzae*, with the corresponding genes being expressed faster and/or to a higher extent in incompatible interactions versus compatible ones (Cooper et al., 2003; Chen et al., 2006). Moreover, recent findings demonstrate that silencing of the 14-3-3 protein gene *GF14e* induces susceptibility to *R. solani* and resistance to *Xoo* (Bruce et al., 2008). A more direct link between BRs and rice innate immunity, however, is provided by Nakashita et al. (2003), who showed that treatment of tobacco or rice with BRs induces resistance against a variety of hemibiotrophic pathogens, among which *M. oryzae* and *Xoo*. Interestingly, this increase in resistance was independent of SA accumulation and *PR* gene expression, suggesting that BRs regulate rice disease resistance through an SA-independent pathway. One explanation for this SA-independency may lie in the particularly complex interplay

of BRs with other plant hormones, such as JA, ET, ABA and auxin (Krishna, 2003). Of particular interest in this regard is that some fungal pathogens are known to synthesize toxins that closely resemble steroid hormones such as zealarenone (Robert-Seillaniantz et al., 2007). Analogous to the situation with ABA and auxin, it is not unlikely that pathogenic microbes employ such BR mimicry as a virulence strategy to tap into the plant's signaling infrastructure to interfere with host defense.

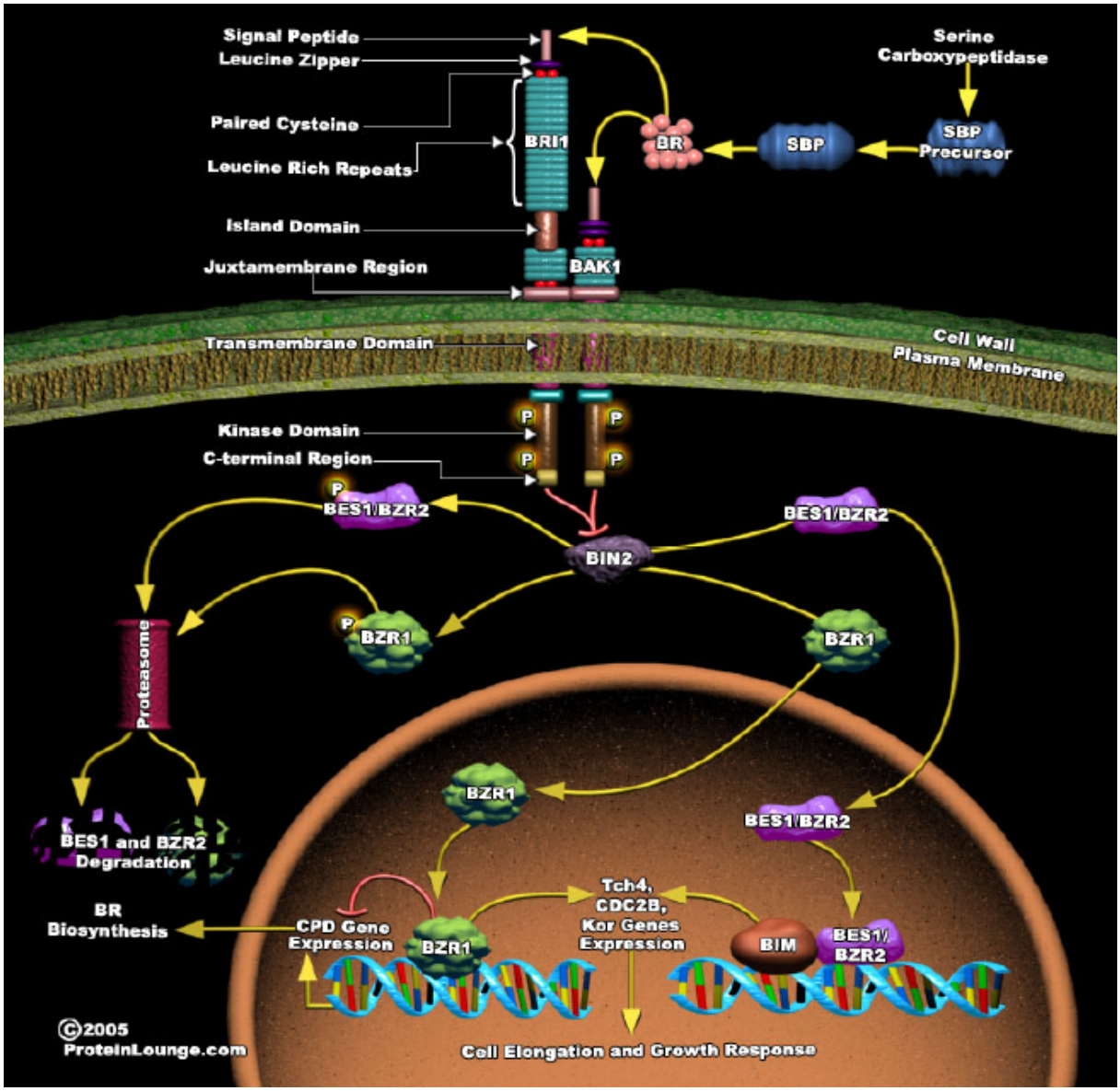

Figure 2.2. Model describing BR signaling (source http://www.ProteinLounge.com).

In the absence of BRs, the kinase domains of the BRI1 homodimer are inhibited by both their own C-terminal domain and by an interaction with BKI1. This allows BIN2 to phosphorylate and inactivate the brassinosteroid response transcription factors (BRFs), including BZR1. Direct binding of BL to BRI1 results in conformational changes of the kinase domain, leading to the phosphorylation of the C-terminal domain of BRI1 and phosphorylation of BIK1, which causes displacement of BKI1 from the plasma membrane and the release of autoinhibition of BRI1. These events lead to BRI1's association with BAK1 and consequent activation of the receptor. The active signaling receptor complex inhibits the activity of BIN2, allowing dephosphorylation of the BRFs by BSU1 and activation or repression of their target genes.

2.3. Additional circuit makers: a role for defense-related MAP kinases, transcription factors and small GTPases

2.3.1. The MAPK signaling paradigm

Integration of the vast array of cellular processes that enable plants to grow, reproduce and fend off microbial pathogens requires the coordinated activity of an elaborate matrix of signal transduction proteins, within which one of the most prominent super-families consists of the protein kinases (Hamel et al., 2006). Within this superfamily, the mitogen-activated protein kinases (MAPKs) form a distinctive and highly conserved subfamily. A particular MAPK cascade minimally consists of three functionally interlinked protein kinases: a MAPK, a MAPK kinase (MAPKK), and a MAPK kinase kinase (MAPKKK). This hierarchical organization allows MAPK cascades to operate as core signal transmission modules capable of efficiently amplifying, integrating and channeling information between the cellular environment and the metabolic and transcriptional response centers (Hamel et al., 2006). Interestingly, mounting biochemical and genetic evidence points to a complex network organization in which kinases at one level can harmonize input signals from more than one upstream effector and can, in turn, act upon more than one target, thereby creating a remarkably versatile matrix of signaling capacities (Cardinale et al., 2002; Nakagami et al., 2005). To date, a total of 20 rice genes encoding MAPK cascade components have been isolated and partially characterized. These include one MAPKKK (OsEDR1) (Kim et al., 2003), two MAPKKs (OsMEK1) (Kim et al., 2000; Wen et al., 2002), and 17 MAPKs (Rohila and Yang, 2007). Most are activated by defense signal molecules and/or pathogen infection and are, directly or indirectly, implicated in the rice defense response (for a comprehensive review see Rohila and Yang, 2007).

In 1999, He et al. isolated the first MAPK in rice as a *M. oryzae*- and wound-inducible protein, and accordingly designated it as OsBWMK1 (blast- and wound-induced MAP kinase). More recently, OsBWMK1 was shown to target and phosphorylate OsEREBP1, an ethylene responsive element binding protein transcription factor (Cheong et al., 2003). Interestingly, the resulting phosphorylated OsEREBP1 exhibited enhanced DNA-binding capacity to the GCC box element (AGCCGCC) present in the promoters of several basic *PR* genes. Moreover, ectopic expression of OsBMWK1 in tobacco resulted in enhanced *PR* transcript accumulation and significantly enhanced resistance to the oomycete *Phytophthora parasitica* var. *nicotianae* and the bacterial pathogen *Pseudomonas syringae* pv. *tabacci*. These findings suggest that OsBWMK1 modulates defense gene activation and disease resistance through phosphorylation of one or more EREPB-like transcription factors (Cheong et al., 2003). However, considering the fact that

the OsBWMK1 promoter contains several *cis*-acting regulatory elements known to be involved in biotic and abiotic stress adaptation (Hong et al., 2007), it is tempting to speculate that OsBWMK1 may exert a more versatile role in governing rice responses to a variety of environmental cues, thereby possibly serving as a node of convergence. Consistent with this hypothesis, *OsBWMK1* expression was recently shown to be differentially regulated by cold, drought, dark and JA, in addition to other signaling molecules, including SA, BTH, and fungal elicitors (Hong et al., 2007).

Presumably the most extensively studied among all of the rice MAPKs, OsMPK5 (also known as OsMSRMK2, OsMAPK2, OsMAPK5, OsBIMK1, or OsMAP1) has been independently isolated by at least five research groups and was shown to be induced at the mRNA level by a variety of biotic and abiotic stresses (Xiong et al., 2001; Agrawal et al., 2002; Huang et al., 2002; Song and Goodman, 2002; Wen et al., 2002). Recently, *OsMPK5* was also linked with spontaneous cell death in the Sekiguchi lesion-mimic mutant (Reyna and Yang, 2006). Interestingly, OsMPK5 seems to function at the intersection of a few different signaling pathways, as suppression of *OsMPK5* expression and its kinase activity abates abiotic stress tolerance while increasing resistance to *M. oryzae* and *Burkholderia glumae* (Xiong and Yang, 2003). Another well-studied rice MAPK with respect to pathogen-induced defense signaling is OsMPK1. In some elegant work, Lieberherr et al. (2005) reported this kinase to be central to PAMP signaling activated upon perception of a *M. oryzae*-derived sphingolipid elicitor. One interesting finding in this study is that RNAi suppression of *OsMPK1* culminates in enhanced expression of *OsMPK5*, suggesting a potential crosstalk and possible functional redundancy between these evolutionary-related MAPKs. The authors also demonstrated that OsMPK1 functions downstream of the small GTPase OsRac1 and heterotrimeric G-protein, both of which are master regulatory proteins in the rice defense response (Suharsono et al., 2002; Thao et al., 2007). In this context, it is worth noting that OsMPK1 is the closest homolog of MPK6, which has recently been identified as a positive regulator of broad-spectrum disease resistance in Arabidopsis (Menke et al., 2004). However attractive, a similar role for OsMPK1 in the rice defense network is rather unlikely since silencing of *OsMPK1* had no marked effect on the response of the silenced plants to either virulent or avirulent isolates of *M. oryzae* (Lieberherr et al., 2005). Most recently, OsMPK6 was added to the list of MAPKs with a putative role in rice innate immunity. Yuan et al. (2007a) reported that inactivation of *OsMPK6* in mutant *mpk6* plants resulted in elevated levels of SA, constitutive expression of OsWRKY03- and OsNH1-dependent defense genes, and spontaneous development of a lesion-mimic/cell death phenotype. Moreover, both *mpk6* knockout and *MPK6* RNAi plants displayed an enhanced

resistance to different races of *Xoo*, suggesting that OsMPK6 functions as a negative regulator of resistance to *Xoo,* possibly via repression of the OsNH1-dependent SA response. In addition to aforementioned MAPKs, various other MAPK cascade components have been shown to be differentially regulated in response to wounding and/or pathogen infection, including interactions with avirulent pathogens (Rohila and Yang, 2007). However, additional research is needed to determine if and how these kinases operate in connection with induced defense pathway regulation and signal crosstalk.

2.3.2. Transcription factors and regulation of defense

Proper context-dependent transcriptional activation of host defense genes is integral to plant defense in response to pathogen infection. Genome-wide transcript profiling has revealed that up to 25% of all *Arabidopsis* genes respond to pathogen attack by altering their transcript levels (Maleck et al., 2000; Tao et al., 2003). Such comprehensive reprogramming implies the existence of a sophisticated regulatory system. Indeed, accumulating evidence indicates that regulation of the defense transcriptome is mediated by a complex network of interconnected circuits linking signaling and gene regulation through well-defined changes in the levels and/or activities of a large number of sequence-specific transcription factors (Eulgem, 2005). Transcription factors (TFs) are divided into several families based upon the characteristics of their respective DNA-binding domains. Out of the more than 45 different TF gene families (Jalali et al., 2006), at least six have been implicated in rice disease resistance, including the comprehensively studied AP2/ERFs, WRKYs, MYBs and bZIPs.

Originally identified as binding factors of ET-responsive GCC box elements, AP2/ERFs (ethylene response factors) have emerged as important regulators of plant responses to various environmental signals, including abiotic stresses (Fujimoto et al., 2001; Park et al., 2001) and pathogen infection (Lorenzo et al., 2003; Gutterson and Reuber, 2004, McGrath et al., 2005, Pre et al, 2008). Rice is estimated to possess at least 139 ERF genes (Nakano et al., 2006; http:/ricetfdb.bio.uni-potsdam.de). Among these, several have been claimed to be involved in the regulation of disease resistance and abiotic stress tolerance on the basis of altered transcript abundance in response to various biotic or abiotic stress treatments (Yang et al., 2002; Cao et al., 2006; Nakano et al., 2006; Lin et al., 2007). However, in many cases, robust genetic evidence supporting these claims is still lacking. One exception, however, is the ethylene-response-element binding protein OsEREBP1 (Cheong et al., 2003). Using an mRNA differential display approach, Kim et al. (2000) originally isolated *OsEREBP1* from suspension-cultured rice cells treated with a *M. oryzae*-derived elicitor. In a more comprehensive study, Cheong et al. (2003) subsequently demonstrated that *in vitro* phosphorylation of OsEREBP1 by the MAP kinase

BWMK1 enhances the ability of the former to bind to the cognate GCC-box motif present in the promoter of various rice *PR* genes, thereby highlighting this TF as important for rice pathogen defense.

Besides AP2/ERF TFs, considerable attention was given in recent years to the participation of WRKY proteins in the plant's transcriptional machinery (Eulgem and Somssich, 2007). As is the case in *Arabidopsis*, rice WRKY TFs form a superfamily consisting of an estimated 102 members that fall into three major groups on a structural basis (Ross et al., 2007). Several *OsWRKY* genes are reported to be activated in response to a plethora of biotic and abiotic stress factors, including exogenously administered SA or JA (Ryu et al., 2006), benzothiadiazole (Shimono et al., 2007), fungal elicitors (Akimoto-Tomiyama et al., 2003; Zhang et al., 2008), mechanical wounding (Guo et al., 2004; Zhang et al., 2008), UV-B radiation (Wang et al., 2007) and infection with *M. oryzae* or *Xoo* (Wen et al., 2003; Ryu et al., 2006). Moreover, OsWRKY03 (Liu et al., 2005) and OsWRKY71 (Liu et al., 2007; Chujo et al., 2008) have been functionally characterized and placed upstream of OsNH1 similar to OsWRKY13, which functions as a molecular switch between the SA and JA signaling pathways (Qiu et al., 2007; Cai et al., 2008). OsWRKY31, on the other hand, appears to be positioned at the crossroads of the auxin signaling pathway and a yet ill-defined disease resistance conduit (Zhang et al., 2008), while plants with reduced amounts of OsWRKY45 failed to develop BTH-inducible resistance to *M. oryzae* (Shimono et al., 2007). Despite these intriguing results, few upstream regulators of rice WRKY factors in defense signaling pathways have yet been characterized, and no WRKY factor had been shown to function directly downstream of a rice receptor-like kinase until Peng et al. (2008) identified OsWRKY62 in a yeast-two-hybrid screen for proteins that interact with the *Xoo* resistance gene *Xa21*. In this study, it was shown that *OsWRKY62* gene encodes two splice variants, *OsWRKY62.1* and *OsWRKY62.2*, both of which partially localize to the nucleus. Interestingly, transgenic plants overexpressing *OsWRKY62*.1 were compromised in basal defense and *Xa21*-mediated resistance to *Xoo*, while overexpression of *OsWRKY62.1* resulted in a significantly reduced accumulation of pathogen defense-related transcripts upon bacterial infection. This implies that OsWRKY62 functions as a negative regulator of innate immunity in rice, modulating both basal and race-specific defense responses. Other rice TFs with a reported regulatory role in rice defense against microbial pathogens include the MYB-like TF JAmyb, the bZIP protein RF2b, and several members of the BELL and NAC TF families (Lee et al., 2001; Dai et al., 2004; Fitzgerald et al., 2005; Luo et al., 2005; Nakashima et al., 2007; Hijhawan et al., 2008).

In conclusion, TFs are widely implicated in the modulation of numerous rice defense responses. Along with additional signaling components, they form the core of a complex transcriptional circuitry that consists of both positive and negative control elements, possibly allowing for an efficient yet balanced amplification and diversification of defense signals. Continued research in this area is predicated on the notion that effective utilization of TF-based technologies in the next generation of biotechnology crops will require a thorough understanding of the various transcriptional networks governing the plant's major biological processes (Century et al., 2008).

2.3.3. The small GTPase OsRAC1: a molecular hub for signal integration and diversification

In addition to MAP kinases and TFs, a number of other regulatory genes controlling downstream defense gene expression have been identified in rice, including *OsEDS5*, *OsDR8*, *NRR*, *OsNDR1*, *Pti1a*, *OsSGT1*, *OsCOI1* and *OsRAR1* (Fitzgerald et al., 2005; Wang et al., 2006b; Takahashi et al., 2007; Thao et al., 2007; Vergne et al., 2007; Wang et al., 2008b). Moreover, an extensive series of studies by Shimamoto's group have provided fascinating insights into the myriad cellular responses regulated by the small GTPase OsRac1. One of the better studied master regulators in the rice defense response, OsRac1 belongs to the plant-specific family of RAC/ROP GTPases, which have recently emerged as a class of versatile signaling molecules orchestrating numerous cellular responses in different plant species (Nibau et al., 2006; Yang and Fu, 2007). Initial studies showed that OsRac1, which is localized in the plasma membrane, transiently stimulates ROS production through activation of an NAPDH oxidase with resultant enhancement of resistance to *M. oryzae* and *Xoo* (Kawasaki et al., 1999; Ono et al., 2001). Interestingly, OsRac1 also suppresses expression of a metallothionein gene that scavenges ROS, thereby further enhancing the ROS-signaled defense response (Wong et al., 2004). Moreover, in concert with the heterotrimeric G-protein subunit RGA1, OsRac1 controls the stability and elicitor-induced activation of the rice MAP kinase OsMPK1 (Lieberherr et al., 2005; Fujiwara et al., 2006). Another target of OsRac1 is cinnamoyl-CoA reductase (CCR), a key enzyme involved in lignin biosynthesis (Wong et al., 2006). Lignin, which is a heterogeneous tridimensional phenolics polymer resulting from the oxidative polymerization of monolignols, is an important factor in plant defense responses, as it presents an undegradable physical barrier to most pathogens (Boerjan et al., 2003). Interestingly, transgenic cell cultures constitutively expressing OsRac1 exhibited enhanced lignin accumulation, which correlated with both increased CCR activity and elevated ROS production. It is therefore not unlikely that OsRac1

has a dual function in lignin biosynthesis to regulate both NAPDH oxidase and CCRs (Wong et al., 2006). Recent evidence connects OsRac1 with yet another range of effector proteins, including the molecular chaperones OsRAR1 and OsSGT1, the scaffolding protein OsRACK1 and the heat shock proteins OsHSP70 and OsHSP90, all of which are well-conserved components of plant innate immunity (Thao et al., 2007; Fujiwara et al., 2008; Nakashima et al., 2008). Emerging from this extensive series of studies is the view that *OsRac1* encodes a master switch for activation of inducible defense responses in rice, functioning at the crossroads of multiple defense-signaling pathways, as well as controlling a vast array of effector proteins involved in various cellular and physiological processes. Manipulation of regulatory genes like these can potentially provide broad-spectrum induced resistance in transgenic plants. Indeed, constitutive overexpression of OsRac1 provides increased resistance to rice blast and bacterial blight infections, whereas a dominant-negative version of OsRac1 compromises basal and *R*-gene-mediated resistance to both these pathogens (Ono et al., 2001; Suharsono et al., 2002). It should be noted, however, that not all studies point to a stimulatory effect of OsRac proteins on rice resistance. Thus, transgenic rice plants overexpressing *OsRacB* showed enhanced disease symptoms upon infection with *M. oryzae*, implicating OsRacB as a negative regulator of basal disease resistance in rice (Jung et al., 2006).

2.4. Collaboration and antagonism: cross-talk in rice defense signaling

Genetic and molecular analyses have so far identified many important components involved in different defense signaling pathways. However, rapidly accumulating evidence indicates that defense signaling is not merely mediated by parallel, linear pathways but rather consists of a complex regulatory network that connects the different pathways enabling each to assist or antagonize the others through a cohort of positive and negative interactions (Kunkel and Brooks, 2002; Pieterse and Dicke, 2007). Such 'crosstalk' between individual pathways is thought to provide the plant with a powerful regulatory potential, which helps the plant to 'decide' on the most appropriate and cost-efficient defensive strategy, depending on the type of attacker it is encountering (Bostock, 2005; Koornneef and Pieterse, 2008). Yet, it may also allow successful pathogens to manipulate the plant's defensive machinery to their own benefit by shutting down biologically effectual defenses through negative crosstalk (for review see Spoel and Dong, 2008).

2.4.1. Crosstalk between SA and JA signaling

Generally speaking, JA signaling contributes to plant resistance against herbivorous insects and necrotrophic pathogens, whereas SA signaling is predominantly associated with resistance against biotrophic pathogens. Although there is evidence for both positive and negative relationships between these pathways in many plant species (Van Wees et al., 2000; Mur et al., 2006; Truman et al., 2007), the primary mode of interaction appears to be mutual antagonism with corresponding trade-offs between biotroph resistance on the one hand, and resistance to necrotrophic pathogens and insect herbivores on the other hand (Bostock, 2005; Pieterse and Dicke, 2007). In rice, crosstalk between SA and JA signaling can be inferred from expression analysis of some defense genes and alterations in endogenous JA and SA levels in response to wounding. For instance, transcript levels of *SaIT*, *OsPR1b*, and *OsIRL* genes are upregulated in response to blast infection, JA, or fungal elicitor treatment, but this effect appears to be negated when plants are treated with both SA and JA or fungal elicitors (Agrawal et al., 2001; Kim et al., 2003, 2004). Moreover, during the early response to wounding, an inverse kinetic pattern is observed in terms of accumulation of endogenous SA and JA, raising the prospect of negative crosstalk in the direction of JA damping SA action (Lee et al., 2004). Additional evidence supporting negative network connections between SA and JA comes from several gene expression studies demonstrating enhanced transcript accumulation of the JA biosynthetic gene *OsAOS2* and the JA-regulated TF gene *JaMYB* in SA-deficient NahG transgenics (Lee et al., 2001; Mei et al., 2006).

Over the past few years, various regulatory components involved in SA/JA crosstalk have been identified (Spoel et al., 2003; Li et al., 2004; Brodersen et al., 2006; Mao et al., 2007; Ndamukong et al., 2007; Koornneef and Pieterse, 2008). These include proteins with stimulatory and repressive functions in both SA-dependent and JA-dependent responses. One of the key players in cross-communication between SA and JA in dicots is NPR1, a master regulator of SA-mediated gene expression and pathogen-induced systemic acquired resistance (SAR). Current models in dicots indicate that upon SAR activation, SA-induced redox perturbation reduces the intermolecular disulphide bonds that normally keep NPR1 in an inactive oligomeric state in the cytosol. This reduction in turn releases monomeric NPR1, which is subsequently translocated to the nucleus, where it interacts with members of the TGA subfamily of bZIP TFs to activate *PR* gene expression (Pieterse and van Loon, 2004; Kesarwani et al., 2007). However, NPR1 not only plays a role in the activation of SA-responsive *PR* genes, but is also required to prioritize SA-dependent responses over JA-dependent responses, as mutants in this protein are impaired in the SA-mediated suppression of JA-marker genes such as *PDF1.2* and *LOX2* (Spoel et al., 2003).

Notably, the function of NPR1 in cross-talk between SA and JA signaling does not require nuclear localization, which is suggestive of a novel function for this master regulatory protein in the cytosol (Spoel et al., 2003).

Interestingly, there is strong evidence for a similar NPR1-mediated mechanism operative in rice. Analogous to the situation in dicots, OsNH1, the closest rice homolog of NPR1, is constitutively present in the cytosol and only migrates to the nucleus following attacker-induced cellular redox changes (Yuan et al., 2007b). Activation of SA-responsive *PR* genes in *OsNH1*-overexpressing plants requires OsNH1 to be targeted to the nucleus, whereas repression of JA-inducible genes in the latter plants only occurs when OsNH1 is localized in the cytoplasm. Moreover, constitutive localization of a site-mutated OsNH1 protein in the nucleus abolishes the enhanced herbivore susceptibility associated with OsNH1-conditioned pathogen resistance (Yuan et al., 2007b), a phenomenon most likely due to de-repression of JA signaling under these conditions. These data elegantly illustrate the importance of OsNH1 in regulating and intertwining the SA- and JA-dependent signaling pathways and underscore the potential of site-mutated OsNH1 as a workable target for engineering broad-spectrum disease resistance in rice without disturbing the JA-regulated insect resistance machinery.

A role in the crosstalk between SA- and JA-signaling also is suggested for the plant-specific transcription factor OsWRKY13 (Qiu et al., 2007). Expression of *OsWRKY13* is induced by each hormone, as well as a range of other signaling molecules including BTH, INA and Ethephon. When ectopically expressed, *OsWRKY13* confers enhanced resistance to bacterial blight and fungal blast, accompanied by activation of SA biosynthesis-related genes and concomitant suppression of genes implicated in JA biosynthesis. This trade-off manifest at the level of hormone synthesis is also apparent at the level of defense gene induction, with *OsWRKY13*-overexpressing lines displaying enhanced accumulation of SA-responsive *PR* transcripts coupled to a dramatic downregulation of JA-controlled genes. OsWRKY13 may thus serve as a point for crosstalk and signal integration – an activator of SA-mediated defense responses and repressor of JA-induced responses.

2.4.2. Crosstalk between ET and ABA signaling

Mounting evidence indicates that ABA and ET function antagonistically during the rice defense response. For instance, exogenous application of ET has been shown to increase the level of resistance to *M. oryzae*, whereas treatment of rice plants with ABA lowers endogenous ET levels and, as a consequence, increases disease susceptibility to *M. oryzae* (Zhou et al., 2006). Remarkably, the ABA- and ET-provoked effects against *M. oryzae* are reverse of those observed against *C. miyabeanus*, tagging ET and ABA as critical modulators of antagonistic defense mechanisms (Koga et al., 2004a; Singh et al., 2004; Chapter 7). A mechanistic understanding of defense-related ABA/ET crosstalk in rice has derived largely from a limited number of studies where, as with SA/JA interactions, mutant and transgenic lines have identified the roles of key transcription factors and effector proteins controlling the possible nodes of convergence between these mutually antagonistic pathways. Elegant work by the Yang lab revealed that RNAi suppression of an ABA-inducible MAP kinase, *OsMPK5*, results in increased levels of endogenous ET, constitutive activation of *PR* genes, and enhanced resistance to *M. oryzae* and *B. glumae* (Xiong and Yang, 2003; Yang, 2007). The same RNAi lines, however, exhibit reduced tolerance to drought, salt, and cold treatments, and are impaired in the ability to develop ABA-inducible resistance to *C. miyabeanus* (Xiong and Yang, 2003; Zhou et al., 2007; Chapter 7). On the other hand, work by Zhou et al. (2006) revealed that suppression of the ET-responsive transcription factor gene *OsEIN2-2* leads to reduced sensitivity to ET and hypersensitivity to ABA. Compared to wild-type rice, the *OsEIN2-2* suppression lines are diminished in their resistance to attack by *M. oryzae* and *B. glumae* but exhibit an enhanced tolerance to abiotic stress treatment (Zhou et al., 2007). Furthermore, consistent with ET inversely regulating blast and brown spot resistance, disease tests with *OsEIN2* antisense plants revealed enhanced resistance to brown spot (Chapter 7). Collectively, these data suggest that *OsMPK5*, *OsEIN2* and *OsEIN2-2* may mediate the antagonistic crosstalk between the ET and ABA pathways, thereby inversely regulating resistance to *M. oryzae* and *B. glumae* on the one hand, and abiotic stress tolerance as well as *C. miyabeanus* resistance on the other hand.

2.4.3. Crosstalk between JA and ABA signaling

In dicotyledoneous plants, the interaction between JA and ABA signaling is rather complex, and both synergistic and antagonistic interactions have been reported, depending on the stress conditions examined. For instance, using PDF1.2, b-CHI and HEL transcript accumulation as markers for JA/ET-responsive gene expression in *Arabidopsis*, Anderson et al. (2004) demonstrated that both basal and JA/ET-induced defense gene expression was suppressed by exogenous ABA treatment but enhanced in the ABA-deficient *aba1* and *aba2* mutants, the latter

resulting in heightened resistance to the necrotroph *Fusarium oxysporium*. On the other hand, induction of JA synthesis by ABA has been widely documented (Adie et al., 2007; Asselbergh et al., 2008) and ABA and JA have been found to cooperate during the wound response through the concerted activation of the transcription factor AtMYC2 (Abe et al., 2003; Lorenzo et al., 2004). In rice, Moons et al. (1997) reported endogenous ABA and JA levels to increase differentially with the dose and duration of salt stress. They also showed that ABA and JA regulate different sets of rice genes. For instance, while ABA treatment was not accompanied by activation of a cationic peroxidase, *PR1a*, *PR10*, and *SalT*, all of which are markedly induced by JA and salt stress, JA proved unable to induce expression of the ABA-responsive *OsLEA3* protein. Moreover, when applied together, ABA and JA inversely affected *SalT* and *OsLEA3* transcript levels, leading the authors to suggest that ABA and JA antagonistically regulate the expression of salt-stress inducible proteins associated with plant response to water deficit or pathogen challenge (Moons et al., 1997). However intriguing, the functional ramifications of such antagonistic ABA/JA crosstalk with respect to pathogen resistance are not clear nor have they been examined. Therefore, further experiments are needed to unequivocally delineate the role, if any, of the ABA-JA connection in regulating gene expression and pathogen defense responses in rice.

2.5. Defense responses underpinning induced defense in rice

Pathogen recognition and consequent signal transduction eventually culminates in the activation of a diverse array of sophisticated effector mechanisms that are instrumental in impeding further pathogen ingress. Direct assessment of the biochemical, cytomolecular, genetic, and physiological alterations during disease development has led to the identification of several defense responses that contribute to the establishment and/or maintenance of the induced defense state in rice. These include production of antimicrobial metabolites and pathogenesis-related proteins, physical reinforcement of the cell walls through production of lignin, and the concerted expression of a battery of defense-related genes, including those involved in rapid and localized cell death. Although the antimicrobial properties of some of these effectors can and have been tested against pathogens in vitro, it has been difficult to assess the efficacy of single effectors during rice-pathogen interactions owing to the general lack of mutant and transgenic lines with defects in specific resistance traits. Another impediment to elucidating the causal roles of individual effector responses arises from the interplay that commonly occurs among defense-related traits in plants.

2.5.1. The hypersensitive response

2.5.1.1. Induced resistance and cell death regulation

One of the most efficient and immediate components of the plant´s inducible defensive repertoire is the hypersensitive response (HR), a form of programmed cell death (PCD) characterized by the rapid collapse and death of a limited number of cells in the vicinity of the invading pathogen (Heath, 2000). This HR cell death, which bears some of the morphological features of the apoptotic cell death processes in other metazoan organisms, primarily occurs in response to avirulent pathogens, in interactions involving race-specific resistance, and in many examples of nonhost resistance (Mysore and Ryu, 2004; Greenberg and Yao, 2004). In addition, HR cell death can also be activated, either directly or indirectly, in genetically susceptible rice plants in response to treatment with resistance-inducing agents (Zhang et al., 2004; Koga et al., 2006; Tanabe et al., 2006; Ahn et al., 2005a,b; Chapter 3). For example, cholic acid, a bile acid elicitor, has been shown to act as a bona fide elicitor, while application of BTH, at least in intact plants, primes rice for higher frequencies of HR-like cell death responses at sites of attempted pathogen entry (Schweizer et al., 1999; Ahn et al., 2005b).

Despite the widespread interest, very little is known about the molecular mechanisms underlying HR and PCD in plants (Lam, 2004). Most of the structural orthologs of the key regulatory proteins of mammalian apoptosis are not encoded by the plant genome, except for BAX-INHIBITOR-1 (BI-1) and DEFENDER AGAINST APOPTOTIC DEATH-1 (Greenberg and Yao, 2004). In rice, Matsumura et al. (2003) identified BI-1 as a key regulator of elicitor-triggered PCD in suspension-cultured rice cells. Cells treated with a cerebroside elicitor from *M. oryzae* showed a dramatic reduction in *BI-1* transcript accumulation with concomitant progress of cell death, whereas over-expression of *BI-1* severely attenuated cerebroside-conferred cell death. Taken together with the ability of cerebroside elicitors to induce HR cell death and increase *M. oryzae* resistance in rice leaves (Koga et al., 1998), these findings raise the possibility that BI-1-modulated cell death control may constitute an important facet of the cerebroside-induced resistance response to the latter pathogen. A potential role for BI-1 and cell death control in induced plant resistance is further substantiated by studies of Hückelhoven et al. (2003, 2004), who found that downregulation of *BI-1* expression closely correlated with the onset of chemical-induced resistance of barley to the powdery mildew *Blumeria graminnis*. Furthermore, over-expression of barley *BI-1* at a single-cell level induced hyper-susceptibility and could reverse the fungal resistance conferred by the loss of MLO, a negative regulator of resistance and HR-like cell death. In *Arabidopsis*, Watanabe and Lam (2006, 2008) recently provided direct genetic evidence of a role for BI-1 as a critical modulator of biotic and abiotic

stress-induced cell death, suggesting that BI-1-mediated cell death control may be a conserved mechanism underlying induced defense in monocot and dicot plants.

2.5.1.2. HR cell death in rice-pathogen interactions: A double-edged sword?

Most of the aforementioned studies relate to the HR induced by hemibiotrophic pathogens, such as the rice blast pathogen, *M. oryzae*. In fact, exciting new data suggest that rice blast defines a novel paradigm for hemibiotrophic plant infection, one in which each successive plant cell invasion is biotrophic but invidual invaded cells are no longer viable by the time the fungus moves into the next cell (Kankanala et al., 2007; Ribot et al., 2008). In contrast, the sheath blight pathogen, *Rhizoctonia solani*, and the brown spot pathogen, *Cochliobolus miyabeanus*, are considered necrotrophs. Both of these fungi kill host cells at very early stages in the infection and cause extensive damage (Ou, 1985). They also produce a variety of phytotoxins that likely promote host cell death (Xiao et al., 1991; Vidhyasekaran et al., 1997; Brooks, 2007). While it is easy to imagine that the HR could result in resistance against (hemi)biotrophic pathogens by restricting pathogen access to water and nutrients, the role of the HR in defense against necrotrophs, which kill host tissues and feed on the remains, is questionable. Indeed, hydroponic feeding of HR-eliciting pyocyanin, a phenazine antibiotic implicated in *P. aeruginosa*-induced systemic resistance to rice blast, promotes susceptibility to *R. solani* and resistance to *M. oryzae* (Chapter 3). In a similar vein, Ahn et al. (2005b) showed a contrasting role of the HR in rice defense against *M. oryzae* and *C. miyabeanus*. In their study, they demonstrated that rapid induction of HR-associated cell death, resulting from either an incompatible gene-for-gene interaction, pre-treatment with conidial germination fluid from *C. miyabeanus* or application of BTH, dramatically increases resistance to *M. oryzae* but fails to protect rice against *C. miyabeanus*. Overall, these findings support the notion that rice requires distinct mechanisms for defense against *M. oryzae*, *C. miyabeanus* and *R. solani* and strengthen the contention that HR cell death can cascade either to the detriment or benefit of the plant depending on the type of host tissues and pathogenic lifestyle of the invading pathogen (Govrin and Levine, 2000; Van Baarlen et al., 2004; Glazebrook, 2005; Spoel and Dong, 2008).

2.5.1.3. The role of reactive oxygen species

The source: a plasma membrane NADPH oxidase

Over the years, data has accumulated indicating that the HR is correlated with a number of physical, physiological and molecular alterations, including deposition of lignin and callose into the plant cell wall (Garcion et al., 2007), and the production of phytoalexins, hydrolytic enzymes

and pathogenesis-related proteins (Greenberg and Yao, 2004). In a range of plant-pathogen interactions, the HR is also intimately associated with a rapid production and accumulation of reactive oxygen species (ROS) during the so-called oxidative burst. The term ROS describes radicals and other nonradical but reactive species derived from molecular oxygen, including the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). In addition to orchestrating HR cell death, ROS can perform multiple other functions in early plant defense responses. For instance, ROS can be directly toxic to pathogens but can also trigger phytoalexin biosynthesis and strengthen plant cell walls through the oxidative cross-linking of cell wall structural proteins. Moreover, ROS can induce arrays of cellular protectant and defense genes and may function as secondary messengers in the induction of systemic acquired resistance (Lamb and Dixon, 1997; Apel and Hirt, 2004). Although alternative mechanisms of ROS production have been described as well (Bolwell et al., 2002), most studies point to a plasma membrane NADPH oxidase as the dominant source of ROS derived from the oxidative burst (Van Breuseghem et al., 2008).

In mammalian phagocytes, NAPDH oxidase forms a multisubunit complex consisting of the cytosolic regulatory components Rac2, $p67^{phox}$, $p47^{phox}$, $p40^{phox}$, and the integral membrane protein flavocytochrome b_{558}, comprising the catalytic subunits $gp91^{phox}$ and $p22^{phox}$ (Babior, 2004). The first plant NADPH oxidase gene to be identified was the rice gene *OsrbohA*, encoding a homolog of the mammalian catalytic subunit $gp91^{phox}$ (Groom et al., 1996). Subsequent studies documented *Rboh* genes (for respiratory burst oxidase homolog) in numerous plant species including *Arabidopsis*, tomato, tobacco and potato (Keller et al., 1998; Torres et al., 1998; Amicucci et al., 1999; Yoshioka et al., 2001, 2003; Simon-Plas et al., 2002). *Arabidopsis*, for instance, encodes 10 *Atrboh* isoforms involved in a diverse range of plant processes. Among these, *AtrbohD* and *AtrbohF* were shown to fine-tune the spatial control of ROS production and the HR during pathogen infection (Torres et al., 2002, 2005). However, rather then driving programmed cell death as originally thought, ROS generated by AtRboh proteins may actually prevent the relay of salicylic acid-dependent pro-death signals to cells surrounding an infection site (Torres et al., 2005). Rice Rbohs are encoded by a nine-member gene family. In an interesting study using RNAi-based knockdown lines, Yoshie et al. (2005) recently provided genetic evidence of a role for *OsrbohA* and *OsrbohE* in pathogen-triggered ROS production in rice cells. Curiously, HR-like cell death was decreased only in the *OsrbohA* knockdown lines and several defense-related genes displayed differential expression patterns in the *OsrbohA* and *OsrbohE* transformants upon inoculation with an avirulent strain of *Acidovorax avenae*. Thus, although the OsRboh proteins are required for ROS production following successful pathogen

recognition, these ROS might serve different signaling functions in rice disease resistance and HR.

Interactions with other plant defense regulators might account for the divergent outcomes in Osrboh-mediated ROS signaling. For example, mounting evidence suggests that coordinated levels of ROS and nitric oxide (NO) are a prerequisite for HR manifestation (Delledonne et al., 2001; Zago et al., 2006; Zaninotto et al., 2006). In line with this, Hu et al. (2003) demonstrated that NO and ROS act in concert to mediate HR responses in elicitor-treated rice cell suspension cultures. Calcium metabolism is also intimately related to ROS signaling. Ca^{2+} fluxes appear to function both upstream and downstream of ROS production, indicating a complex spatiotemporal Ca^{2+} regulation of these signaling networks (Levine et al., 1996; Kurusu et al., 2004, 2005; Torres and Dangl, 2005). Other possible signaling intermediates that may decode the ROS signals are kinases and the MAPK module, as well as the phytohormones SA, JA, ABA and ET (Ganesan and Thomas, 2001; Del Pozo et al., 2004; Yang et al., 2004; Torres et al., 2005; Desikan et al., 2005; Love et al., 2005; Mur et al., 2006; Asselbergh et al., 2007).

Regulation of rice NAPDH oxidase: a role for Rac GTPase

Despite the accumulating evidence that Rboh proteins have a diversified functional portfolio, mediating the production of apoplastic ROS that operate in various elaborate signaling networks, the regulation of plant Rbohs remains largely unknown (Apel and Hirt, 2004; Torres and Dangl, 2005). Unlike the mammalian $gp91^{phox}$, all plant Rboh proteins carry an extended N terminus that contains two Ca^{2+} binding EF-hands, which could account for the direct activation of these oxidases by Ca^{2+} (Keller et al., 1998; Sagi and Fluhr, 2001). Although required, calcium-dependent protein kinase (CDPK)-mediated phosphorylation is not sufficient for full activation of Rboh, indicating that other signaling components are needed as well (Nuhse et al., 2007; Kobayashi et al., 2007). In the absence of other homologs of mammalian NAPDH oxidase polypeptides, the small GTPase Rac/Rop is a likely candidate for being a regulator of plant NAPDH oxidases. Indeed, a recent breakthrough suggests that rice RbohB activation is mediated by binding of its N-terminal extension to the small GTPase OsRac1 (Wong et al., 2007). Shimamoto's group originally identified OsRac1 as a positive regulator of cell death and ROS production in transformed rice cells and plants (Kawasaki et al., 1999; Ono et al., 2001; Suharsono et al., 2002). Using yeast two-hybrid screens and a range of protein-protein interaction assays, they further demonstrated that direct interaction between Rac GTPase and the N-terminal region of Rboh is ubiquitous and that a substantial part of the N-terminal region, including the two EF-hand motifs, is needed for the interaction. Unexpectedly, Ca^{2+} binding by

the EF-hand motifs was not required for the Rac-Rboh interaction and cytosolic Ca^{2+} was even found to negatively modulate Rboh regulation. An intriguing model integrates the positive role of Ca^{2+} and CDPKs in the initiation of Rboh-mediated ROS production with the suppression of Rac-Rboh interaction by cytosolic Ca^{2+} (Wong et al., 2007; Fig. 2.3). First, the initial cytosolic Ca^{2+} influx activates CDPK, which phosphorylates the N-terminal region of Rboh, leading to a conformational change. In the second stage, the conformational change facilitates Rac GTPase binding of Rboh, leading to induction of ROS formation. Finally, the ROS produced induces a second phase of cytosolic Ca^{2+} accumulation, which abolishes Rac binding, indicating a negative feedback loop that allows for termination of the oxidative burst. Collectively, these results delineate that cytosolic Ca^{2+} transients control Rboh activity by modulating the interaction between Rac GTPase and Rboh in a subtle yet dynamic manner.

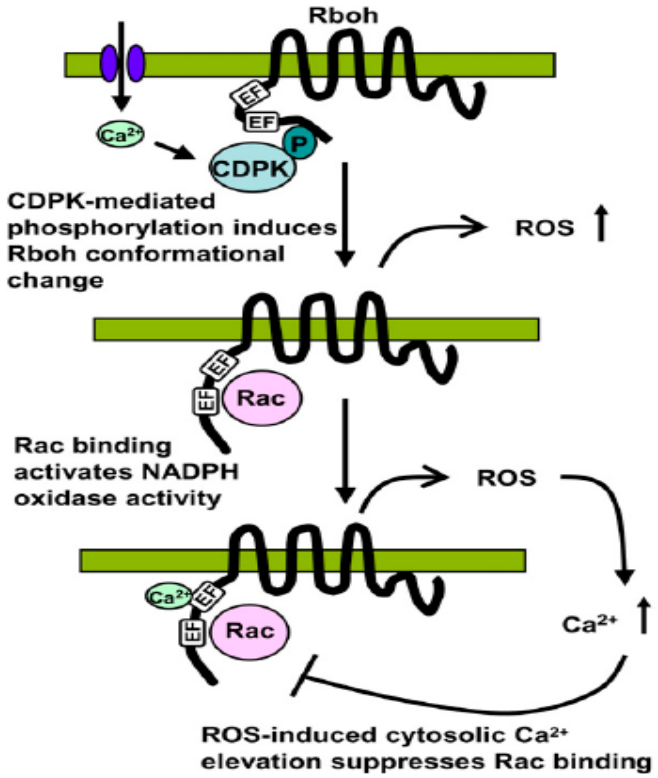

Figure 2.3. Model of plant NAPDH oxidase regulation (Wong et al., 2007).

Initial cytosolic Ca^{2+}-influx activates CDPK (calcium-dependent protein kinase), which phosphorylates the N-terminal region of Rboh, leading to a conformation change that facilitates Rac GTPase binding of Rboh, culminating in activation of ROS production. Subsequently, the ROS produced may induce cytosolic Ca^{2+} elevation, which inhibits Rac binding, thereby terminating the oxidative burst.

Significance of the oxidative burst in rice pathogen defense

There is ample evidence indicating that rapid and extensive generation of ROS during the oxidative burst is a supreme defense response against the hemibiotrophs *M. oryzae* and *Xoo*. Analysis of superoxide and H_2O_2 in compatible and incompatible rice-*M. oryzae* interactions revealed that a rapid and profound oxidative burst specifically occurs in the incompatible interaction (Ganesan and Thomas, 2001; Vergne et al., 2007). A role for H_2O_2 generation in gene-for-gene resistance to *Xoo* was implied by experiments using transgenic cell lines harboring a fusion gene that comprised the extracellular LRR (Leucine Rich Repeats) and transmembrane

domains of the *Arabidopsis* receptor kinase BRI1 and the serine/threonine kinase domain of the bacterial leaf blight resistance gene *Xa21* (He et al., 2000). Treatment with BRs resulted in accumulation of H_2O_2, demonstrating the involvement of the oxidative burst, and probably H_2O_2 itself, in the *Xa21*-mediated signal transduction pathway. Consonant with this is the finding that induction of H_2O_2 in transgenic rice plants expressing a fungal glucose oxidase gene leads to the concerted induction of defense-related gene expression, HR-like cell death and increased resistance to *M. oryzae* and *Xoo* (Kachroo et al., 2003a). Analysis of transgenic plants constitutively expressing *OsSBP*, a cerebroside elicitor-responsive gene homologous to mammalian selenium-binding proteins, further strengthened the link between ROS formation and (hemi)biotroph resistance in rice (Sawada et al., 2004). Overexpression of *OsSBP* led to suppression of the plant's antioxidant machinery, with resultant accumulation of Rboh-generated ROS, HR-like cell death and resistance to blast, whereas plants expressing an *OsSBP* antisense construct were rendered more disease-sensitive. Rboh-controlled ROS formation preceding HR cell death has also been described in rice seedlings treated with the blast resistance elicitors alpha-picolinic acid and N-acetylchitooligosaccharide (Zhang et al., 2004; Ning et al., 2004), while BTH and probenazole, two of the most potent elicitors of resistance to both blast and leaf blight, were shown to prime for enhanced ROS accumulation following pathogen infection (Iwata et al., 2004; Chen et al., 2007). Taken together, these findings strongly suggest that a timely and localized production of ROS may be a crucial early signal leading to activation of defense responses and associated pathogen resistance in rice.

In addition to the above, there is evidence for an indirect role of ROS in rice defense as priming agents (Chapter 3). Root colonization with the rhizobacterium *Pseudomonas aeruginosa* 7NSK2 reduces blast disease in foliar tissues through induction of a defense state that is commonly referred to as induced systemic resistance or ISR (van Loon et al., 1998; Bakker et al., 2007). Extensive bacterial mutant analysis revealed that 7NSK2 mounts ISR via secretion of the redox-active pigment pyocyanin, which in turn induces local H_2O_2 generation on the root surface. Curiously, this root-localized primary oxidative burst was shown to cue the formation of low-frequency reiterative H_2O_2 microbursts in naïve leaves, the latter being indispensable for the onset of pyocyanin-mediated ISR. Given the apparent intimacy between redox sensing and signaling leading to SAR expression (Alvarez et al., 1998; Choi et al., 2007), and the role of cellular redox in the control of many physiological processes, including the response to pathogens (Foyer and Noctor, 2005a,b; Pavet et al., 2005; Ge et al., 2007; Koornneef et al., 2008; Van Breusegem et al., 2008), one may hypothesize that pyocyanin-induced H_2O_2 microbursts may contribute to the ISR-induced state by priming the plant for potentiated activation of cellular

defense responses following challenge infection. In support of this assumption, microscopic analysis of the early infection events in pyocyanin-supplied plants revealed a hyperactivation of HR-like cell death at sites of fungal attack, leading to rapid containment of the invading pathogen (Chapter 3).

Notwithstanding the compelling evidence demonstrating the central importance of plant-produced ROS in rice resistance to hemibiotrophic pathogens such as *M. oryzae*, Egan et al. (2007) recently brought to the fore that *M. oryzae* undergoes an oxidative burst of its own during plant infection. In this fascinating work, the authors demonstrate that generation of superoxide by the fungal NADPH oxidase-encoding genes *Nox1* and *Nox2* is a prerequisite for appressorium-mediated cuticle penetration. This Nox-derived superoxide is believed to accumulate within the appressorium to facilitate oxidative cross-linking of proteins, thereby strengthening the appressorium cell wall. Although hitherto unsuspected, these findings demonstrate that initiation of rice blast disease requires ROS production by the invading pathogen and underscore how ROS produced during the early phase of plant-pathogen interactions can mediate multiple responses, sometimes with opposite effects, in different cellular and spatial contexts or in response to different pathogens.

2.5.2. Pathogenesis-related (PR) proteins

2.5.2.1. PRs in rice: current status

The seminal experiments performed in the 1970s on tobacco plants reacting hypersensitively to *Tobacco mosaic virus* (TMV) first demonstrated the appearance of novel proteins accumulating in response to the infection (Van Loon and Strien, 1999 and reviews therein). In the years to follow, a large number of so-called pathogenesis-related (PR) proteins were shown to occur in plant species from at least 13 families upon infection with oomycetes, bacteria, fungi, viruses, viroids, as well as insect or nematode attack (Van Loon et al., 2006b). The recognized PRs, which are functionally defined as host-encoded proteins induced in infected tissues as well as systemically, have been extensively reviewed and currently comprise 17 families of induced proteins (Van Loon and Strien, 1999; Van Loon et al., 2006b). To date, a number of *PR*-like genes have been cloned from rice and a role of several of these in restricting pathogen activity, growth, and spread fits with the identification of the PR-2 and PR-3 families as antifungal ß-1,3-endoglucanases and chitinases, respectively, and the PR-13 family as thionins, known to have broad antifungal and antibacterial properties (see Jwa et al., 2006 for a recent and detailed review).

During the past seven years, differential display, DNA microarrays, and other high-throughput approaches have considerably broadened our knowledge on rice genes induced upon pathogen infection or treatment with resistance-eliciting agents (*M. oryzae*: Kim et al., 2001; Lu et al., 2004; Jantasuriyarat et al., 2005; Vergne et al., 2007; Gowda et al., 2007; *Xoo*: Chu et al., 2004; Li et al., 2006; *R. solani*: Venu et al., 2007; Zhao et al., 2008; Rice Yellow Mottle Virus: Ventelon-Debout et al., 2008; Rice dwarf virus: Shimizu et al., 2007; BTH: Shimono et al., 2007; probenazole: Nishiguchi et al., 2004; chitin elicitor: Akimoto-Tomiyama et al., 2003). Whilst being informative about the complexity of signaling networks, identifying subsets of genes that are coregulated to a given stress or phytohormone and often revealing unexpected or previously uncharacterized interactions in signal-response coupling and associated metabolic pathways, all of these approaches have invariantly shown large numbers of induced genes that often do not fit the classical list of *PRs*. These genes can be broadly assigned to the following processes: secondary metabolism, cell-wall metabolism, oxidative burst, transport, protein metabolism, antimicrobial proteins, activators of defense reactions and photosynthesis. The advent of reverse genetic tools in rice with, amongst others, sequence-indexed populations of T-DNA, *Ac/Ds* and Tos17-tagged lines (An et al., 2005; Miyao et al., 2007; Hsing et al., 2007; Jung et al., 2008) has opened new doors for large-scale assessment of the importance of such candidate genes in disease resistance. Given its commercial potential, there is considerable interest in this area.

2.5.2.2. Induced PR genes: evidence from transgenic plants

The importance of antimicrobial PR proteins in rice resistance has been repeatedly tested by over-expression of the corresponding genes in transgenic plants. A recent example includes the over-expression of the stress-inducible 1,3;1,4-ß-glucanase gene *Gns1* resulting in increased resistance to a virulent strain of *M. oryzae*. This protection was speculated to result from a combined action of the over-expressed *Gns* and the earlier activation of the defense-related genes *PR1* and *PBZ1* in transgenic plants compared to control plants (Nishizawa et al., 2003). Previously, several groups already demonstrated that transgenic plants constitutively expressing infection-related chitinases, ß-1,3-glucanases, or thaumatin-like protein genes, alone or in combination, are rendered more resistant to *M. oryzae*, *R. solani*, and *Sarocladium grisea* (Nishizawa et al., 1999; Datta et al., 1999, 2001; Kalpana et al., 2006). Enhanced resistance to *M. oryzae* has also been reported in transgenic rice transformed with genes encoding antifungal proteins of bacterial or fungal origin (Krishnamurthy et al., 2001; Moreno et al., 2005). Furthermore, in some interesting recent work, Shao et al. (2008) demonstrated that ectopic expression of a *Xoo*-derived harpin-encoding gene (*hrf1*) not only triggers enhanced accumulation of several PR-protein

transcripts, but also results in elevated silicon levels. Most importantly, hrf1-transformed plants and their progenies were highly resistant to all major *M. oryzae* races in rice-growing areas along the Yangtze River, an area with arguably the highest diversity of the blast pathogen in the world. The enormous potential of bioengineering for sustainable, broad-spectrum disease protection of rice is also evident from the work of Gomez et al. (2007). In this study, the authors demonstrate that ectopic expression of the maize *PRms* gene, encoding a fungal-inducible PR protein, confers heritable resistance against a fairly broad range of bacterial and fungal pathogens, with examples of the latter representing various parasitic habits (i.e. biotrophs, hemibiotrophs and necrotrophs). Intriguingly, *PRms* plants were found to be primed for potentiated expression of *M. oryzae*-inducible defense genes, an effect that the investigators attribute to the heightened levels of sucrose associated with the transgenic phenotype. Implicit here is the view that these plants use altered sucrose levels as a molecular sensor to activate pathogen defense mechanisms. This concept is corroborated by a large body of work in *Arabidopsis* suggesting extensive crosstalk between sugar signaling, *PR* gene expression and induced resistance pathways (Yoshida et al., 2002; Cartieaux et al., 2003; Thibaud et al., 2004; Cartieaux et al., 2008). Although the exact mechanism(s) by which altered sugar sensitivity and/or increased sugar signaling leads to activation of biotic stress responses and subsequent enhancement of disease resistance is (are) unresolved, these studies draw important inferences connecting sugar sensing and pathogen-induced defense signaling in both monocot and dicot plants.

2.5.3. Phytoalexins

Phytoalexins are plant defensive compounds of low molecular weight that are synthesized *de novo* in response to microbial infection (van Etten et al., 1994). Extensive phytochemical investigation has demonstrated that rice produces up to 15 distinct phytoalexins (Peters, 2006). With exception of the flavonoid sakuranetin, all of these fall into the large family of labdane-related diterpenoids. According to their carbon skeletons, diterpenoid phytoalexins have been classified into four groups: phytocassanes A-E (Koga et al., 1995, 1997; Yajima and Mori, 2000), oryzalexins A-F (Akatsuka et al., 1985; Kato et al., 1993, 1994), momilactones A and B (Kato et al., 1973; Cartwright et al., 1981), and the stemarane-type oryzalexin S (Tamogami et al., 1993). Over the past decade, a number of laboratories have devoted considerable effort towards elucidation of the complex metabolic networks underlying diterpenoid phytoalexin biosynthesis and all of the genes encoding the diterpene synthases/cyclases responsible for the various rice diterpenoid phytoalexins have now been identified (Cho et al., 2004; Prisic et al., 2004; Peters, 2006; Shimura et al., 2007). In contrast, relatively little work has been carried out on sakuranetin,

which is thought to derive in a single step from the core flavonoid intermediate (2*S*)-naringenin by an as yet undefined 7-*O*-methyltransferase (Rakwal et al., 1996, 2000).

There is a vast amount of correlative evidence suggesting that phytoalexin accumulation may be a key component of the rice defensive machinery. Most tellingly, phytoalexin biosynthesis is activated in response to treatment with a variety of biotic and abiotic resistance-inducing agents including fungal cell wall elicitors (Koga et al., 1998; Umemura et al., 2002), cholic acid (Koga et al., 2006), methionine (Nakazato et al., 2000), jasmonic acid (Nojiri et al., 1996; Tamogami and Kodama, 2000), and UV-B irradiation (Kodama et al., 1992; Dillon et al., 1997). Furthermore, phytoalexins generally accumulate more rapidly and to larger quantities in *R* gene-mediated incompatible interactions when compared to compatible ones (Koga et al., 1995). Likewise, consistent qualitative and quantitative differences in phytoalexin production were found among rice cultivars of different susceptibility to blast and there was a strong correlation between the accumulation of sakuranetin, momilactone A and oryzalexin S, and resistance to *M. oryzae* (Dillon et al., 1997). Critically, in the course of these studies production of phytoalexins was found to be localized to the blast disease lesions, rather then being systemic, which is consistent with the observed direct antifungal activity of these compounds (Koga et al., 1995). Elevated levels of phytoalexins have also been found in silicon-induced resistant rice plants (Rodrigues et al., 2003) and various lesion mimic mutants that exhibit improved disease resistance (Takahashi et al., 1999; Jung et al., 2005). Another illustration of a possible role for phytoalexins in disease resistance of rice comes from studies on ATP-binding cassette (ABC) transporter genes in *M. oryzae*. ABC transporters are transmembrane proteins that play important roles as energy-dependent efflux pumps, providing resistance to a variety of metabolic poisons including phytoalexins (Sipos and Kuchler, 2006). Based on mutational analysis of *ABC1*, *ABC2* and *ABC4* genes, several groups have suggested that these multidrug extrusion systems contribute significantly to *M. oryzae* pathogenesis, providing the fungus with a powerful tool to withstand the host-specific adverse environment (Urban et al., 1999; Sun et al., 2006; Gupta and Chattoo, 2008). In line with this assumption, multidrug efflux pumps are now emerging as a major phytoalexin tolerance mechanism in various microbes, similar to antibiotic multi-resistance in human pathogens (Garcion et al., 2007).

2.6. Conclusions and prospects

Rice diseases are among the major constraints for rice production worldwide. Although significant progress has been made in cloning rice disease resistance genes and functional genomics in general, still relatively little is known about the signaling pathways and defense effector responses involved in determining rice disease resistance, especially compared to the relative wealth of information available in dicots. Employing a holistic approach comprising genomics, proteomics and metabolomics, future studies should not only focus on analyzing individual signaling components and specific defense effectors, but also emphasize elucidation of the large and complex interplay among various defense pathways. From a practical point of view, such conceptual advances will eventually serve in the development of resistant rice varieties, should it be by genetic engineering, classical breeding or through improvement of cultural practices for effective utilization of chemically and biologically induced resistance in certain agricultural contexts.

Chapter

3

Redox-active pyocyanin secreted by *Pseudomonas aeruginosa* 7NSK2 triggers systemic resistance to *Magnaporthe oryzae* but enhances *Rhizoctonia solani* susceptibility in rice

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Published in: *Molecular Plant-Microbe Interactions*, **19**:1406-1419

Pseudomonas aeruginosa 7NSK2 induces resistance in dicots through a synergistic interaction of the phenazine pyocyanin and the salicylic acid-derivative pyochelin. Root inoculation of the monocot model rice with 7NSK2 partially protected leaves against blast disease (*Magnaporthe oryzae*) but failed to consistently reduce sheath blight (*Rhizoctonia solani*). Only mutations interfering with pyocyanin production led to a significant decrease in induced systemic resistance (ISR) to *M. oryzae*, and *in trans* complementation for pyocyanin production restored the ability to elicit ISR. Intriguingly, pyocyanin-deficient mutants, unlike the wild-type, triggered ISR against *R. solani*. Hence, bacterial pyocyanin plays a differential role in 7NSK2-mediated ISR in rice. Application of purified pyocyanin to hydroponically grown rice seedlings resulted in significantly increased H_2O_2 levels on the root surface, which in turn cued the formation of reiterative H_2O_2 microbursts in naïve leaves. Co-application of pyocyanin and the antioxidant sodium ascorbate alleviated the opposite effects of pyocyanin on rice blast and sheath blight pathogenesis, suggesting that the differential effectiveness of pyocyanin with respect to 7NSK2-triggered ISR is mediated by transiently elevated H_2O_2 levels *in planta*. The cumulative results suggest that reactive oxygen species act as a double-edged sword in the interaction of rice with the hemibiotroph *M. oryzae* and the necrotroph *R. solani*.

Introduction

Rice is the most important staple food grain for more than two billion people living in the rural and urban areas of humid and subhumid Asia. Diseases are among the most important limiting factors that affect rice production, causing annual yield loss conservatively estimated at 5% (Mew et al., 2004). More than 70 diseases caused by fungi, bacteria, viruses or nematodes have been recorded on rice (Ou, 1985), among which rice blast (*Magnaporthe oryzae*) and sheath blight (*Rhizoctonia solani*) are the most serious fungal constraints on high productivity.

The filamentous ascomycete *Magnaporthe oryzae* (Hebert) Barr (anamorph *Pyricularia oryzae* (Cooke) Sacc.) is the most devastating pathogen of rice worldwide due to its widespread distribution and destructiveness (Talbot, 2003). The rice- *M. oryzae* interaction is a well-documented gene-for-gene system (Jia et al., 2000; Silue et al., 1992), and the fungus is a hemibiotroph since successful infection requires an initial biotrophic phase in which the pathogen forms bulbous invasive hyphae within apparently healthy plant cells (Koga, 1994). Once established in the plant, the fungus switches to necrotrophic growth, killing plant cells and ramifying throughout the tissue. Rice sheath blight is caused by *Rhizoctonia solani* Kühn (sexual stage: *Thanetophorus cucumeris* (Frank) Donk), a soil- and water-borne fungal pathogen enjoying a very wide host range. The pathogen has a necrotrophic lifestyle and is able to produce a host-specific carbohydrate-based phytotoxin (Vidhyasekaran et al., 1997).

Resistant cultivars and application of pesticides have been used for disease control. However, the useful life span of most blast resistant cultivars is only a few years, due to the breakdown of the resistance in face of the high pathogenic variability of the pathogen population (Song and Goodman, 2001). Though partial genetic resistance to sheath blight has been reported, no major gene-governed resistance has been found so far despite screening of more than 3000 accessions of germplasm worldwide (Mew et al., 2004). As chemical means of management are often expensive, currently no economically viable or sustainable control measures are available to tackle the diseases.

Thus, there is a need to develop alternative disease control strategies providing durable, broad-spectrum resistance. Among such new strategies, induced resistance has emerged as a potential supplement in international crop protection measures. Induced resistance can be defined as the phenomenon by which plants exhibit increased levels of resistance to a broad spectrum of pathogens by prior activation of genetically programmed defense pathways. The most extensively studied type of induced resistance is systemic acquired resistance (SAR). SAR is expressed locally and systemically after a localized infection by a necrotizing pathogen and is characterized by the accumulation of salicylic acid and pathogenesis-related (PR) proteins

(Durrant and Dong, 2004). Colonization of roots with selected plant growth-promoting rhizobacteria (PGPR) can also lead to a type of systemic resistance, commonly denoted as induced systemic resistance (ISR) (Bakker et al., 2003; Van Loon et al., 1998). Generally, the onset of ISR, unlike SAR, is not accompanied by the concomitant activation of PR genes (Van Wees et al., 1999). Instead, recent research revealed that ISR-expressing plants are primed to react faster to pathogen attack (Verhagen et al., 2004).

Bacterial determinants of ISR that have been identified are cell surface components, such as outer membrane lipopolysaccharides (LPS) or flagella, iron-regulated metabolites with siderophore activity, benzylamine derivatives, volatile compounds and certain antibiotics (Bakker et al., 2003; Iavicoli et al., 2003; Meziane et al., 2005; Ongena et al., 2005; Ryu et al., 2004). In general, most rhizobacteria show redundancy in ISR-triggering traits and their effects can be complementary or additive. Moreover, the mechanisms involved in rhizobacteria-mediated ISR tend to vary among bacterial strains and pathosystems, indicating a great degree of flexibility in the molecular processes leading to ISR, which makes it difficult to derive a general model for PGPR-induced ISR.

To date, molecular biology research aimed towards understanding induced resistance mechanisms has focused mainly on dicotyledoneous model plant species such as *Arabidopsis thaliana* and tobacco. Conversely, in the class of *Monocotyledoneae*, including the most important agronomic cereals, molecular information on chemically and biologically induced resistance mechanisms is largely missing (Kogel and Langen, 2005). One of the most compelling examples of a rice SAR-like response is the enhanced resistance to *M. oryzae* that was demonstrated in response to an infection with the non-host pathogen *Pseudomonas syringae* pv. *syringae* (Smith and Metraux, 1991). However, Reimmann et al. (1995) failed to reproduce these results indicating that conditions for SAR are critical. Although the synthetic salicylic acid analogue benzo(1,2,3)-thiadiazole-7-carbothioc acid (BTH) has been shown to induce SAR in wheat (Görlach et al., 1996) and disease resistance in rice (Rohilla et al., 2002; Schweizer et al., 1999) and maize (Morris et al., 1998), reports about the induction of systemic resistance in monocots using beneficial microorganisms are scarce. These include one in barley, where pre-inoculation with the root-colonizing fungus *Piriformospora indica* induced systemic resistance to several fungal diseases (Waller et al., 2005). In rice, colonization of the rhizosphere with the PGPR strains *Pseudomonas fluorescens* PF1 and FP7 enhanced resistance against sheath blight disease (Nandakumar et al., 2001). Someya et al. (2002, 2005) reported induced resistance to rice blast and sheath blight by the antagonistic bacterium *Serratia marcescens* B2.

In the present study, we assessed the PGPR strain *Pseudomonas aeruginosa* 7NSK2 for its capacity to elicit systemic resistance to *M. oryzae* and *R. solani* in a rice-based model system. Previously, 7NSK2 was shown to induce resistance in several dicot plant species such as bean, tobacco and tomato (Audenaert et al., 2002b; Bigirimana and Höfte 2002; De Meyer and Höfte, 1997; De Meyer et al., 1999a,b). Its ability to trigger ISR has been linked to the production of salicylic acid (Bigirimana and Höfte 2002; De Meyer and Höfte, 1997; De Meyer et al., 1999a). Furthermore, 7NSK2-mediated ISR was shown to be dependent on a functional salicylic acid (SA) response in the plant as 7NSK2 no longer induced resistance in transgenic *NahG* tomato or tobacco plants which are unable to accumulate SA (Audenaert et al., 2002b; De Meyer et al., 1999a). However, recent evidence indicates that, at least for the wild-type, the phenazine pigment pyocyanin and the SA-derived siderophore pyochelin, rather than SA itself, are the essential determinants responsible for ISR elicitation (Audenaert et al., 2002b).

Here, we demonstrate that root treatment of rice seedlings with *P. aeruginosa* 7NSK2 significantly reduces rice blast but fails to mount ISR against sheath blight and provide evidence that this differential effectiveness is due to the production of the phenazine compound pyocyanin. Furthermore, our results provide new insight into the role of reactive oxygen species in the interaction of rice with hemibiotrophic and necrotrophic pathogens.

Results

Pseudomonas aeruginosa 7NSK2 triggers ISR in rice to *Magnaporthe oryzae* but not to *Rhizoctonia solani*

We first tested if root colonization by *Pseudomonas aeruginosa* 7NSK2 could be consistently obtained with the combined seed- and root-inoculation assay used in this study. The amount of bacteria recovered from root surfaces was determined 17 days after the last soil drench (i.e. one week after challenge inoculation). Pooled over three independent experiments, colonization of the roots was reflected by a bacterial titer of 1.43×10^5 CFU per g of root fresh weight ($\pm$ 3.4×10^4; n = 12). This population density, which is well above the threshold density of 10^5 CFU per g of root for *P. fluorescens* WCS374-mediated ISR in radish (Raaijmakers et al., 1995), was consistently obtained throughout all experiments performed in this study.

Next, we investigated whether colonization of the rhizosphere of rice seedlings with *P. aeruginosa* 7NSK2 had a protective effect against leaf blast disease, caused by the ascomycete *Magnaporthe oryzae*. In several preliminary experiments, *P. aeruginosa* 7NSK2 significantly reduced leaf blast symptoms, producing a resistance phenotype resembling this of genetically determined,

intermediate resistance. This type of resistance is characterized by the formation of many small dark-brown spots (diameter < 2 mm) 2 to 3 days after inoculation (Fig. 3.1B). In contrast, on non-treated control leaves, large susceptible-type lesions (diameter 3 tot 6 mm) with a gray centre appeared, often surrounded by chlorotic or necrotic tissue (Fig. 3.1A). These susceptible-type lesions appeared no earlier than 4 days post inoculation. As colonization of the rhizosphere by *P. aeruginosa* 7NSK2 did not completely abolish the formation of susceptible-type lesions, we quantified the disease by counting the number of susceptible-type lesions 6 to 7 days post inoculation. This method is both rapid and quantitative. In general, variation in disease severity between independent inoculation experiments, ranging from approximately 20 to 75 lesions per control leaf, is a phenomenon inherent in the rice-*M. oryzae* pathosystem (Schweizer et al., 1998). Therefore, and since no clear correlation between disease severity and the level of induced resistance could be observed, inoculation data are presented as relative infection values compared to non-treated controls. Pooled over five independent experiments, 7NSK2 reduced rice blast severity by 37.19% (± 6.64; n = 81).

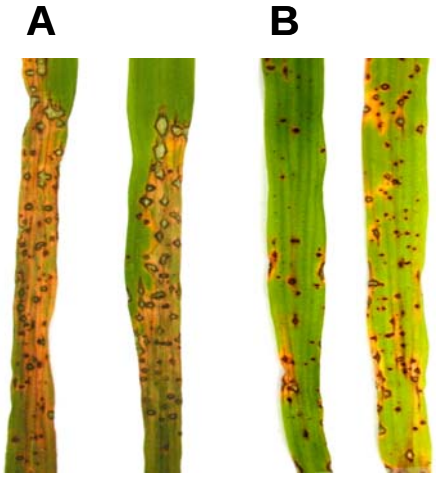

Figure 3.1. **Phenotype of *Pseudomonas aeruginosa* 7NSK2-mediated induced systemic resistance in rice to *Magnaporthe oryzae*.**

A, Control plants were treated with water. **B**, *P. aeruginosa* 7NSK2 was grown on King's medium B (KB) and applied to rice seeds, roots and soil. Four-week-old plants (5-leaf stage) were challenge-inoculated by spraying a spore suspension of virulent *M. oryzae* VT5M1 at 1 x 10^4 spores per ml. Photographs depicting representative symptoms were taken six days after fungal inoculation.

To test the spectrum of 7NSK2-mediated ISR in rice, we then assayed for induction of resistance against sheath blight, which ranks next to blast in causing yield losses, especially in intensified production systems. Although small protective effects were observed in single experiments, 7NSK2 proved unable to consistently reduce the length of lesions caused by the sheath blight fungus *Rhizoctonia solani* (data not shown).

In vitro dual culture experiments revealed a clear antagonistic potential of *P. aeruginosa* 7NSK2 to both *M. oryzae* and *R. solani* (data not shown). To exclude direct antagonism between the inducing agent *P. aeruginosa* 7NSK2 and the challenging leaf pathogens *M. oryzae* and *R. solani*, possible systemic plant colonization by the bacterium was checked. However, 7NSK2 and the derived mutants were never detected in sheath and leaf extracts of root-treated rice seedlings at distinct time points, indicating that bacterial plant colonization remained confined to the root zone (data not shown). The detection limit of this assay is about 10 CFU per stem or leaf. Given the spatial separation between the inducing bacterium (root) and the challenging pathogen (leaf or leaf sheath), the observed disease reduction can be attributed to induced systemic resistance.

The phenazine compound pyocyanin is an essential determinant of 7NSK2-mediated ISR to *Magnaporthe oryzae* in rice

Preliminary experiments with 7NSK2 inoculum prepared from iron-rich medium revealed that 7NSK2-triggered resistance to *M. oryzae* is not dependent on the iron nutritional state of the inoculum, suggesting that siderophores such as pyochelin do not play a crucial role in ISR to *M. oryzae* (data not shown). In order to identify the bacterial factors operative in triggering systemic resistance to *M. oryzae*, the potency of *P. aeruginosa* 7NSK2 to induce ISR was compared with that of a collection of mutants deficient in the production of pyocyanin and/or pyochelin. All bacterial strains were routinely grown on iron-poor KB medium. Figure 3.2A shows that the pyochelin-negative mutant KMPCH (also pyoverdin deficient) induced resistance to an extent similar to that induced by the wild type, hereby excluding an essential role of the siderophores pyoverdin and pyochelin in ISR in rice to *M. oryzae*. Treatment with the newly generated pyocyanin-negative mutants 7NSK2-phzM and KMPCH-phzM no longer caused disease reduction, indicating the involvement of the phenazine antibiotic pyocyanin in ISR. Both mutant strains were constructed by gene replacement of the *phzM* gene, encoding an *O*-methyl transferase which is necessary for the conversion of phenazine-1-carboxylate to the pyocyanin precursor 5-methylphenazine-1-carboxylic acid betaine (Mavrodi et al., 2001). A deficiency in root colonization could be ruled out, since bacterial counts in the rhizosphere of plants inoculated with strains 7NSK2-phzM and KMPCH-phzM were similar to those of 7NSK2-treated plants (data not shown). *In trans* complementation of 7NSK2-phzM for pyocyanin production (strain 7NSK2-phzMc) restored the capacity to induce resistance to *M. oryzae*, confirming the essential role of pyocyanin in 7NSK2-mediated ISR (Fig. 3.2B). Root colonization with the pyocyanin-overproducing strain 7NSK2-phz2, which is mutated in the regulatory gene *retS*, encoding a hybrid sensor kinase, yielded variable results. A significant reduction in the

number of susceptible-type lesions was observed in one experiment [relative infection (RI) value = 76%], whereas treatment with 7NSK2-phz2 generated a higher infection rate (RI = 129%) in another trial. In the remaining two experiments no statistically significant differences could be observed between control plants and plants colonized with 7NSK2-phz2 (RI = 92% and 106%). *In vitro* experiments monitoring production of pyocyanin by 7NSK2 and respective mutant strains revealed that 10^9 CFU 7NSK2-phz2 produces about 25.05 µg pyocyanin per ml culture supernatant (± 1.166; n = 7), thereby showing a 5-fold increase compared to the wild type.

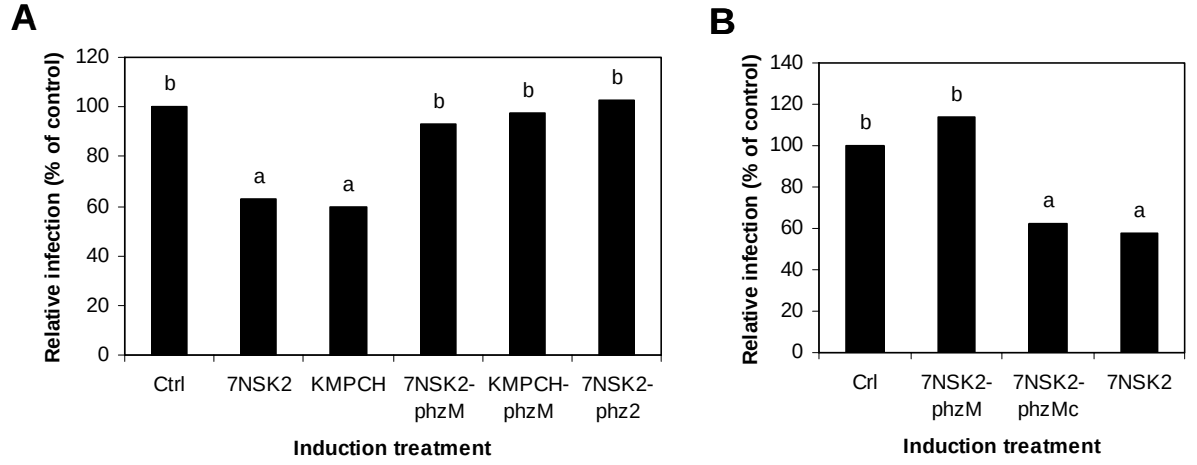

Figure 3.2. Influence of root treatment with *Pseudomonas aeruginosa* 7NSK2 and various mutants on rice blast (*Magnaporthe oryzae*) severity.

P. aeruginosa 7NSK2 and derived mutants were grown on King's medium B (KB) and applied to rice seeds, roots and soil. Control plants were treated with water. Four-week-old plants (5-leaf stage) were challenge-inoculated by spraying a spore suspension of virulent *M. oryzae* VT5M1 at 1 x 10^4 spores per ml. Six days after challenge infection, disease was rated by counting the number of susceptible-type lesions per leaf 4 and expressed relative to challenged control plants. Statistical analysis was performed on pooled data, as interaction between treatment and experiment was not significant at $\alpha = 0.05$ by analysis of variance. Different letters indicate statistically significant differences between treatments according to Kruskal-Wallis followed by Mann-Whitney comparison tests ($P = 0.05$). Mutants derived from strain 7NSK2 have the following characteristics: KMPCH (pyoverdin and pyochelin deficient), 7NSK2-phzM (*phzM*, nonproducing pyocyanin), KMPCH-phzM (pyoverdin and pyochelin deficient; *phzM*, nonproducing pyocyanin) and 7NSK2-phzMc = strain 7NSK2-phzM complemented with functional *phzM* gene of 7NSK2, restoring pyocyanin production.

Pyocyanin-negative mutants of 7NSK2 trigger ISR in rice to *R. solani*

In spite of its resistance-inducing potential against *M. oryzae*, *P. aeruginosa* 7NSK2 proved unable to consistently mount ISR to the sheath blight fungus *R. solani* in several preliminary experiments. These data notwithstanding, we tested the same set of mutant strains as described before in a series of infection assays with *R. solani* as challenging pathogen. All strains were routinely grown on KB medium. Pooled over three independent experiments, neither the wild-type strain 7NSK2 nor the pyochelin-negative mutant KMPCH significantly reduced sheath blight severity (Fig. 3.3). However, inoculation of the rhizosphere of rice seedlings with the

corresponding pyocyanin-deficient strains (7NSK2-phzM and KMPCH-phzM) resulted in significantly higher protection levels to *R. solani* compared to wild type-treated and control plants. Conversely, no statistically significant differences could be observed between treatment with the pyocyanin-overproducing strain 7NSK2-phz2 and control plants. The inability of the pyocyanin-positive strains to mount ISR to *R. solani* was not due to insufficient root colonization of the rice seedlings, since bacterial counts in the rhizosphere of plants inoculated with the respective strains showed no marked differences (data not shown).

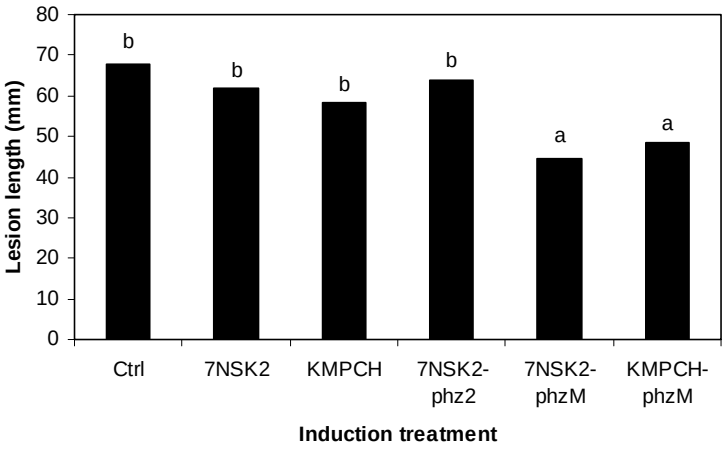

Figure 3.3. **Influence of root treatment with *Pseudomonas aeruginosa* 7NSK2 and various mutants on sheath blight (*Rhizoctonia solani*) severity.**

P. aeruginosa 7NSK2 and derived mutants were grown on King's medium B (KB) and applied to rice seeds, roots and soil. Control plants were treated with water. Four-week-old plants (5-leaf stage) were challenge-inoculated by placing a 1-cm toothpick colonized by *R. solani* inside the sheath of the second youngest fully developed leaf. Disease severity was assessed by measuring the length of *R. solani* lesions four days after challenge infection. Data presented are means from at least three independent experiments with 12 replications per treatment in each experiment. Statistical analysis was performed on pooled data, as interaction between treatment and experiment was not significant at $a = 0.05$ by analysis of variance. Bars with the same letter are not significantly different by non-parametric Kruskal-Wallis and Mann-Whitney comparisons at $P = 0.05$. Mutants derived from strain 7NSK2 have the following characteristics: KMPCH (pyoverdin and pyochelin deficient), 7NSK2-phz2 (overproducing pyocyanin), 7NSK2-phzM (*phzM*, nonproducing pyocyanin) and KMPCH-phzM (pyoverdin and pyochelin deficient, *phzM*, nonproducing pyocyanin).

Pyocyanin induces resistance to *Magnaporthe oryzae* but enhances infection by *Rhizoctonia solani*

The observation that pyocyanin-deficient mutants, unlike wild-type strains, triggered resistance to *R. solani*, whereas the same mutants lost their ability to mount ISR to *M. oryzae* (Figs. 3.2A and 3.3), suggested that the secretion of pyocyanin might account for the differential effectiveness of 7NSK2-mediated ISR to the latter pathogens. Therefore, and since it has been reported before that high concentrations of purified pyocyanin can induce resistance to *Botrytis cinerea* in bean (Abeysinghe, 1999), we wanted to further explore the role of bacterially produced pyocyanin in 7NSK2-mediated ISR in rice. To this purpose, we isolated pyocyanin from the

pyocyanin-overproducing strain 7NSK2-phz2 using a chloroform-based extraction assay (Kanner et al., 1978) and applied the purified compound to the roots of rice seedlings. To avoid excessive immobilization of the metabolite through adsorption on soil particles and/or spontaneous degradation or bio-degradation in soil, a soil-less gnotobiotic rice-growing system was developed. In this system, 24-day-old rice seedlings were hydroponically fed with a dilution series of pyocyanin by adding the desired concentration to the nutrient solution. In order to include a pyocyanin concentration in the experimental set-up that is equivalent to the production by *P. aeruginosa* 7NSK2, we spectrophotometrically quantified the *in vitro* pyocyanin production by the latter strain. Pooled over two independent experiments, 10^9 CFU 7NSK2 produced 4.958 μg of pyocyanin per ml culture supernatant (± 0.483 μg; n = 7). Provided that pyocyanin production by 7NSK2 is proportional to the amount of 7NSK2, 10^5 CFU 7NSK2 should produce about 0.5 ng of pyocyanin. This is similar to the amount of pyocyanin applied in a 25 pM pyocyanin solution (1000 ml of 25 pM pyocyanin ≈ 5.2 ng of pyocyanin per tray or 0.43 ng per seedling). Thus, feeding a 25 pM pyocyanin solution in the hydroponic system resembles the production of pyocyanin in the rhizosphere of soil-grown rice plants colonized by 7NSK2. No signs of phytotoxicity were observed in leaves of plants after pyocyanin feeding at any of the concentrations tested. In the 25 pM to 100 nM pyocyanin range, ISR to *M. oryzae* was evident for all concentrations tested. However, no significant protection could be observed at 50 μM pyocyanin (Fig. 3.4A). Furthermore, pyocyanin did not reduce the number of virulent lesions to the same extent as treatment of the roots with 7NSK2. Taken together, these results indicate that *P. aeruginosa* 7NSK2-mediated ISR to *M. oryzae* can be partially mimicked by application of pyocyanin to roots. Conversely, pyocyanin feeding favored subsequent infection by *R. solani*, irrespective of the applied concentration (Fig. 3.4B). Since pyocyanin is known for its antibiotic properties (Hassan and Fridovich, 1980), we checked whether pyocyanin-feeding leads to translocation of the compound to distal plant tissues such as leaves. However, pyocyanin remained undetected in leaves of root-feeded plants using HPLC analyses (detection limit: 13 ng per g of FW). Summarized, these data suggest a dual role of pyocyanin in 7NSK2-mediated ISR and corroborate the results obtained in the ISR assays with the pyocyanin-negative mutants 7NSK2-phzM and KMPCH-phzM.

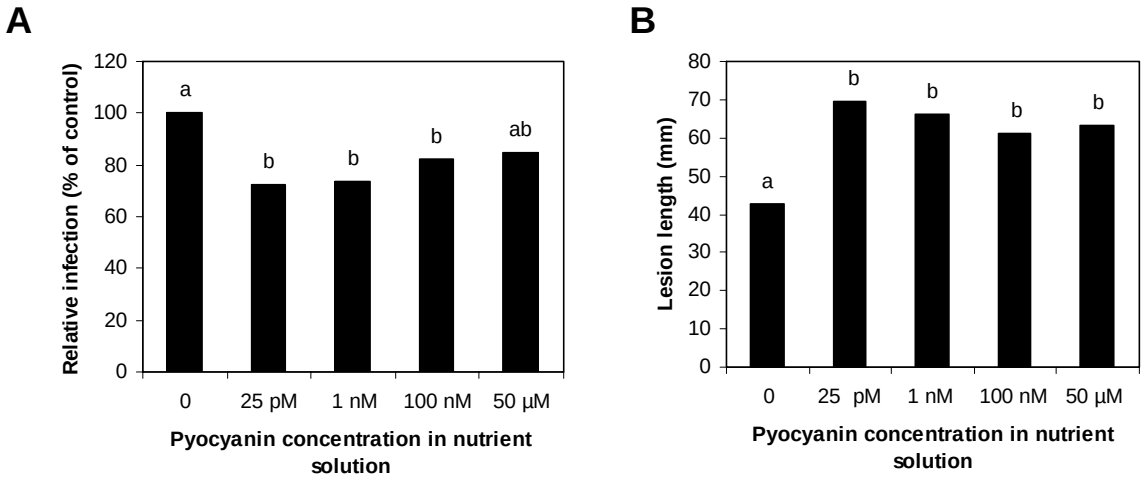

Figure 3.4. **Effect of adding pyocyanin to the nutrient solution on A, leaf blast severity and B, the length of *Rhizoctonia solani* lesions on hydroponically grown rice plants.**

Pyocyanin was purified from *P. aeruginosa* 7NSK2-phz2 cultures and added to the half-strength Hoagland nutrient solution at different concentrations; 4 days later, plants were inoculated by **A**, spraying a spore suspension of virulent *M. oryzae* VT5M1 at 1 x 10^4 spores per ml or **B**, placing a 1-cm toothpick colonized by *R. solani* inside the sheath of the second youngest fully developed leaf. Six and four days after pathogen inoculation respectively, disease was rated by **A**, counting the number of susceptible-type blast lesions per leaf 4 or **B**, measuring the total length of sheath blight lesions. In the case of *M. oryzae* infections, results were expressed relative to challenged control plants. The values presented are from a representative experiment that was repeated three times with similar results. Bars with the same letter are not significantly different by non-parametric Kruskal-Wallis and Mann-Whitney comparisons at $P = 0.05$.

Pyocyanin triggers enhanced levels of H_2O_2 in local and systemic tissue upon root treatment

Given the fact that pyocyanin has the capacity to undergo redox cycling under aerobic conditions with resulting generation of superoxide and hydrogen peroxide *in vitro* (Hassan and Fridovich, 1980), we asked whether pyocyanin also would be capable of producing ROS in our gnotobiotic system. To this end, we monitored the levels of H_2O_2, which is the major and most long-living reactive oxygen species, both on the roots and in the leaves of hydroponically grown rice seedlings in response to pyocyanin feeding. Detection of H_2O_2 on roots was carried out by means of an endogenous peroxidase-dependent staining procedure with 3,3´-diaminobenzidine (DAB). Roots of rice seedling treated with 100 nM pyocyanin showed strong DAB staining compared to Hoagland-treated control roots. However, DAB staining was not observed on roots in the presence of the H_2O_2 scavenger, ascorbic acid, confirming the specificity of the staining (Fig. 3.5). The *in planta* accumulation of H_2O_2 was determined following the titanium (IV) chloride method as described by Wu et al. (1995). Inclusion of 100 nM pyocyanin in the nutrient solution revealed a transient rise in H_2O_2 levels in systemic leaves at 8 h post-application compared to control plants, followed by decay to control levels (Fig. 3.6A). A second more pronounced rise in H_2O_2 was observed at 48 h post-treatment and persisted for at least 24 h.

Since regulation of H_2O_2 levels in plant tissue is brought about by the coordinated activities of H_2O_2 generating and degrading enzymes, we sought to extend our analysis of the ROS generating potential of pyocyanin in the gnotobiotic system by monitoring the level of various antioxidant enzymes in response to pyocyanin feeding. Changes in total superoxide dismutase (EC 1.15.1.1) activity were concomitant with the biphasic generation pattern of H_2O_2, reaching 1.8 fold higher levels after 48 h of incubation, suggesting that pyocyanin-induced H_2O_2 accumulates sequentially from superoxide as the primary origin (Fig. 3.6B). Comparative analysis of the kinetics of several H_2O_2 degrading enzymes such as catalase (EC 1.11.1.6), ascorbate peroxidase (EC 1.11.1.11) and guiacol peroxidase (EC 1.11.1.7) revealed distinct enzyme-specific activity patterns (Figs. 3.6C, D and E). For instance, the first phase of H_2O_2 accumulation coincided with declined activities of catalase and guiacol peroxidase, while no significant alteration of ascorbate peroxidase activity could be observed. In addition, catalase activity showed a declining trend between 24 h and 72 h of incubation, whereas both ascorbate and guiacol peroxidase activity progressively increased within this timeframe, indicating a balanced interplay between H_2O_2 detoxifying enzymes in response to pyocyanin feeding. Taken together, these data clearly demonstrate the ability of bacterial pyocyanin to generate ROS on the root surface of rice seedlings as well as in systemic leaves.

Figure 3.5. Detection of pyocyanin-derived H_2O_2 by 3,3′-diaminobenzidine (DAB) staining.

Roots of hydroponically grown rice seedlings were immersed for 2 h in nutrient solution **A**, with or **B**, without pyocyanin (1 nM), rinsed several times with distilled water and subsequently incubated in DAB solution (1 mg/ml) for 12 h at room temperature. The specificity of the staining was verified by adding 10 mM ascorbic acid to the DAB solution (**C** and **D**, respectively).

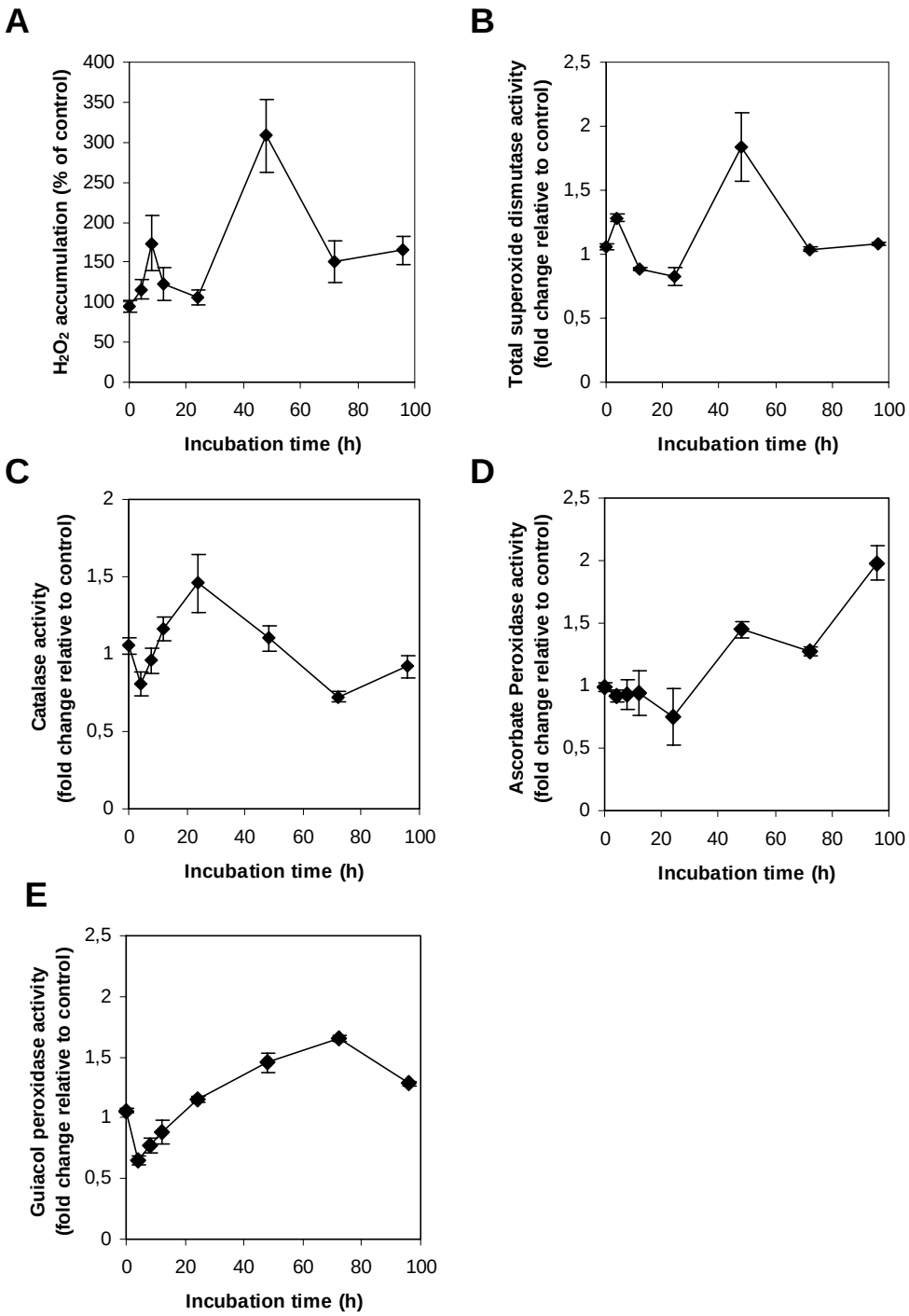

Figure 3.6. Effect of pyocyanin on A, H_2O_2 levels, and the activities of B, superoxide dismutase C, catalase D, guiacol and E, ascorbate peroxidase in the fourth leaf of hydroponically grown rice seedlings.

Pyocyanin was purified from *P. aeruginosa* 7NSK2-phz2 cultures and added to the half-strength Hoagland nutrient solution to a concentration of 100 nM. Data are means (± SE) of four replicates of a representative experiment. Each replicate consisted of one pooled sample from six individual plants. Two series of independent experiments were carried out giving reproducible results.

Ascorbic acid attenuates the *M. oryzae* resistance-inducing and *R. solani* infection-promoting potential of pyocyanin

The observation that application of purified pyocyanin to the roots of hydroponically grown rice seedlings triggers enhanced levels of H_2O_2 and antioxidant enzymes in distal leaves, prompted us to test whether ROS generated by pyocyanin *in planta* account for the dual role of the latter compound in 7NSK2-mediated ISR. To this end, we investigated the effect of adding ascorbate, which is one of the major natural quenching agents, to the pyocyanin solution on the subsequent challenge with *M. oryzae* and *R. solani*. Figure 3.7A shows clearly that co-application of 50 µM sodium ascorbate and 100 nM pyocyanin attenuated the pyocyanin-triggered resistance to *M. oryzae*. Similarly, addition of 50 µM ascorbate to the pyocyanin feeding solution alleviated the stimulation of *R. solani* infection by pyocyanin (Fig. 3.7B). Ascorbate itself at this concentration had no detectable effect on disease development. However, application of higher concentrations of ascorbate (2.5 mM to 10 mM) to the roots reduced sheath blight severity (data not shown).

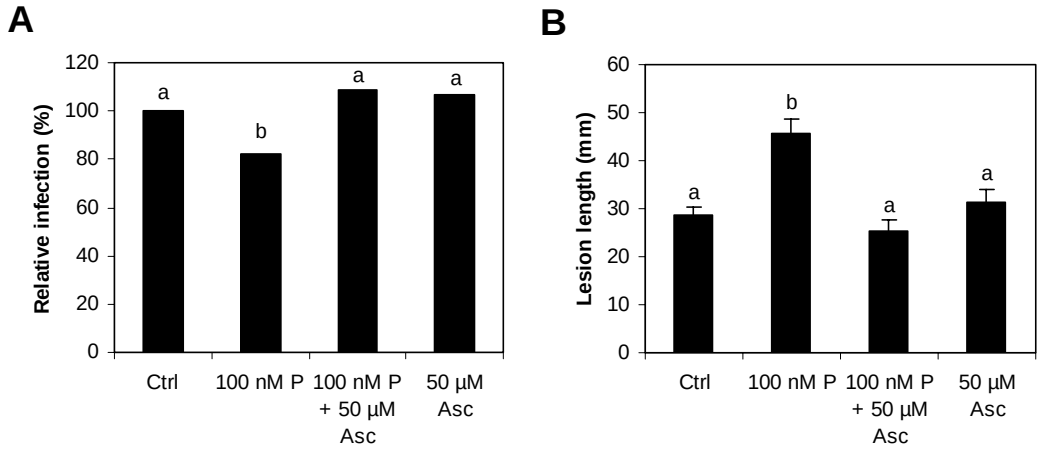

Figure 3.7. Effect of adding ascorbate (Asc) to the pyocyanin feeding solution on A, the blast resistance-inducing potential and B, the *R. solani* infection-promoting ability of pyocyanin.

Pyocyanin (P) was purified from *P. aeruginosa* 7NSK2-phz2 cultures and added to the half-strength Hoagland nutrient solution containing 50 µM sodium ascorbate (Asc); 4 days later, plants were inoculated by **A**, spraying a spore suspension of virulent *M. oryzae* VT5M1 at 1 x 10^4 spores per ml or **B**, placing a 1-cm toothpick colonized by *R. solani* inside the sheath of the second youngest fully developed leaf. Six and four days after pathogen inoculation respectively, disease was rated by **A**, counting the number of susceptible-type blast lesions per leaf 4 or **B**, measuring the total length of sheath blight lesions. In the case of *M. oryzae* infections, results were expressed as relative infection values compared to control plants. The values presented are from representative experiments that were repeated three times with similar results. Bars with the same letter are not significantly different by **A**, non-parametric Kruskal-Wallis and Mann-Whitney comparisons at $P = 0.05$ or **B**, Fisher's least significant difference test ($\alpha = 0.05$).

Cytological comparison of fungal infection and host cellular reactions between control and pyocyanin-treated plants

To further elucidate the mechanisms of pyocyanin-mediated ISR, cytological studies combining differential interference contrast (DIC) and incident fluorescence microscopy were conducted. To establish compatibility, it appears important for *M. oryzae* to keep the invaded epidermal cell of a susceptible rice line alive in the early stages of infection before switching to necrotrophic growth (Koga, 1994). Since the evidence placing reactive oxygen species as central signals in the elicitation of certain types of cell death is compelling (Van Breusegem and Dat, 2006), we first investigated whether pyocyanin feeding provokes cell death before challenge infection. However, pyocyanin alone did not cause any cell death, neither in local nor in systemic tissue (data not shown). Nevertheless, pyocyanin-treated plants expressed potentiated HR-like cell death in response to infection with *M. oryzae*. In control plants, fungal hyphae grew vigorously within penetrated epidermal cells (Fig. 3.8A). In pyocyanin-treated plants, 43.6% (± 8.7%; n = 200) of attacked epidermal cells reacted to fungal ingress through the development of HR-like cell death as indicated by the granulation of the cytoplasm and a bright autofluorescence of epidermal cell walls (Figs. 3.8B and 8E). These reactions were not observed in control plants up to 36 hai (Figs. 3.8A and 8D). Addition of ascorbate to the pyocyanin feeding solution attenuated the above-mentioned effects (Fig. 3.8C), while ascorbate feeding alone did not significantly interfere with the infection process (data not shown).

In both control and pyocyanin-treated plants, germinated sclerotia of *R. solani* colonized the inner surface of the leaf sheath within 12h of inoculation. Penetration of the sheath surface was observed 24 hai regardless of pyocyanin treatment. The most frequent penetration was by hyphal tips (Figs. 3.8G and 8H), although other infection structures such as lobate appressoria (Fig. 3.8I) and infection cushions (Fig. 3.8J) were also observed. Hyphal tips or infection pegs produced from lobate appressoria either penetrated directly into the epidermis, or first colonized sub-cuticularly before entering epidermal cells. Colonization of epidermal and mesophyll cells occurred both inter- and intra-cellular, and was often associated with intense browning of penetrated and neighboring cells. In control plants, discrete groups of epidermal cells showing intense browning were commonly observed 5-15 cell layers ahead of fungal invasion, presumably due to secretion of phytotoxins by *R. solani* (Fig. 3.8K). By contrast, in sheaths of pyocyanin-treated plants, enlarged zones of dying cells preceding fungal colonization, were frequently observed (Fig. 3.8L). Ascorbate largely abrogated this pyocyanin-provoked runaway cell death in response to challenge with *R. solani* (data not shown).

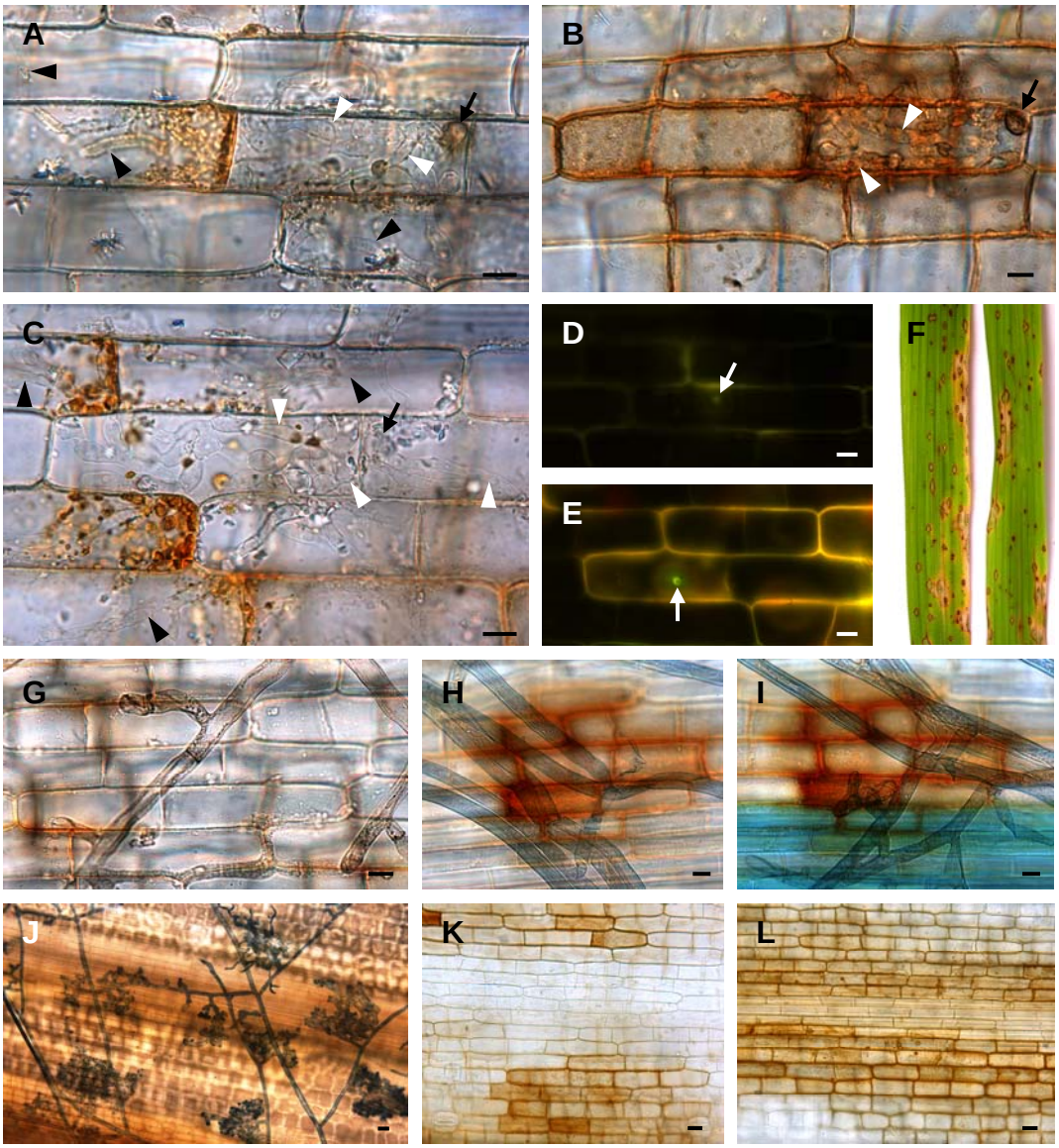

Figure 3.8. Interaction phenotypes of pyocyanin-mediated cytological responses of *Oryza sativa* line CO-39 to *Magnaporthe oryzae* and *Rhizoctonia solani*.

A through **F**, Infection sites inoculated with *M. oryzae*. Scale bars represent 10 µm. **A**, Vigorous invasion of living tissues in control plants. Upon penetration by a dome-shaped appressorium (black arrow), the fungus colonizes the first penetrated epidermal cell as well as neighboring cells by 36 hours after infection (hai) producing primary (white arrowheads) and secondary invading hyphae (black arrowheads), respectively. **B**, Expression of HR-like cell death blocks *M. oryzae* in hydroponically grown rice plants amended with 100 nM pyocyanin (36 hai). Intracellular hyphae (white arrowheads) originating from an appressorium (arrow) are restricted to the initially penetrated epidermal cell and stopped from infecting adjacent plant tissue. Note the granulation of the cytoplasm in both first invaded and neighboring epidermal cells. **C**, Addition of 50 µM ascorbate to the pyocyanin feeding solution abrogates pyocyanin-induced HR-like cell death. By consequence, fungal growth is not arrested and invading hyphae form an extensively branched mycelium both in the first invaded (white arrowheads) and surrounding epidermal cells (black arrowheads). Arrow points to the site of fungal penetration. **D**, Faint autofluorescent halo surrounding point of penetration (white arrow) as well as weak local autofluorescence in control plants under blue light excitation (24 hai). **E**, Epifluorescence image of epidermal cells of pyocyanin-feeded plants responding to *M. oryzae* infection (24 hai). The penetrated epidermal cell and the cell walls of three surrounding cells exhibit bright autofluorescence under blue light excitation. White arrow indicates position of the fungal appressorium. **F**, Symptoms of *M. oryzae* on the fourth leaf of control plants (left, 7 days post inoculation [dpi]) and pyocyanin-treated plants (right, 7 dpi). **G** through **J**, Micrographs of infection sites of control plants inoculated with *R. solani* (similar observations were made in pyocyanin-treated plants). **G**, Direct penetration by hyphae of *R. solani* and associated browning of epidermal cell walls at the site of contact (24 hai). Bar = 10 µm. **H**, Browning of epidermal cells underlying *R. solani* hyphae as observed by 24 hai. Fungal hyphae were stained using trypan blue dye. Bar = 10 µm. **I**, Penetration attempt by a lobate appressoria-like structure of *R. solani* and associated host cell death. Extracellular mycelium is stained with trypan blue. Bar = 10 µm. **J**, Colonization of sheath surface by hyphae and formation of infection cushions by 40 hai. Fungal mycelium was stained with trypan blue. Bar = 30 µm. **K** and **L**, Browning of non-penetrated epidermal cells preceding fungal invasion as observed in control plants (**K**) and pyocyanin-treated plants (**L**) respectively by 32 hai. Bars = 20 µm.

Discussion

In this study, we have analyzed *Pseudomonas aeruginosa* 7NSK2-mediated ISR in the monocot model plant rice against leaf blast (*Magnaporthe oryzae*) and sheath blight (*Rhizoctonia solani*). In a standardized assay, root treatment with *P. aeruginosa* 7NSK2 effectively protected rice against blast but failed to consistently reduce sheath blight severity. Because inducing bacteria and challenging pathogens remained spatially separated throughout the experiment, antagonism by direct interactions could be ruled out, demonstrating that 7NSK2-induced protection is plant mediated. ISR against *M. oryzae* was phenotypically manifested by a reduction in the number of susceptible-type blast lesions (Fig. 3.1), thereby resembling the resistance phenotype of quantitative trait loci-governed partial resistance (Zahirul et al., 2005).

Recent evidence by Audenaert et al. (2002b) suggests that, whereas ISR elicited by *P. aeruginosa* 7NSK2 in dicots requires the SA signaling pathway, the bacterial trigger of ISR is the combination of the SA-derived siderophore pyochelin and the phenazine pyocyanin, rather than SA itself. In view of pharmacological studies demonstrating that ferripyochelin-catalyzed hydroxyl generation from pyocyanin-derived $O_2^{\cdot-}/H_2O_2$ contributes to microvasculature injury which occurs as a consequence of pulmonary infections with *P. aeruginosa* (Britigan et al., 1992, 1997), the authors proposed that the generation of hydroxyl radicals by the Fe-pyochelin-pyocyanin interaction might constitute the basis of 7NSK2-mediated ISR. In this work, however, we found no evidence for the involvement of iron-regulated pyochelin in 7NSK2-mediated ISR in rice. This pyochelin-independency of 7NSK2-triggered ISR was borne out by the observation that bacterial inoculum prepared from iron-rich medium was as effective as inoculum prepared from iron-poor KB in controlling rice blast disease, and was further confirmed by the ISR-inducing potential of the pyochelin-negative mutant KMPCH (also pyoverdin-deficient) (Fig. 3.2A). At inoculation, 7NSK2 grown on iron-rich medium had an internal iron pool that was visible in the red color of the bacterial pellet whereas an internal iron pool was not observed for KB-grown 7NSK2, since siderophore-mediated iron acquisition is strictly regulated. These observations make a role for iron-regulated metabolites of *P. aeruginosa* in ISR to *M. oryzae* highly unlikely.

Similar to 7NSK2-mediated ISR in tomato, the pyocyanin-deficient mutant 7NSK2-phzM lost the capacity to trigger ISR against rice blast. A similar phenomenon was observed for the pyocyanin and pyochelin double negative mutant KMPCH-phzM (Fig. 3.2A). Because the inability of these strains to induce resistance to *M. oryzae* did not result from insufficient rhizosphere populations, these data strongly suggest that pyocyanin production by *P. aeruginosa* 7NSK2 is necessary for ISR to *M. oryzae* in rice. Additional support was provided by

complementation experiments (Fig. 3.2B), as well as by the protective effect obtained upon hydroponic feeding with pure pyocyanin (Fig. 3.4A). Surprisingly, treatment with the pyocyanin-overproducing strain 7NSK2-phz2, which produces about 5x more pyocyanin compared to the wild type, failed to consistently mount ISR to *M. oryzae*. Provided that the pyocyanin production *in vitro* is an adequate indication of the capacity to produce pyocyanin in the rhizosphere by the respective strains, these results suggest that only a balanced production of pyocyanin triggers ISR to *M. oryzae*. However, we found no clear dose effect for pyocyanin in our gnotobiotic system, at least in the physiologically relevant pico- and nanomolar range (Fig. 3.4A). These conflicting observations could be reconciled when considering the distinct pyocyanin application in soil-based and hydroponic assays. Contrary to the putative sustained pyocyanin production by the bacterial strains in the rhizosphere, purified pyocyanin was fed only once in the hydroponic system. *In vitro* studies have shown that pyocyanin has multiple deleterious effects on mammalian cells, such as inhibition of cell respiration, ciliary function, epidermal cell growth, and prostacyclin release, disruption of calcium homeostasis, and inactivation of catalase and vacuolar ATPase (Lau et al., 2004). Moreover, pyocyanin induces apoptosis in neutrophils (Allen et al., 2005) and modulates the glutathione redox cycle (Muller, 2002) in lung epithelial and endothelial cells. Hence, it is conceivable that sustained exposure of rice roots to substantial levels of pyocyanin, as secreted by the overproducing mutant 7NSK2-phz2, causes toxic effects that might negatively interfere with the induction of ISR. Such a concept would be consistent with previous findings by Abeysinghe (1999) who reported that only balanced doses of pyocyanin trigger resistance to *Botrytis cinerea* in bean. Similar results were obtained by Iavicoli et al. (2003) when studying the involvement of the antibiotic 2,4-diacetylphloroglucinol in *P. fluorescens* CHAO-mediated ISR to *Peronospora parasitica* in *Arabidopsis*. On the other hand, the mutation in *phz2* is likely to have a pleiotropic effect since it results in the inactivation of the hybrid sensor kinase RetS. While we selected this mutant as a pyocyanin hyperproducer, others demonstrated that the same mutation affects type III secretion, motility and virulence, and promotes biofilm formation (Goodman et al., 2004; Ventre et al., 2006; Zolfaghar et al., 2005). Therefore, we cannot exclude the possibility that the effect observed with 7NSK2-phz2 is not only due to pyocyanin overproduction.

One of the most peculiar events in the early phase of plant-pathogen interactions is the rapid and transient production of reactive oxygen species by the plant, namely the oxidative burst. Because pyocyanin is a redox-active compound (Hassan and Fridovich, 1979, 1980), and has been demonstrated before to be capable of generating ROS in an animal system (Britigan et al., 1997), we investigated whether pyocyanin production in the rhizosphere modulates the oxidative

machinery of rice seedlings. By means of a combination of histochemical DAB stainings and *in planta* measurements of H_2O_2, we demonstrated that pyocyanin feeding of hydroponically grown rice seedlings leads to enhanced H_2O_2 levels both on the root surface of rice seedlings and subsequently in distal leaves (Figs. 3.5 and 3.6). The pyocyanin-elicited H_2O_2 burst in the systemic leaves adopted a biphasic generation pattern, similar to the two-phase kinetics frequently observed during an avirulent pathogen-induced oxidative burst (Levine et al., 1994; Shirasu et al., 1997). This biphasic response might indicate a capacity for multiple reiterations of pyocyanin-triggered ROS generation in order to maintain the induced state. Such a mechanism would either require signal amplification for reiteration of the pyocyanin-triggered ROS generation or, alternatively, could result from successive redox cycles of pyocyanin as studies by Rezka et al. (2004) demonstrated that pyocyanin has the capacity to undergo redox cycling without extensive modification of the pigment's phenazine chromophore, thus leaving the pigment intact.

There is ample evidence indicating that ROS, and H_2O_2 in particular, generated in the oxidative burst, perform multiple important functions in early plant defense responses. ROS are directly protective, activate phytoalexin biosynthesis and also drive peroxidase-mediated cross-linking of proline-rich cell wall glycoproteins (Lamb and Dixon, 1997). Moreover, ROS induce arrays of cellular protectant and defense genes and also cue the collapse of challenged cells (Foyer and Noctor, 2005; Neill et al., 2002). In addition to these intracellular or local intercellular signal functions, some studies have highlighted the potential role for local ROS accumulation in systemic signaling leading to the establishment of SAR (Fobert and Després, 2005). Elegant research by Alvarez et al. (1998) demonstrated redox changes in systemic tissues following SAR induction. These changes were observed as well-timed, transient microbursts of H_2O_2 production that were required for SAR manifestation. Taking these facts into account, we speculate that the transient enhancement of H_2O_2 levels, observed in systemic leaves of pyocyanin-treated rice seedlings, might likewise function in 7NSK2-mediated ISR to *M. oryzae* by low-level activation of defense responses throughout the plant, thereby contributing to the ISR-induced state. Critical to the formation of a hypothesis of transiently increased H_2O_2 levels as the central event in 7NSK2-mediated ISR in rice was the observation that inclusion of H_2O_2-quenching ascorbate into the pyocyanin containing nutrient solution abrogated both pyocyanin-induced H_2O_2-generation and pyocyanin-triggered ISR to *M. oryzae* (Figs. 3.5 and 3.7A). Although the exact nature of the quenching effect of ascorbate, and in turn, the attenuation of the ISR performance by ascorbate cannot be explained at this stage, since it is not known whether ascorbate merely has a quenching effect and/or might interfere further downstream of the signalling pathways leading to ISR, the involvement of H_2O_2 production by redox-active pyocyanin in relation to ISR is apparent. Hence,

induction of pyocyanin-mediated H_2O_2 microbursts most likely constitutes the *in situ* mechanism of 7NSK2-mediated ISR against *M. oryzae*. In line with this concept, there is substantial evidence demonstrating the defensive capacity of H_2O_2 in rice-*M. oryzae* interactions. Induction of elevated levels of H_2O_2 in transgenic rice expressing a fungal glucose oxidase gene triggered the expression of several defense genes, cell death and enhanced blast resistance in response to wounding and pathogen-infection (Kachroo et al., 2003a). Likewise, increasing endogenous levels of H_2O_2 by expression of constitutively active OsRac1, a small GTP-binding protein homologous to human Rac, triggered cell death and enhanced blast resistance in transgenic rice plants. Conversely, dominant negative OsRac1 suppressed elicitor-induced ROS production in transgenic cell cultures, and in plants suppressed R-gene-mediated resistance to *M. oryzae* (Kawasaki et al., 2006; Ono et al., 2001). Moreover, the increased blast resistance of several rice lesion mimic mutants is linked with elevated H_2O_2 production (Ueno et al., 2003; Takahashi et al., 1999).

Pyocyanin-negative mutants, unlike the wild-type bacterium, significantly reduced sheath blight severity (Fig. 3.3), whereas the same mutant strains lost the capacity to mount ISR to *M. oryzae*. In concordance with these observations, pyocyanin-treated rice seedlings exhibited increased susceptibility to *R. solani* (Fig. 3.4B), suggesting that pyocyanin acts as a negative regulator of disease resistance responses towards *R. solani*. Similar to pyocyanin-induced ISR to *M. oryzae*, addition of ascorbate to the nutrient solution alleviated pyocyanin-stimulated susceptibility to *R. solani* (Fig. 3.7B). Hence, the cumulative results suggest that the differential beneficial effect of pyocyanin in ISR to *M. oryzae* and *R. solani* is due to its capacity to generate H_2O_2 *in planta*.

In keeping with our results, ROS have been thought previously to play a dual role in plant resistance to pathogens. Despite the numerous lines of evidence demonstrating the involvement of ROS in the induction of various defense reactions, including orchestration of hypersensitive cell death, which is a highly effective defense mechanism against biotrophic pathogens, their accumulation has also been reported to be involved in successful pathogenesis of necrotrophic pathogens (Glazebrook, 2005; Hennin et al., 2001; Govrin and Levine, 2000). Although the role of H_2O_2 in cell death induction is widely accepted (Apel and Hirt, 2004; Delledonne et al., 2001), its benefit for resistance strategies varies with the type of pathosystem and host tissues. For instance, transgenic expression of animal cytoprotective antiapoptotic genes in tobacco conferred heritable resistance to several necrotrophic pathogens (Dickman et al., 2001). Similarly, the HR-deficient *Arabidopsis* mutant *dnd1* was highly resistant to the necrotrophic fungi *Botrytis cinerea* and *Sclerotinia sclerotiorum*, whereas treatments of *A. thaliana* with pro-oxidantia, or a HR-causing *P. syringae* strain, prior to infection with *B. cinerea* or *S. sclerotiorum* enhanced disease severity,

suggesting that HR-associated cell death facilitates pathogenesis by necrotrophic pathogens (Govrin and Levine, 2000). Although root treatment with pyocyanin, at least in the pico- and nanomolar range, did not induce visible cell death, neither in local nor in systemic tissue, a marked increase in the number of HR-expressing epidermal penetration sites was observed in response to infection with *M. oryzae* (Fig. 3.8B). Furthermore, pyocyanin feeding, despite not interfering with the penetration process of *R. solani*, provoked intense browning of epidermal cells ahead of fungal invasion (Fig. 3.8L). As ascorbate treatment inhibited these pyocyanin-mediated cellular responses (Fig. 3.8C), it could be reasoned that the pyocyanin-induced generation of H_2O_2 microbursts might lower the threshold for initiating programmed cell death, and by consequence facilitate subsequent infection with *R. solani*, which as a necrotrophic pathogen depends on host cell death as a prerequisite for successful pathogenesis.

In summary, the dual role of the phenazine antibiotic pyocyanin in *P. aeruginosa* 7NSK2-mediated ISR suggests that rice requires distinct mechanisms for defense against *M. oryzae* and *R. solani*. On one hand, root treatment with pyocyanin was effective against *M. oryzae*, triggering reiterative H_2O_2 microbursts, and causing rapid HR-associated cell death in response to fungal infection, which most likely leads to breakdown of the biotrophic phase of the *M. oryzae* infection cycle. On the other hand, treatment with pyocyanin significantly promoted subsequent infection by the necrotrophic pathogen *R. solani* by facilitating pathogen-triggered host cell death. Hence, the oxidative burst and related hypersensitive response might act as a double-edged sword in the interaction of rice with hemibiotrophic (*M. oryzae*) and necrotrophic (*R. solani*) pathogens. This conclusion is substantiated with recent research by Ahn et al. (2005b), demonstrating the differential beneficial effect of the HR as defense mechanism against *M. oryzae* and the necrotrophic rice pathogen *Cochliobolus miyabeanus*. Considering that the effect of the oxidative burst and HR-associated cell death depends on the type of invading pathogen, the widespread cultivation of resistant blast varieties that rely upon major resistance genes may contribute to the increase in sheath blight incidence. In this respect, our recent observation that *R. solani* colonization and sheath blight development is favoured by pre-inoculation with a HR-triggering incompatible *M. oryzae* isolate is of particular interest (De Vleesschauwer et al., unpublished results) and might explain why there are no HR-triggering gene-for-gene phenomena known for *R. solani*-rice interactions. Our work underscores the importance of utilizing appropriate innate defense mechanisms in plant breeding programs and might contribute to the development of new strategies for disease control.

Materials and Methods

Bacterial strains and plasmids

Bacteria and plasmids used in this study are listed in Table 3.1. Mutant strains 7NSK2-*phzM* and KMPCH-*phzM* were constructed as described by Rabaey et al. (2005) by homologous recombination using plasmid pZM1-Gm as suicide vector. In trans complementation of 7NSK2-*phzM* for pyocyanin production was performed as described by Audenaert et al. (2002b).

Evaluation of plant colonization by *Pseudomonas aeruginosa* 7NSK2 and mutants

To assess whether root colonization with *P. aeruginosa* leads to bacterial colonization of distal plant parts, leaves and stems of 20- and 35-day-old plants (end of bioassay) were checked for bacterial colonization. For four plants per treatment, leaves and stems were pooled before maceration in 1.5 ml of sterile demineralized water and plated out on KB amended with the appropriate antibiotics. Bacterial counts were made after 24 and 48 h of incubation at 37 °C and the experiment was performed twice.

Pathogen inoculation and disease rating

The Vietnamese *M. oryzae* isolate VT5M1 was used for all infection trials. Inoculum production and inoculation was performed exactly as described by Ninh Thuan et al. (2006). Each plant was sprayed with 1 ml of inoculum (1 x 10^4 spores ml^{-1} in a 0.5% gelatin solution). Six days after inoculation, disease was assessed by counting the number of susceptible-type lesions, which are defined as elliptical to round-shaped lesions characterized by a gray centre indicative of sporulation of the fungus (Schweizer et al., 1997).

Rhizoctonia solani

The virulent *R. solani* isolate MAN-86 (AG-1, IA), obtained from symptomatic plants (cv. IR-50) in rice fields in the state of Karnataka (India) and kindly provided by Dr. Sam Gnanamanickam, was used to inoculate the plants. Inoculum was obtained as described by Rodrigues et al. (2003b). Inoculated plants were kept for 72 h inside the humid inoculation chambers (≥ 92% R.H.) at 30 ± 4 ºC and thereafter transferred to greenhouse conditions. Ninety-six h after inoculation, disease was evaluated by measuring the length of the water-soaked lesions as described by Singh et al. (2002).

Pyocyanin extraction and quantification

The pyocyanin-overproducing strain 7NSK2-phz2 was grown for 48 h on Pseudomonas P agar medium (Difco, Le Pont de Claix, France) at 37 ºC. Purification of pyocyanin was performed as described by Abeysinghe (1999). For quantification, the weight of the purified pyocyanin crystals was determined. For all experiments reported herein, pyocyanin was suspended in sterile demineralized water and it was filter-sterilized prior to use.

Table 3.1. ***Bacteria and plasmids used in this study with their relevant characteristics***

Strains or plasmids	Relevant characteristics[a]	Reference or source
P. aeruginosa		
7NSK2	Pyo$^+$, Pvd$^+$, Pch$^+$, SA$^+$, wild type	Iswandi et al., 1987
KMPCH	Pyo$^+$, Pvd$^-$, Pch$^-$, SA$^+$, chemical mutant of the pyoverdin-negative mutant MPFM1; Km^r	Höfte et al., 1993
7NSK2-phzM	Pyo$^-$, Pvd$^+$, Pch$^+$, SA$^+$, *phzM* mutant of 7NSK2, obtained by gene replacement using plasmid pZM1-Gm; Gm^r	This study
7NSK2-phzMc	Pyo$^+$, Pvd$^+$, Pch$^+$, SA$^+$, 7NSK2-phzM containing pHZM (functional *phzM* gene on plasmid pBBR1MCS) that restores pyocyanin production, Gm^r, Cm^r	This study
7NSK2-phz2	Pyo$^+$, Pvd$^+$, Pch$^+$, SA$^+$, *retS* (PA4856) mutant of 7NSK2 obtained by miniTn*pho*A3 mutagenesis, overproduces pyocyanin, Gm^r	This study
KMPCH-phzM	Pyo$^-$, Pvd$^-$, Pch$^-$, SA$^+$, *phzM* mutant of KMPCH, obtained by gene replacement using plasmid pZM1-Gm; Gm^r	This study
Plasmids		
pBR322	Suicide vector in *Pseudomonas*, Cm^r/Cb^r/Tc^r	Bolivar, 1978
pZM1-Gm	2054-bp PCR-amplified fragment of primer pair 4209A-B (*phzM*) of *P. aeruginosa* PAO1, inactivated by a site-specific insertion of a 803-bp *NotI*-blunted Gm cassette, cloned in pBR322	Rabaey et al., 2005
pBBR1MCS	Broad host-range cloning vector for *Pseudomonas*, Cm^r	Kovach et al., 1994
pHZM	A 2054-bp PCR-amplified fragment of primer pair 4209A-B (*phzM*) of 7NSK2 cloned in the *EcoRV* site of pBBR1MCS, Cm^r	Audenaert et al., 2002b

[a] Pyo = Pyocyanin, Pvd = Pyoverdin, Pch = Pyochelin, SA = salicylic acid, Km = Kanamycin, Gm = Gentamycin, Cm = Chloramphenicol, Cb = Carbenicilin, Tc = Tetracycline

In vitro production of pyocyanin by bacterial strains

To monitor *in vitro* production of pyocyanin by the distinct strains, bacteria were grown for 48 h on Pseudomonas P agar medium at 37 ºC. Bacteria were scraped off the plates, suspended in sterile distilled water and centrifuged for 10 min at 4 ºC. Pyocyanin present in the supernatant was extracted twice with chloroform and determined spectrophotometrically in the presence of 0.1 M HCl (optical density at 510 nm) as described by Essar et al. (1990). The experiment was set up in 6 replicates and repeated twice.

Hydroponic plant growth

For experiments in which purified pyocyanin was applied to rice seedlings, plants were grown in a hydroponic gnotobiotic system. Surface-sterilized rice seeds were germinated for 5 days on wet filter paper in Petri dishes. After incubation, germinated seeds were sown in perforated plastic trays (23x16x6 cm) filled with sterilized vermiculite, and supplemented with half-strength Hoagland solution (Hoagland and Arnon, 1938). Every three days, 0.5 litre of the half-strength Hoagland solution was added to each tray containing 12 seedlings. In this model, 4 days before challenge inoculation, various concentrations of pyocyanin and sodium ascorbate were applied to the plants by including the desired concentration in the nutrient solution without ethylenediaminetetraacetic acid ferric sodium salt (Acros, Geel, Belgium). This, to avoid possible ferric-catalyzed $HO^{\cdot}$ generation from pyocyanin-derived $O_2^{-\cdot}/H_2O_2$.

Histochemical detection of H_2O_2

To assess whether pyocyanin was able to produce H_2O_2 in the gnotobiotic system, rice roots were dipped in half-strength Hoagland nutrient solution containing 1 nM pyocyanin for 2 h, rinsed thoroughly with demineralized water and subsequently incubated for 12 h at room temperature in water with 0.01% Triton-X-100 and 3,3'-diaminobenzidine (DAB). DAB (Sigma-Aldrich, Bornem, Belgium) polymerizes in the presence of H_2O_2 to form a brownish-red precipitate that can be visualized. The specificity of the staining was verified by adding 10 mM ascorbic acid.

***In planta* determination of H_2O_2**

The *in planta* accumulation of H_2O_2 was determined following the $TiCl_4$-based technique as described by Mur et al. (2005). H_2O_2 accumulation was expressed relative to values obtained in control samples. Each experiment consisted of 6 replicates per treatment and was repeated twice to generate the data presented.

Enzyme extraction and activity assays

Frozen leaf samples were crushed to a fine powder in a mortar under liquid nitrogen. Soluble proteins were extracted by resuspending the powder in four volumes of 50 mM sodium phosphate buffer (pH 7.5), containing 1 mM EDTA, 1 mM PMSF, 5 mM sodium ascorbate and 5% (w/v) PVPP. The homogenate was centrifuged at 17000 *g* for 10 min. The supernatant was divided into aliquots, frozen in liquid nitrogen and stored at – 80 ºC for further analysis. All above operations were carried out at 0-4 ºC. Activity levels of the various antioxidant enzymes (namely catalase [CAT], guiacol-dependent peroxidase [GPX], ascorbate peroxidase [APX], and superoxide dismutase [SOD]) in plant extracts were measured spectrophotometrically as described by Garcia-Limones et al. (2002).

The CAT reaction medium consisted of 50 mM sodium phosphate buffer pH 7.0, 20 mM H_2O_2 and between 10 and 50 µl of enzyme extract. The reaction was started by adding H_2O_2 and the decrease in A_{240} (ε = 39.4 mM^{-1} cm^{-1}), produced by H_2O_2 breakdown was recorded. One CAT unit is defined as the amount of enzyme necessary to decompose 1 µmol min^{-1} H_2O_2 under the above assay conditions.

To assay GPX activity, the reaction mixture (3.0 ml) consisted of 100 mM potassium phosphate buffer pH 6.5, 15 mM guiacol, 0.25% (v/v) H_2O_2 (200 mM) and different volumes of enzyme extract. The reaction was started by adding H_2O_2 and the oxidation of guiacol was determined by the increase in A_{470} (ξ = 26.6 mM^{-1} cm^{-1}). One GPX unit is defined as the amount of enzyme that produces 1 µmol min^{-1} oxidized guiacol under the above assay conditions.

For APX activity assays, the reaction mixture consisted of 50 mM potassium phosphate buffer pH 7.0, 0.25 mM sodium ascorbate, 5 mM H_2O_2 and 50 µl of enzyme extract. The reaction was started by adding H_2O_2 and the oxidation of ascorbate was measured by the decrease in A_{290} (ξ = 2.8 mM^{-1} cm^{-1}). One unit of APX activity is defined as the amount of enzyme that oxidizes 1 µmol min^{-1} ascorbate under the above assay conditions.

SOD activity was determined from the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) in the presence of riboflavin. The reaction mixture (1.5 ml) consisted of 50 mM potassium phosphate buffer pH 7.8, 0.1 mM EDTA, 13 mM methionine, 75 µM NBT, 2 µM riboflavin and between 10 and 50 µl of enzyme extract. The reaction was started by adding riboflavin and A_{560} was recorded after 12 min incubation at room temperature under continuous light (70W). One SOD unit was defined as the amount of enzyme that inhibits the rate of NBT reduction by 50% under the above assay conditions.

In all assays the blank consisted of the components of the reaction mixture except for the enzyme extract, which was replaced by an equal volume of the assay buffer. In the SOD assay, the enzyme blank was taken as the 100% rate of NBT photochemical reduction. In the remaining cases the enzyme blanks were subtracted from the assay measurements. Protein levels in enzyme extracts were determined by the Bradford method (Bradford, 1976) with BSA as a standard.

Detection of pyocyanin by HPLC analysis

Hydroponically grown 28-day-old CO-39 seedlings were fed with a 50 µM pyocyanin solution as indicated above. At various time points post-application, leaves were excised, grinded and homogenized in chloroform. The soluble material was subjected to pyocyanin extraction according to Abeysinghe (1999). The extract was evaporated *in vacuo* to dryness and subsequently solubilized in 50% methanol. Samples were analyzed by high-performance liquid chromatography using a Genesis C18 column as described by Rabaey et al. (2005).

Cytological investigation by using bright-field and fluorescence microscopy

M. oryzae inoculation of intact leaf sheaths and preparation of specimens for microscopy were conducted as described by Koga et al. (2004b). For inoculation with *R. solani*, sheaths were opened carefully and a small piece (circa 1 mg) of sclerotium placed inside the sheath. A few drops (100 µl) of sterile water were added to the inoculated sheath. Cytological observations were made using an Olympus model BX51 microscope (Olympus, Aartselaar, Belgium) equipped with differential interference contrast optics. The autofluorescence of epidermal cell walls or the whole-epidermal cells of each appressorial site

examined was recognized by incident fluorescence microscopy (Olympus U-MWB2 GFP filter set-excitation: 450-480 nm, dichroic beamsplitter: 500 nm, barrier filter BA515). Images were acquired digitally (Olympus Color View II camera, Aartselaar, Belgium) and further processed with the Olympus analySIS cell^F software.

Chapter

4

Root colonization of rice by *Serratia plymuthica* IC1270 triggers a hydrogen peroxide-fueled resistance response with divergent effects against hemibiotrophic and necrotrophic leaf pathogens

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Accepted with revisions in BMC Plant Biology

Induced systemic resistance (ISR) is a state of enhanced defensive capacity developed by a plant colonized by selected strains of nonpathogenic rhizobacteria. Using a combined cytomolecular and pharmacological approach, we analyzed the host defense mechanisms associated with the establishment of ISR in rice by the rhizobacterium *Serratia plymuthica* IC1270. In a standardized soil-based assay, root treatment with IC1270 rendered foliar tissues more resistant to the hemibiotrophic pathogen *Magnaporthe oryzae*, causal agent of the devastating rice blast disease. Analysis of the cytological and biochemical alterations associated with restriction of fungal growth in IC1270-induced plants revealed that IC1270 primes rice for enhanced attacker-induced accumulation of reactive oxygen species (ROS) and autofluorescent phenolic compounds in and near epidermal cells displaying dense cytoplasmic granulation. Similar, yet more abundant, phenotypes of hypersensitively dying cells in the vicinity of fungal hyphae were evident in a gene-for-gene interaction with an avirulent *M. oryzae* strain, indicating that, at the cytological level, IC1270-mediated ISR is a partial phenocopy of effector-triggered immunity. Yet, this IC1270-mediated ISR response seems to act as a double-edged sword within the rice defense network as bacterized plants displayed an increased vulnerability to the necrotrophic pathogens *Rhizoctonia solani* and *Cochliobolus miyabeanus*. Artificial enhancement of ROS levels in inoculated leaves faithfully mimicked the opposite effects of IC1270 bacteria on aforementioned pathogens, suggesting a central role for oxidative events in the IC1270-induced resistance mechanism. Besides tagging ROS as modulators of antagonistic defense mechanisms in rice, this work reveals the mechanistic similarities between *S. plymuthica*-mediated ISR and effector-triggered immunity and underscores the importance of using appropriate innate defense mechanisms when breeding for broad-spectrum rice disease resistance.

Introduction

Plants have evolved a powerful immune system to resist their potential colonization by microbial pathogens and parasites. Over the past decade, it has become increasingly clear that this innate immunity is, in essence, composed of two interconnected branches, termed PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl, 2006; Eulgem and Somssich, 2007). PTI is triggered by recognition of pathogen- or microbial-associated molecular patterns (PAMPs/MAMPs), which are conserved molecular signatures decorating many classes of microbes, including non-pathogens. Perception of MAMPs by pattern recognition receptors (PRRs) at the cell surface activates a battery of host defense responses leading to a basal level of resistance (Chisholm et al., 2006). As a result of the evolutionary arms-race between plants and their intruders, many microbial pathogens acquired the ability to dodge PTI-based host surveillance via secretion of effector molecules that intercept MAMP-triggered defense signals (Gohre and Robatzek, 2008). In turn, plants have adapted to produce cognate R-(resistance) proteins by which they recognize these pathogen-specific effector proteins, resulting in a superimposed layer of defense variably termed effector-triggered immunity (ETI), gene-for-gene resistance or *R*-gene-dependent resistance (Jones and Dangl, 2006).

In many cases, effector recognition culminates in the programmed suicide of a limited number of challenged host cells, clearly delimited from the surrounding healthy tissue. This hypersensitive response (HR) is thought to benefit the plant by restricting pathogen access to water and nutrients and is correlated with an integrated set of physiological and metabolic alterations that are instrumental in impeding further pathogen ingress, among which a burst of oxidative metabolism leading to the massive generation of reactive oxygen species (ROS) (Greenberg and Yao, 2004; Glazebrook, 2005). Apart from local immune responses, ETI-associated HR formation also mounts a long-distance immune response termed systemic acquired resistance (SAR), in which naïve tissues become resistant to a broad spectrum of otherwise virulent pathogens (Durrant and Dong, 2004). It should be noted, however, that PTI, when activated by PAMPs that activate the SA signaling pathway, can trigger SAR as well (Mishina and Zeier, 2007).

An archetypal inducible plant defense response, SAR requires endogenous accumulation of the signal molecule salicylic acid (SA) and is marked by the transcriptional reprogramming of a battery of SA-inducible genes encoding pathogenesis-related (PR) proteins. By contrast, there is ample evidence for induced disease resistance conditioned by molecules other than SA, as illustrated by rhizobacteria-mediated induced systemic resistance [ISR; (Van Loon et al., 1998)]. ISR, which delivers systemic protection without the customary pathogenesis-related protein

induction, is a resistance activated upon root colonization by specific strains of plant growth-promoting rhizobacteria (PGPRs) (Bostock, 2005). In a series of seminal studies using the reference strain *Pseudomonas fluorescens* WCS417r, Pieterse and associates (Pieterse et al., 1996; Pieterse et al., 1998; Pieterse et al., 2000) demonstrated that, at least in Arabidopsis, ISR functions independently of SA, but requires components of the jasmonic acid (JA) and ethylene (ET) response pathways. Even though colonization of the roots by ISR-triggering bacteria leads to a heightened level of resistance against a diverse set of intruders, often no defense mechanisms are activated in aboveground plant tissues upon perception of the resistance-inducing signal. Rather, these tissues are sensitized to express basal defense responses faster and/or more strongly in response to pathogen attack, a phenomenon known as priming (Conrath et al., 2002). As demonstrated recently, priming of the plant's innate immune system confers broad-spectrum resistance with minimal impact on seed set and plant growth (van Hulten et al., 2006). Hence, priming offers a cost-efficient resistance strategy, enabling the plant to react more effectively to any invader encountered by boosting infection-induced cellular defense responses (Conrath et al., 2006; Beckers and Conrath, 2007).

In contrast to the overwhelming amount of information on inducible defenses in dicotyledonous plant species, our understanding of the molecular mechanisms underpinning induced disease resistance in rice (*Oryza sativa*) and other cereals is still in its infancy (Kogel and Langen, 2005). Evidence demonstrating that central components of the induced resistance circuitry, including the master regulatory protein NPR1, are conserved in rice has only recently been presented (Chern et al., 2001; Chern et al., 2005b; Shimono et al., 2007; Yuan et al., 2007). Moreover, reports on SAR-like phenomena in rice are scarce. Most tellingly in this regard, a 17-year-old report of systemically enhanced resistance against the rice blast pathogen *M. oryzae* triggered by a localized infection with the non-rice pathogen *P. syringae* pv. *syringae* remains one of the most compelling examples of a SAR-like response in rice to date (Smith and Metraux, 1991). In contrast, there is a sizeable body of evidence demonstrating systemic protection against various rice pathogens resulting from ISR elicited by, amongst others, *Pseudomonas* (Nandakumar et al., 2001; Nagarajkumara et al., 2005), *Bacillus* (Jayaraj et al., 2004) and *Serratia* strains (Someya et al., 2005). However, none of these studies managed to portray an accurate picture of ISR in terms of mechanistic issues.

In a previous study, we demonstrated that rice plants of which the roots were colonized by the fluorescent pseudomonad *P. aeruginosa* 7NSK2 developed an enhanced defensive capacity against infection with *M. oryzae*. Bacterial mutant analysis revealed that this 7NSK2-mediated ISR is based on secretion of the redox-active pigment pyocyanin. Perception of pyocyanin by the

plant roots was shown to cue the formation of reiterative micro-oxidative bursts in naïve leaves, thereby priming these leaves for accelerated expression of HR-like cell death upon pathogen attack (Chapter 3). Aiming to gain further insight into the molecular mechanisms underpinning rhizobacteria-modulated ISR in rice, we tested the ability of the biocontrol agent *Serratia plymuthica* IC1270 to induce systemic resistance against various rice pathogens with different modes of infection. Originally isolated from the rhizosphere of grapes, *S. plymuthica* IC1270 is a well-characterized PGPR strain producing a broad palette of antimicrobial compounds (Chernin et al., 1995; Ovadis et al., 2004; Meziane et al., 2005; De Vleesschauwer and Höfte, 2007). In addition to its potential as a direct antagonist of a wide array of plant pathogens, preliminary experiments in bean and tomato revealed that IC1270 is equally capable of reducing disease through activation of a plant-mediated defense response (De Vleesschauwer and Höfte, 2007). Here, we demonstrate that colonization of rice roots by IC1270 renders foliar tissues more resistant to *M. oryzae*. Using a combined cytological and pharmacological approach, evidence is provided that IC1270 locks plants into a pathogen-inducible program of boosted ROS formation, culminating in the prompt execution of HR cell death at sites of attempted pathogen entry. Similar, yet even more pronounced, phenotypes of hypersensitively dying cells in the vicinity of fungal hyphae were observed in a genetically incompatible rice-*M. oryzae* interaction, indicating that, at the cytological level, IC1270-mediated ISR is a partial phenocopy of *R*-gene-mediated ETI. However, this IC1270-inducible and ETI-resembling resistance mechanism seems to play an ambivalent role within the rice disease resistance network, as bacterized plants were rendered hypersusceptible to the necrotrophic pathogens *R. solani* and *C. miyabeanus*

Results

Differential effectiveness of ISR triggered by *S. plymuthica* IC1270

To assess the ISR-triggering capacity of *S. plymuthica* IC1270, susceptible rice plants were grown in soil containing IC1270 bacteria, and subsequently challenged with several fungal pathogens exhibiting different modes of infection. In these ISR bioassays, the resistance-inducing potential of IC1270 was compared to that of *P. aeruginosa* 7NSK2, a well-studied PGPR strain which we previously uncovered as a potent activator of induced resistance responses in rice (Chapter 3).

We first tested whether root colonization by *S. plymuthica* IC1270 exerts a protective effect against infection by the hemibiotrophic ascomycete *M. oryzae*, causal agent of the devastating rice blast disease and a major threat to food security worldwide (Caracuel-Rios and Talbot, 2007). By 4 days post-inoculation (dpi), leaves of control, non-bacterized plants displayed typical water-soaked, diamond-shaped lesions, developing conidia at the center of each lesion by 6 dpi. In contrast, IC1270-bacterized plants exhibited a marked reduction in the number of these susceptible-type lesions, producing a resistance phenotype mimicking that of quantitative trait loci-governed intermediate resistance (Fig. 4.1A). This resistance type is characterized by the abundance of small necrotic non-sporulating lesions, less than 2 mm in diameter, 60 to 72 h post-inoculation (hpi). Consistent with our previous findings (Chapter 3), treatment with *P. aeruginosa* 7NSK2 resulted in a substantial reduction of disease as well. No significant differences in the number of susceptible-type lesions could be observed between IC1270- and 7NSK2-treated plants, indicating that IC1270 and 7NSK2 are equally effective in suppressing *M. oryzae*.

Because IC1270 clearly inhibited the growth of *M. oryzae* in dual culture experiments (data not shown), possible systemic plant colonization by the rhizobacteria was checked. However, in all bioassays performed, IC1270 bacteria were absent from sheaths or leaves of root-induced plants, indicating that bacterial colonization remained confined to the root zone (data not shown). Although such spatial separation does not rule out the possibility that IC1270-conferred protection might result from long-distance translocation of bacteria-produced allelochemicals to systemic leaves, the latter is rather unlikely as pilot experiments revealed that mutants defective in the synthesis of various antimicrobial metabolites were as effective as wild-type IC1270 in reducing blast disease severity (De Vleesschauwer and Höfte, unpublished results). The cumulative data therefore strongly suggest that the beneficial protective activity exerted by *S. plymuthica* IC1270 is based on activation of the plant's defensive repertoire, rather then being caused by microbial antagonism.

To test the spectrum of effectiveness of this IC1270-mediated ISR, we next assayed for induction of resistance against the sheath blight pathogen, *Rhizoctonia solani*, and the brown spot pathogen, *Cochliobolus miyabeanus*, both of which are considered necrotrophic fungi. In contrast to *M. oryzae*, which sequentially invades living cells (Kankanala et al., 2007), *R. solani* and *B. oryzae* kill host cells at very early stages in the infection, leading to extensive tissue damage (Ou, 1985). As shown in Figure 4.1B, both IC1270 and 7NSK2 failed to reduce disease caused by *R. solani*. This impaired ISR response was not due to insufficient root colonization as bacterial counts in the rhizosphere of bacterized rice seedlings were comparable to those obtained in the *M. oryzae* bioassays ($1.14 \pm 0.19 \times 10^5$ CFU. g^{-1}). Interestingly, in all four independent experiments, IC1270 pretreatment favored subsequent infection by *R. solani*, causing an average 39.6% increase in disease severity relative to non-induced controls. A similar trend was observed when challenging with *C. miyabeanus*, with IC1270 consistently promoting vulnerability to the latter pathogen (Fig. 4.1C). Root colonization by 7NSK2, however, yielded variable results. No significant differences between control and 7NSK2-treated plants could be observed in three bioassays (Fig. 4.1D), whereas in the two remaining assays, root treatment with 7NSK2 rendered rice seedlings substantially more susceptible to brown spot (Fig. 4.1E).

In all experiments, mock-inoculated control plants remained healthy, and no apparent differences in appearance, size, or weight of control, 7NSK2 or IC1270-treated plants were observed prior to challenge infection (data not shown). Thus, under the experimental conditions used in this study, root treatment with the ISR-inducing bacteria did not lead to detectable effects on plant growth that could have affected the growth or development of the respective pathogens. Collectively, these findings demonstrate that *S. plymuthica* IC1270 plays an ambivalent role in the rice induced resistance network, acting as a positive regulator of resistance to the hemibiotroph *M. oryzae* while promoting susceptibility to the necrotrophs *C. miyabeanus* and *R. solani*.

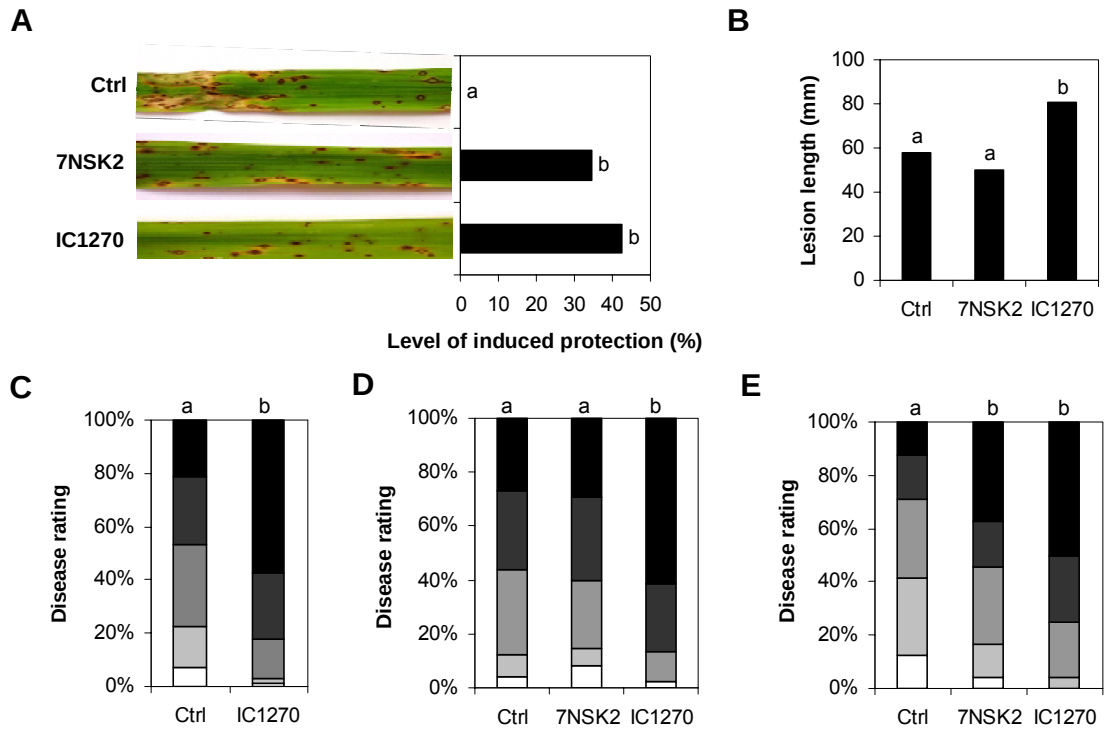

Figure 4.1. Spectrum of effectiveness of *Pseudomonas aeruginosa* 7NSK2- and *Serratia plymuthica* IC1270-triggered ISR in rice.

ISR was induced by growing the plants in soil containing 7NSK2 or IC1270 bacteria. Control plants were treated with water. **A,** Quantification of ISR against *M. oryzae*. Plants were challenged when 4 weeks old (5-leaf stage) by spraying a spore suspension of virulent *M. oryzae* VT7 at 1 x 10^4 conidia ml^{-1}. Six days after challenge inoculation, disease was rated by counting the number of susceptible-type lesions per leaf 4 and expressed relative to non-bacterized control plants. Photographs depicting representative symptoms were taken 7 days post inoculation. **B,** Quantification of ISR against *R. solani*. Four-week-old plants were challenged by placing a 1 cm-toothpick colonized by *R. solani* inside the sheath of the second youngest fully developed leaf; 4 days later, disease severity was assessed by measuring the total length of sheath blight lesions. **C – E,** Quantification of ISR against *C. miyabeanus*. Plants were challenge-inoculated when five weeks old by spraying a conidial suspension at 1 x 10^4 conidia ml^{-1}. Disease evaluation was performed 4 d postinoculation, using a 1-to-5 disease severity scale as described in the Methods section. For all graphs, statistical analysis was performed on pooled data from at least four independent experiments, because interaction between treatment and experiment was not significant at $\alpha = 0.05$ and variances were homogeneous. Figures **D** and **E**, however, represent data pooled from three and two independent experiments, respectively. Different letters indicate statistically significant differences between treatments according to non-parametric Kruskall-Wallis and Mann-Whitney tests ($n \geq 42$; $\alpha = 0.05$).

S. plymuthica IC1270 triggers HR-like responses at the sites of pathogen attack

To begin to unravel the defense mechanism(s) underpinning IC1270-mediated ISR, we analyzed the cytological alterations associated with restriction of *M. oryzae* in IC1270-induced plants using the intact leaf sheath method designed by Koga and associates (2004b). In this system, intact leaf sheaths of non-bacterized and IC1270-induced plants of the highly susceptible rice variety CO39 were routinely inoculated by injecting a conidial suspension of the virulent blast isolate VT7. For comparison with *R* gene-mediated ETI, we also included the VT7-resistant variety C101LAC, the latter being a near-isogenic line of CO39 carrying the blast resistance genes *Pi-1* and *Pi-33* (Mackill and Bonman, 1992; Berruyer et al., 2003).

No obvious alterations in cell physiology due to IC1270 treatment were observed prior to infection. Similarly, quantitative recording of attempted blast infections revealed no significant differences in the number of unsuccessful penetration events, indicating that both IC1270-mediated ISR and *R*-gene-conditioned ETI are unlikely to impede pre-penetration development by *M. oryzae* (data not shown). On the other hand, epidermal cells were found to respond to fungal ingress through various cellular reaction types depicted at 48 hpi in Figure 4.2A. A susceptible reaction was manifested as a type 1 phenotype in which extensively branched invasive hyphae vigorously invaded living epidermal cells with little or no host response. Interaction phenotype 2, on the other hand, was characterized by prompt arrest of fungal growth in the first-invaded epidermal cell, a phenomenon associated with enhanced vesicular activity and browning of the anticlinal cell walls, while a type 3 reaction represented infection sites in which fungal invasion was curtailed shortly after penetration due to development of HR-like cell death, as indicated by the characteristic aggregation of the cytoplasm and a bright autofluorescence of the anticlinal cell walls (Koga, 1994; Koga et al., 2004b). As expected, sheath cells of non-induced, susceptible CO39 plants inoculated with virulent VT7 predominantly mounted a type 1 reaction, whereas HR was the prevailing plant response in the incompatible interaction between VT7 and C101LAC. Most conspicuously, IC1270-induced CO39 sheath cells displayed an interaction profile resembling that observed in VT7-invaded sheaths of genetically resistant C101LAC, with type 3 reactions accounting for approximately 60% of all interactions at 48 hpi (Fig. 4.2B).

At later stages of infection, *M. oryzae* had massively colonized the epidermis and mesophyll of CO39 sheaths causing extensive host damage as evidenced by the ubiquitous presence of cellular debris and fragmented remnants of host cell walls around invasive hyphae in the mesophyll (data not shown). By contrast, in resistant C101LAC, as well as in IC1270-induced CO39, invading hyphae were largely trapped within hypersensitively dying cells in the epidermal layer, preventing fungal passage to the underlying tissue.

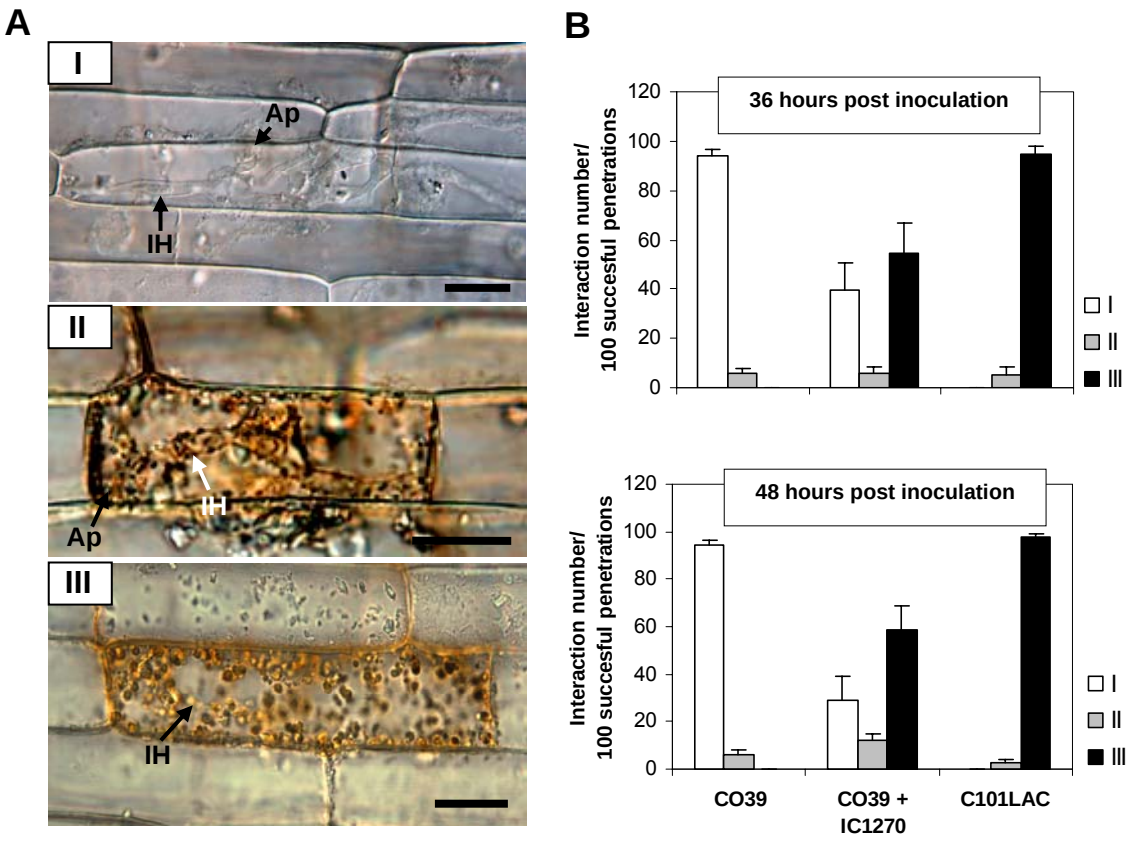

Figure 4.2. Influence of root treatment with *S. plymuthica* IC1270 on *M. oryzae*-induced cellular responses in rice.

A, Intact leaf sheaths of the susceptible cv. CO39 and its resistant near-isogenic line C101LAC were challenged by injecting a conidial suspension of *M. oryzae* VT7. Left, Micrographs depicting representative interaction phenotypes: (I), Vigorous invasion of living tissues in the absence of visible host responses. (II), Fungal arrest in the first-invaded cell associated with browning of anticlinal cell walls and enhanced vesicular activity. (III), Abrupt arrest of fungal invasion in hypersensitively reacting epidermal cell as indicated by dense cytoplasmic aggregation. Ap, appressorium or appressorial site. IH = invading hyphae. Scale bars = 20 μm. **B,** Frequencies of abovementioned interaction phenotypes at 36 and 48 hours post inoculation. Each bar represents the mean and SD of six replications stemming from three plants. At least 50 single-cell interaction sites originating from representative sheath sections were examined per replication. Data from one experiment is presented. Repetition of experiments led to results very similar to those shown.

Because rapid accumulation of phenolic compounds is a hallmark of rice defense against *M. oryzae* (Koga, 1994; Rodrigues et al., 2005), we also examined the effect of IC1270 pre-treatment on the level of autofluorescence. Autofluorescence was detectable as early as 18 hpi, irrespective of IC1270 treatment or the level of resistance of the cultivars used (Fig. 4.3A). However, similar to what was observed in resistant C101LAC, root treatment of CO39 with IC1270 caused the frequency of autofluorescent appressorial sites to increase rapidly from 18 hpi onward, reaching a level of 60 and 100% of all interactions by 24 and 36 hpi, respectively (Fig. 4.3B). By contrast, in non-induced CO39 cells, less than 6% of the appressorial sites showed autofluorescence by 24 hpi, indicating that root colonization by IC1270 primes rice sheath cells for accelerated

deposition of autofluorescent phenolic compounds at sites of attempted pathogen invasion. Along with the high frequency of hypersensitively reacting cells, these observations indicate that, at the cytological level, IC1270-mediated ISR is a partial phenocopy of *R*-gene-conditioned ETI to *M. oryzae*.

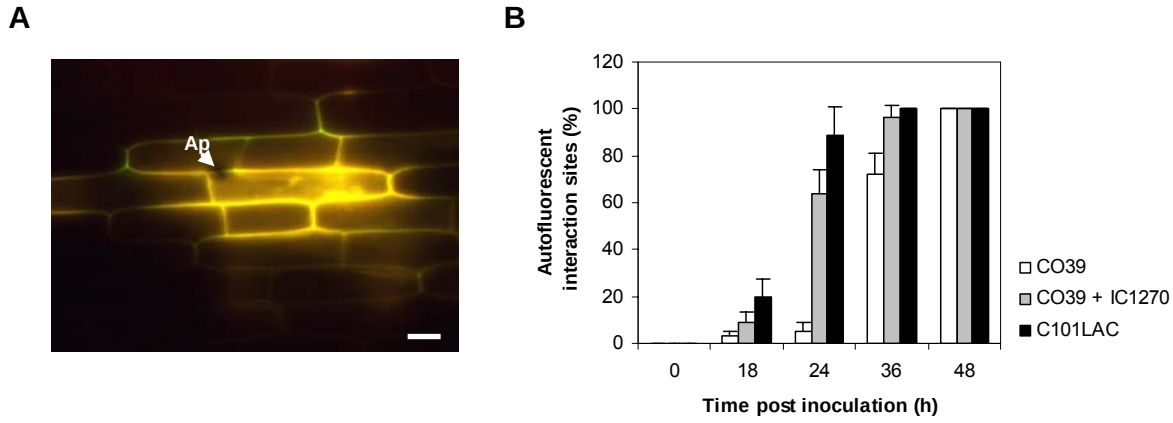

Figure 4.3. *S. plymuthica* IC1270 primes rice for enhanced accumulation of autofluorescent phenolics upon challenge inoculation.

Intact leaf sheaths of the susceptible cv. CO39 and its resistant near-isogenic line C101LAC were challenged by injecting a conidial suspension of *M. oryzae* VT7. **A,** Epifluorescence image of IC1270-induced sheath cells at 24 hpi. Ap = appressorium. Scale bar = 20 µm. **B,** Comparative kinetic analysis of autofluorescence in water-treated susceptible (CO39), susceptible yet ISR-expressing (CO39 + IC1270), and genetically resistant (C101LAC) plants. Each bar represents the mean and SD of six replications stemming from three plants. Data from one experiment is presented. Repetition of experiments led to results very similar to those shown.

S. plymuthica IC1270-mediated ISR to *M. oryzae* involves priming for enhanced attacker-induced H_2O_2 generation

There is ample evidence demonstrating the active involvement of reactive oxygen species (ROS), and H_2O_2 in particular, in the induction, signaling and execution of blast resistance in rice (Ganesan and Thomas, 2001; Ono et al., 2001; Kawasaki et al., 2006; Vergne et al., 2007). Furthermore, in the course of previous studies, we demonstrated that pyocyanin-induced H_2O_2 microbursts are primordial for the onset of *P. aeruginosa* 7NSK2-mediated ISR against *M. oryzae* (Chapter 3). Taking these facts into account, we sought to extend our cytological analysis of ISR elicited by IC1270 by monitoring the spatiotemporal patterns of pathogenesis-related H_2O_2 production. *In planta* accumulation of H_2O_2 was visualized using an endogenous peroxidase-dependent staining procedure with 3,3'-diaminobenzidine (DAB). In these DAB assays, reddish-brown precipitates are deposited at the sites of H_2O_2 accumulation (Thordal-Christensen et al., 1997). No DAB accumulation was observed in mock-inoculated controls, regardless of IC1270 treatment or the inherent level of resistance of the cultivars used. However, comparative kinetic

analysis of H_2O_2 production in pathogen-inoculated seedlings revealed the occurrence of a wide range of distinct DAB staining patterns that could be grouped into five categories (Fig. 4.4A). The first type comprised interaction sites in which DAB accumulation was not detectable despite massive fungal colonization of both penetrated and neighboring epidermal cells. Conversely, interaction sites displaying H_2O_2 accumulation in the primary invaded epidermal cell following spread of the invasive hyphae into neighboring cells were classified as a type II reaction. Type III interaction sites were characterized by the ubiquitous occurrence of DAB-positive vesicle-like bodies targeted to the invading hyphae. A type IV reaction referred to intracellular DAB staining tightly associated with the characteristic cytoplasmic aggregates of HR-expressing cells (type IV), while interaction sites displaying whole-cell DAB accumulation were scored as a type V reaction. Importantly, when the DAB solution was supplemented with ascorbate, staining was abolished, indicating that the staining was due to H_2O_2 (data not shown).

Leaf sheath cells of susceptible CO39 were characterized by the high ratio of H_2O_2-negative type I reactions, accounting for 78% and 67% of all interaction sites by 36 and 48 hpi, respectively (Fig. 4.4B). In some incidences (21% of all interaction sites at 48 hpi), H_2O_2 accumulated in the initially penetrated epidermal cell following the formation of an extensively branched mycelium in the neighboring cells. Yet, this type II reaction seemingly occurred too late to effectively stall the pathogen. IC1270-induced CO39 cells, on the other hand, exhibited a strikingly different set of responses in that type I reactions, reaching a level of 33% at 36 hpi, were no longer discernible by 48 hpi. The rapid decline in the frequency of type I reactions from 36 hpi onward corresponded to an approximately 15% increase in the frequency of both type III and type V reactions. HR-like cell death of attacked epidermal cells, seen at approximately 52% of all interaction sites, was always associated with H_2O_2 accumulation in the cytoplasmic aggregates, beginning 32 hpi. Although not identical, the H_2O_2 signature of IC1270-treated CO39 plants 48 hpi showed substantial similarity to that observed in the incompatible interaction between C101LAC and VT7, thereby further emphasizing the possible mechanistic parallels between IC1270-mediated ISR and R-protein-dictated ETI.

Starting 50 hpi, a strong accumulation of H_2O_2 was found in CO39 mesophyll cells that appeared to collapse, whereas in samples from IC1270-induced CO39 or C101LAC sheaths, DAB staining in the mesophyll layer was seldom observed (data not shown). However, at these late infection stages, massive H_2O_2 accumulation is most likely a consequence of progressive cellular destruction and overtaxed anti-oxidative capacities, and hence, a chaotic reaction associated with susceptibility, rather than a controlled defense response restricting cellular

accessibility for *M. oryzae*. Together these results clearly demonstrate the potential of IC1270 to prime rice for augmented generation of epidermis-localized H_2O_2.

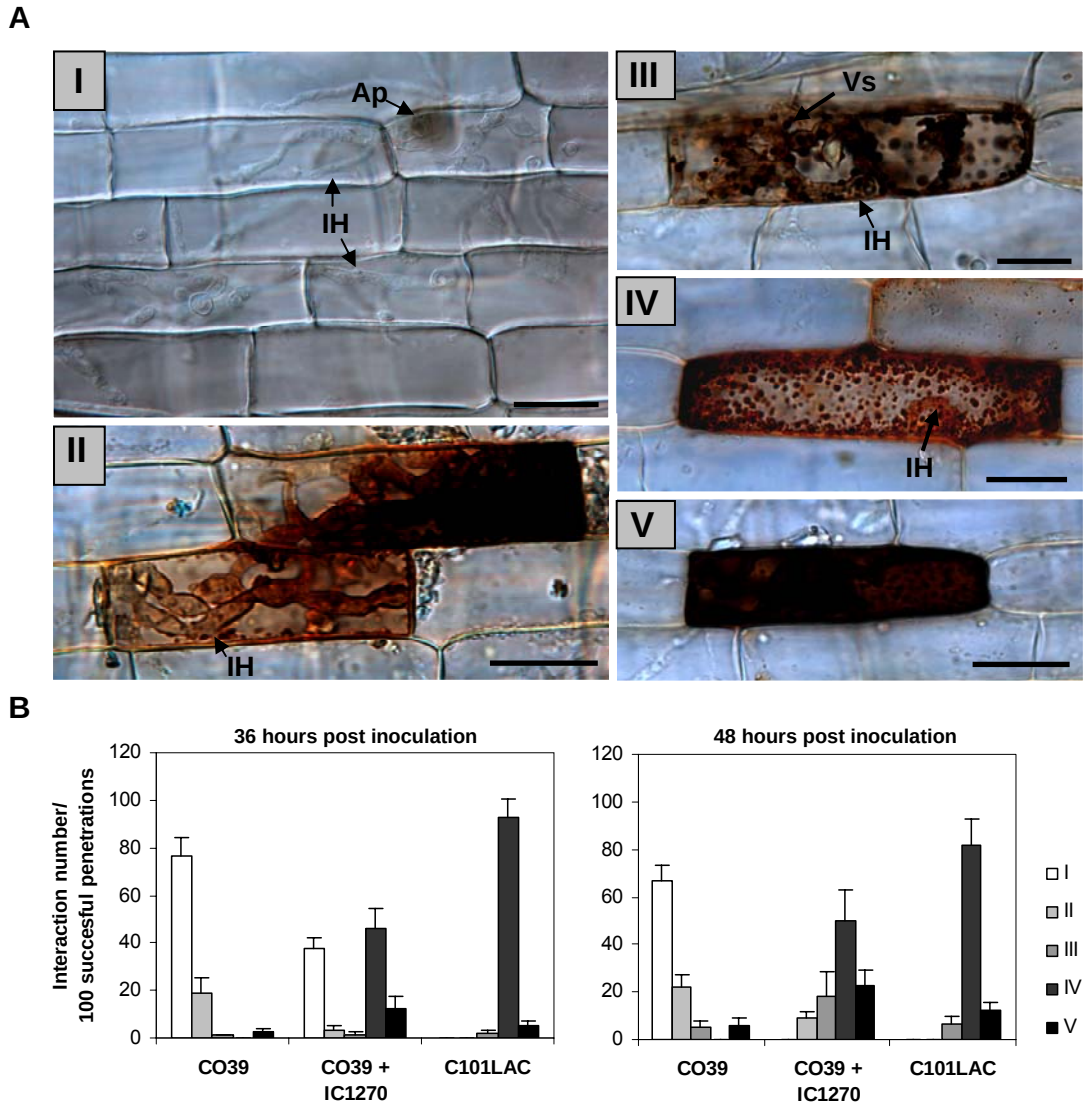

Figure 4.4. Influence of treatment with *S. plymuthica* IC1270 on *M. oryzae*-induced H_2O_2-generation in epidermal sheath cells.

Intact leaf sheaths of the susceptible rice cv. CO39 and its resistant near-isogenic line C101LAC were challenged by injecting a conidial suspension of *M. oryzae* VT7. **A**, Micrographs depicting distinct H_2O_2 accumulation patterns in inoculated leaf sheaths supplied with 3,3'-diaminobenzidine (DAB): (I), successful fungal colonization of living epidermal cells in the absence of DAB staining; (II) DAB accumulation in the first-invaded cell following fungal invasion of adjacent cells; (III) accumulation of DAB-positive vesicle-like bodies in the vicinity of the invasive hyphae; (IV) DAB-positive cytoplasmic granules in hypersensitively reacting cells ; (V) whole-cell DAB staining. Ap, appressorium or appressorial site; IH, invading hyphae; Vs, vesicles. Scale bars = 20 µm. **B,** Frequencies of abovementioned DAB patterns at 36 and 48 hours post inoculation. In all graphs, bars represent the mean and SD of six replications originating from three plants. At least 50 single-cell interaction sites originating from representative sheath sections were examined per replication. Data from one experiment is presented. Repetition of experiments led to results very similar to those shown.

Manipulation of oxidative stress in inoculated leaves

In light of the well-documented ability of ROS to serve multiple defense-related signaling functions, sometimes with opposite effects in different contexts (Torres et al., 2006; Van Breuseghem et al., 2008), we asked whether the ability of IC1270 to boost pathogenesis-related H_2O_2 generation might account for the differential effectiveness of IC1270-ISR against *M. oryzae*, *R. solani* and *C. miyabeanus*. To address this question, we examined the effect of manipulating the oxidative stress in pathogen-inoculated leaves on subsequent disease development. To artificially raise the level of ROS in inoculated leaves, detached leaves were pressure-infiltrated with mixtures of glucose plus glucose oxidase (G/GO) and xanthine plus xanthine oxidase (X/XO). Similar to what has been observed in other plant species (Alvarez et al., 1998; Orozco-Cardenas et al., 2001), supplying rice leaves with G/GO resulted in the sustained production of H_2O_2 within the apoplast, whereas a mixture of xanthine and xanthine oxidase was found to generate both superoxide and H_2O_2, the latter by dismutation (data not shown). Treatment with either compound (xanthine, glucose, gluconate) or with the enzymes alone had no significant effect on disease development compared to buffer-treated control leaves. However, infiltration of G/GO or X/XO dramatically reduced the size of the necrotic lesions incited by *M. oryzae* infection (Figs. 4.5A, D). By contrast, pre-treatment with G/GO or X/XO mixtures strongly stimulated necrosis induced by *R. solani* (Fig. 4.5B). By 60 hours after infection, the majority of ROS-treated and *Rhizoctonia*-inoculated leaves showed extensive necrosis and were almost completely deteriorated (Fig. 4.5D). Enhanced ROS generation also greatly enhanced lesion formation by *C. miyabeanus*, suggesting a common pathogenicity mechanism for both these necrotrophs (Figs. 4.5C, D). Extensive lesions were also observed when manipulating plant-intrinsic catalase activity. Although exogenous catalase did not significantly alter lesion development, infiltration of rice leaves with a specific catalase inhibitor, 3-aminotriazole, prior to inoculation, was indistinguishable from the G/GO- or X/XO-treated leaves. No lesions were detected in leaves infiltrated with ROS-producing mixtures, catalase or 3-AT alone, as previously reported (Govrin and Levine, 2000).

Building on our earlier work on 7NSK2-mediated ISR to *M. oryzae*, we sought to extend our analysis of the proposed dual role of ROS in rice defense by feeding the pro-oxidative pigment pyocyanin to hydroponically grown rice plants and observe any effects on plant resistance. Opposite to the enhanced resistance observed against *M. oryzae*, pyocyanin feeding favored subsequent infection by both *C. miyabeanus* and *R. solani* (Fig. 4.6). Amending the pyocyanin solution with ascorbate, which has long been recognized as a major antioxidant buffer and free-radical scavenger (Pignocchi and Foyer, 2003), severely attenuated the pyocyanin-provoked

resistance or susceptibility, corroborating our previous findings (Chapter 3). Taken together, these results clearly demonstrate that enhanced ROS levels in inoculated leaves positively influence resistance to *M. oryzae* while exerting a negative effect on resistance to *C. miyabeanus* and *R. solani*, implying a role for ROS as critical modulators of antagonistic rice defenses.

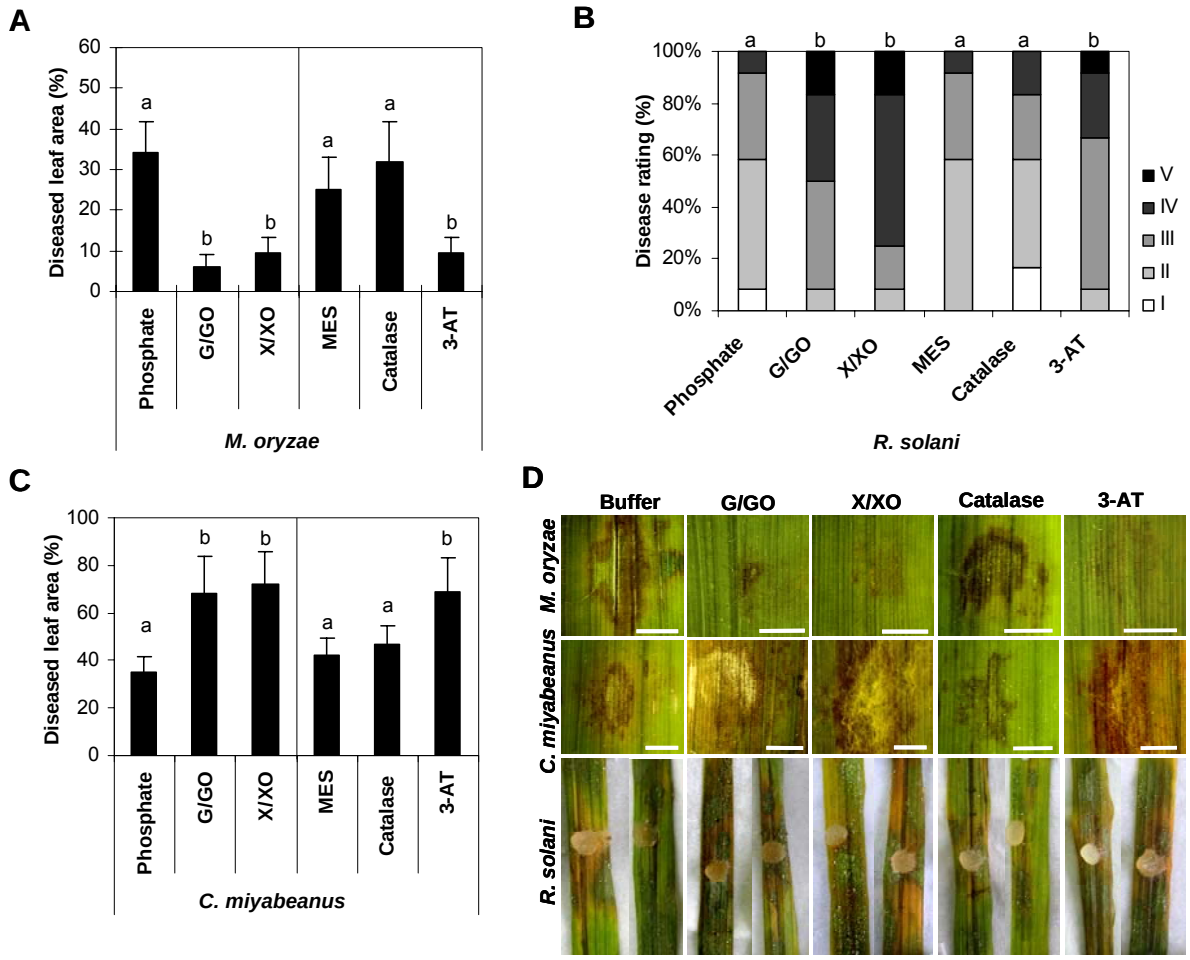

Figure 4.5. Effect of artificial ROS manipulation on *M. oryzae*, *C. miyabeanus* and *R. solani* infection.

For continuous generation of H_2O_2 *in situ*, detached leaves were infiltrated with mixtures of glucose oxidase (100 units ml^{-1}) plus glucose (2 mM), or xanthine oxidase (0.1 units ml^{-1}) plus xanthine (1 mM). Control plants were treated with buffer solution only (50 mM phosphate, pH = 6.5). Alternatively, plants were infiltrated with 3-aminotriazole (10 mM) or catalase (1100 units ml^{-1}) with MES buffer-treated plants as corresponding controls. Two hours later, 10 µl droplets of conidial suspension of *M. oryzae* (5 x 10^4 sp ml^{-1}) or *C. miyabeanus* (5 x 10^4 sp ml^{-1}) were carefully applied to the center of the infiltrated area. For infection with *R. solani*, 8-mm mycelium-overgrown agar plugs were used. After 4 days of incubation under laboratory conditions, *M. oryzae* and *C. miyabeanus* symptom development was assessed using digital image analysis for quantification of necrotic leaf areas. The intensity of the *R. solani* symptoms was evaluated 60 h post-inoculation and graded into five categories based on the leaf area affected: 1 = no infection, 2 = 1 to 10%, 3 = 11 to 25 %, 4 = 26 to 50%, and 5 = more than 50% affected leaf area. In all graphs, bars represent the mean and SD of twenty-four leaf segments. The experiment was repeated twice with very similar results. Different letters indicate statistically significant differences between treatments (*M. oryzae* and *C. miyabeanus*, Fisher's LSD test, $\alpha = 0.05$; *R. solani*, Mann-Whitney, $\alpha = 0.05$). Photographs depicting representative symptoms were taken 96 hpi in case of *M. oryzae* and *C. miyabeanus* challenge, and 60 hpi in case of challenge with *R. solani*. G = glucose, GO = glucose oxidase, X = xanthine, XO = xanthine oxidase, 3-AT = 3-aminotriazole.

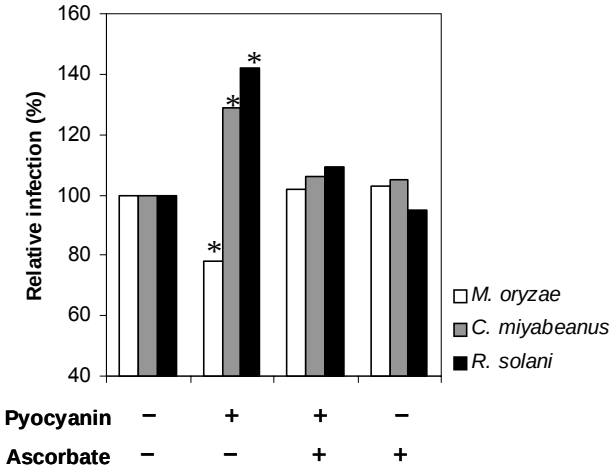

Figure 4.6. Effect of ascorbate on resistance to *M. oryzae*, *C. miyabeanus* and *R. solani* in pyocyanin-amended hydroponically-grown rice plants.

Purified pyocyanin (100 nM) and/or ascorbate (50 μM) were added to the half-strength Hoagland nutrient solution 4 days prior to challenge inoculation. For details on *M. oryzae*, *C. miyabeanus* and *R. solani* bioassays, see legend to Figure 4.1. The values presented are from representative experiments that were repeated three times with similar results. Asterisks indicate statistically significant differences with the non-treated control (Kruskall-Wallis and Mann-Whitney, $\alpha = 0.05$, $n = 24$).

Discussion

Despite the emergence of rice as a pivotal model for molecular genetic studies of disease resistance in cereal crops, molecular information regarding chemically and biologically induced defenses is still largely missing. In an effort to broaden our understanding of the rice induced resistance machinery, we analyzed the host defense responses underpinning ISR triggered by the biocontrol agent *S. plymuthica* IC1270. The results presented in this study demonstrate that effective root colonization by IC1270 predisposes rice to undergo a massive oxidative burst and related HR-like cell death at sites of attempted pathogen invasion, culminating in a heightened resistance to the hemibiotrophic blast pathogen, *M. oryzae*. The same treatment, however, rendered plants more susceptible to attack by the necrotrophic pathogens *R. solani* and *C. miyabeanus*. Besides tagging ROS and the associated HR-like cell death as two-faced players in the rice defense response, these findings strengthen the argument that rice requires distinct mechanisms for defense against *M. oryzae* and the necrotrophs *R. solani* and *C. miyabeanus*.

Mounting evidence indicates that generation of systemic resistance does not necessarily require direct activation of defense mechanisms, but can also result from a faster and stronger activation of basal defenses in response to pathogen attack (Conrath *et al.,* 2006). For instance, unlike pathogen-induced SAR, classic rhizobacteria-mediated ISR in Arabidopsis triggered by the

model strain *P. fluorescens* WCS417r is not marked by a direct induction of defense genes. Rather, ISR-expressing plants are primed for enhanced expression of a specific subset of JA/ET-responsive genes upon pathogen attack (van Wees et al., 1999; Verhagen et al., 2004). Other ISR-inducing PGPRs also have been found to enhance the plant's defensive capacity by hyper-activating pathogen-induced defenses (Benhamou et al., 1996; Ahn et al., 2002; Kim et al., 2004a; Tjamos et al., 2005; Ahn et al., 2007), suggesting that priming for enhanced defense is a common mechanism in PGPR-mediated ISR. The results presented in this study provide further support to this concept since root colonization by IC1270 did not cause a strong constitutive resistance phenotype, but rather primed plants to hyper-respond to subsequently inoculated pathogens, resulting in excessive defense activation and enhanced resistance to *M. oryzae*. This priming effect of IC1270 was borne out by the observation that challenge inoculation of IC1270-bacterized plants with *M. oryzae* entailed a rapid accumulation of autofluorogenic phenolic compounds in and around epidermal cells displaying dense cytoplasmic granulation (Figs. 4.2 and 4.3), two features that are considered as hallmarks of an ETI-associated HR (Koga, 1994; Rodrigues et al., 2005).

Comparative profiling of pathogenesis-related H_2O_2 accumulation in blast susceptible, yet ISR-expressing, and genetically resistant leaf sheath cells, further strengthened the parallels between R protein-mediated ETI and IC1270-triggered ISR priming (Fig. 4.4). Hence, IC1270 appears to protect rice from *M. oryzae* by reprogramming pathogen-attacked epidermal cells to undergo a rapid HR-like response, thereby providing a possible functional interface between rhizobacteria-mediated ISR and avirulent pathogen-induced ETI. Such mechanistic similarities between ISR and ETI are compatible with the idea that defense signals from multiple 'entry points' can converge and target overlapping sets of defense effectors (Eulgem, 2005; Knoth and Eulgem, 2008; Tsuda et al., 2008). Of relevance to considerations here is the substantial overlap between gene expression changes and alterations in SA content induced during an avirulent pathogen-triggered ETI response, and those induced by treatment with flg22, an 22-amino-acid epitope of the archetypal MAMP elicitor flagellin (Navarro et al., 2004; Mishina and Zeier, 2007; Tsuda et al., 2008). Although unequivocal evidence is still lacking, the striking homologies with the sensitive perception mechanisms for pathogen-derived MAMPs that function in PTI suggest that ISR-triggering rhizobacteria are perceived in a similar manner (Meziane et al., 2005; Bakker et al., 2007). It is thus not inconceivable that the mechanistic parallels between IC1270-mediated ISR and ETI can be traced back to converging MAMP- and R-protein-induced defense responses. Furthermore, consistent with the view of ETI as an accelerated and amplified PTI response (Tao et al., 2003; Katagiri, 2004; Jones and Dangl, 2006), such MAMP-orchestrated ISR

elicitation may also explain the partial nature of the IC1270-induced resistance against *M. oryzae* (Figs 4.1, 4.2 and 4.4).

The rapid production of ROS via consumption of oxygen during the so-called oxidative burst is a hallmark of the plant's defense response. Although ROS are generally viewed as initiating agents in the disease resistance network (Apel and Hirt, 2004), an expanding body of evidence indicates that ROS formation can cascade either to the detriment or benefit of the plant depending mainly on the type of host tissues and the lifestyle of the invading pathogen (Glazebrook, 2005; Bostock, 2005). Hence, ROS can play a dual role in pathogen defense, acting as key players in resistance to biotrophic pathogens on the one hand (Levine et al., 1994; Van Breusegem and Dat, 2006), but weakening necrotroph resistance by assisting pathogen-induced host cell death on the other hand (Govrin and Levine, 2000; Kumar et al., 2001; Glazebrook, 2005; Govrin et al., 2006). Taking these facts into account, we propose that priming for potentiated ROS generation may likewise function in IC1270-mediated ISR, thereby accounting for the differential effectiveness of this resistance against hemibiotrophic and necrotrophic pathogens. Critical to the formation of a hypothesis of primed ROS generation as a key event in ISR by IC1270 was the observation that artificially increased H_2O_2 levels, either resulting from infiltration of ROS-generating mixtures, application of redox-active pyocyanin, or inhibition of endogenous catalase activity, faithfully mimicked IC1270 in conditioning resistance to *M. oryzae* but promoting susceptibility to *C. miyabeanus* and *R. solani*. Although we are aware that final proof for primed ROS generation as the causal resistance mechanism underpinning IC1270-mediated ISR requires the use of inhibitor compounds able to abrogate the oxidative burst in bacterized plants (e.g. DPI), such scavenger experiments could not be performed since detached leaves, needed for effective infiltration of chemicals in rice, somehow failed to develop ISR. Feeding ROS quenching agents to hydroponically grown plants suffered from the same experimental vagaries in that IC1270 bacteria, in spite of sufficient root colonization, also lost the ability to mount ISR in our well-established hydroponic system, a phenomenon presumably due to small differences in the availability of iron and other nutrients in this system compared to standard soil-based ISR assays. Therefore, we can not rule out the possibility that the altered pathogen response of IC1270-induced plants may result in part from ROS-independent processes. Nonetheless, the involvement of boosted ROS generation in the establishment of IC1270-mediated ISR is apparent.

In accordance with previous studies (Govrin and Levine, 2000; Mur et al., 2005), continuous generation of H_2O_2 in situ by infiltration of G/GO or 3-AT did not induce any detectable cell death per se, indicating that additional pathogen-induced signals are needed for expression of

HR-like cell death. Indeed, current concepts suggest that death of host cells during the HR requires the poised production of nitric oxide (NO) and ROS, coupled to simultaneous suppression of the plant's antioxidant machinery (Delledonne et al., 2001; Zago et al., 2006; Zaninotto et al., 2006). In view of these data, it could be reasoned that IC1270-mediated priming for potentiated ROS generation might lower the threshold for activation of programmed cell death, thereby blocking the hemibiotroph *M. oryzae* in its initial biotrophic phase. In line with this concept, there is ample evidence demonstrating that early-produced H_2O_2 is a central signal leading to the elicitation of a wide range of blast-effective defenses, among which rapid programmed cell death. Most tellingly, Kachroo and associates (2003a) reported a fungal glucose oxidase gene to sequentially induce H_2O_2 generation, rapid HR-like cell death and enhanced resistance against *M. oryzae* when ectopically expressed in young rice plants. On the other hand, it is not inconceivable that IC1270-mediated priming for H_2O_2 may tilt the ROS-controlled cellular life-or-death balance toward death, thereby facilitating subsequent tissue colonization by *R. solani* and *C. miyabeanu*s which, as pathogens with a necrotrophic lifestyle, depend on host cell-killing for successful pathogenesis. This notion is corroborated by recent observations demonstrating that IC1270 pretreatment has no marked impact on the early infection events in *C. miyabeanus*- or *R. solani*-challenged plants except for a substantial increase in the number of dying cells preceding the fungal growth front (De Vleesschauwer and Höfte, unpublished results). Whether this increased cell death ahead of the developing hyphae is caused by stimulation of toxin-provoked tissue necrotization remains to be elucidated. However, given the myriad defense-related responses modulated by ROS (Torres and Dangl, 2005; Van Breusegem et al., 2008), other yet unidentified mechanisms also may play a role.

In summary, our results favor a model whereby effective root colonization of rice by IC1270 locks colonized plants into a pathogen-inducible program of boosted ROS generation and prompt execution of HR-like cell death, a mechanism which shows remarkable similarity with R-protein-mediated ETI responses. Although highly effective against the hemibiotroph *M. oryzae*, halting the pathogen in its biotrophic phase, IC1270 pretreatment enhanced infection by the necrotrophs *R. solani* and *C. miyabeanus*, possibly by facilitating pathogen-triggered host cell death. Considering that defense responses effective against *M. oryzae* may not be effective against or even facilitate infection by *R. solani* and *C. miyabeanus*, our work underscores the importance of utilizing appropriate innate defense mechanisms when breeding for broad-spectrum rice disease resistance.

Materials and Methods

Cultivation of rhizobacteria and pathogens

Bacterial strains used in this study were *Serratia plymuthica* IC1270, which was originally described as *Enterobacter agglomerans* (Chernin et al., 1995) and *Pseudomonas aeruginosa* 7NSK2 (Iswandi et al., 1987). For inoculation experiments, IC1270 and 7NSK2 were grown on iron-limiting King's B medium [KB; (King et al., 1954)] for 24 h at 28ºC and 37ºC, respectively. Bacterial cells were scraped off the plates and suspended in sterile saline (0.85% NaCl). Densities of the bacterial suspensions were adjusted to the desired concentration based on their optical density at 620 nm.

Magnaporthe oryzae isolate VT7, a field isolate from rice in Vietnam (Thuan et al., 2006), was grown at 28ºC on half-strength oatmeal agar (Difco, Sparks, USA). Seven-day-old mycelium was flattened onto the medium using a sterile spoon and exposed to blue light (combination of Philips TLD 18W/08 and Philips TLD 18W/33) for seven days to induce sporulation. Conidia were harvested as described in Chapter 3, and inoculum concentration was adjusted to a final density of 1 x 10^4 spores ml^{-1} in 0.5% gelatin (type B from Bovine skin; Sigma-Aldrich G-6650).

Rhizoctonia solani isolate MAN-86, belonging to anastomosis group AG-1 IA (Taheri *et al.*, 2007), was maintained on potato dextrose agar (PDA; Difco Laboratories, Detroit, USA). Inoculum was obtained according to Rodrigues et al. (2003b) with minor modifications. After autoclaving, 15 toothpicks, 1 cm in length, and five agar plugs (5 mm in diameter), obtained from the margin of an actively growing colony of *R. solani*, were transferred to PDA plates. These plates were then incubated for 8 days at 28ºC so *R. solani* could colonize the toothpicks.

Cochliobolus miyabeanus strain 911, obtained from diseased rice in field plots at the International Rice Research Institute (Manila, The Philippines), was grown for sporulation at 28ºC on PDA. Seven-day-old mycelium was flattened onto the medium using a sterile spoon and exposed to blue light for three days under the same conditions mentioned above. Upon sporulation, conidia were harvested exactly as stated in Thuan et al. (2006) and re-suspended in 0.5% gelatin to a final density of 1 x 10^4 conidia ml^{-1}.

Pathogen inoculation and disease rating

Four-week-old rice seedlings (5-leaf stage) were challenge-inoculated with *Magnaporthe oryzae* as described in Chapter 3. Six days after inoculation, disease severity on the fourth leaves of each plant was rated by counting the number of elliptical to round-shaped lesions with a sporulating gray center, and expressed relative to non-bacterized control plants.

R. solani bioassays were performed essentially as described in Rodrigues et al. (2003b). Plants were challenged when four weeks old by placing a 1-cm toothpick colonized by *R. solani* inside the sheath of the second youngest fully expanded leaf. Inoculated plants were maintained inside humid inoculation chambers (≥ 92% relative humidity; 30 ± 4ºC) for 72 h, and, thereafter, transferred to greenhouse

conditions. Four days after challenge infection, disease severity was assessed by measuring the length of the water-soaked lesions.

C. miyabeanus bioassays were performed as described in Ahn *et al.* (2005b) with minor modifications. Five-week-old seedlings (6.5-leaf stage) were misted with a *C. miyabeanus* spore suspension containing 1 x 10^4 conidia ml^{-1} in 0.5% gelatin. Inoculated plants were kept in a dew chamber (≥ 92% relative humidity; 30 ± 4ºC) for 18 h to facilitate fungal penetration, and subsequently transferred to greenhouse conditions for disease development. Disease symptoms were scored at four days after inoculation for about 48 leaves per treatment. Disease ratings were expressed on the basis of diseased leaf area and lesion type: I, no infection or less than 2% of leaf area infected with small brown specs less than 1 mm in diameter; II, less than 10% of leaf area infected with brown spot lesions with gray to white center, about 1-3 mm in diameter; III, average of about 25% of leaf area infected with brown spot lesions with gray to white center, about 1-3 mm in diameter; IV, average of about 50% of leaf area infected with typical spindle-shaped lesions, 3 mm or longer with necrotic gray center and water-soaked or reddish brown margins, little or no coalescence of lesions; V, more than 75% of leaf area infected with coalescing spindle-shaped lesions.

Induction treatments

Induced systemic resistance (ISR) assays were performed as described in Chapter 3 with minor modifications. Briefly, rice plants (*Oryza sativa* spp. *indica* line CO39) were grown under greenhouse conditions (30 ± 4ºC, 16-h photoperiod) in commercial potting soil (Structural; Snebbout, Kaprijke, Belgium) that had been autoclaved twice on alternate days for 21 min. Rice seeds first were surface sterilized with 1% sodium hypochlorite for two min, rinsed three times with sterile, demineralized water and incubated for five days on a wet sterile filter paper in sealed Petri dishes at 28ºC. Prior to sowing in perforated plastic trays (23 by 16 by 6 cm), roots of germinated seeds were dipped in a bacterial suspension of the ISR-inducing strains [5 x 10^7 colony-forming units (cfu) ml^{-1}] for 10 min. The autoclaved soil was thoroughly mixed with bacterial inoculum to a final density of 5 x 10^7 cfu ml^{-1}. To ensure consistent root colonization by the eliciting bacteria, rice plants were soil-drenched a second time with bacterial inoculum (5 x 10^7 cfu ml^{-1}) at ten days after sowing. In control treatments, soil and rice plants were treated with equal volumes of sterilized saline.

For experiments in which purified pyocyanin was applied to the roots of rice seedlings, plants were grown in a hydroponic gnotobiotic system as described before (Chapter 3). In this system, plants were fed with various concentrations of pyocyanin and ascorbate 4 days before challenge inoculation by adding the desired concentration to the half-strength Hoagland nutrient solution. Pyocyanin extraction, quantification and application were performed exactly as stated in Chapter 3.

Evaluation of plant colonization by *S. plymuthica* IC1270 and *P. aeruginosa* 7NSK2

Bacterial colonization of the plant roots was determined by the time the bioassays were discontinued. Roots of three plants of each treatment were rinsed to remove most of the soil, weighed, and 1 g of root was macerated in sterile demineralized water. Serial dilutions were plated on KB agar supplemented with rifampicin (40 μg/ml) for IC1270, and KB agar for 7NSK2. After overnight incubation at 28ºC and 37ºC for IC1270- and 7NSK2-bacterized roots, respectively, the number of colony-forming units per gram of root fresh weight was determined. Possible spreading of root-inoculated bacteria to distal leaves was checked as described before (Chapter 3). The detection limit of this assay is approximately 10 CFU per sheath or leaf blade.

Cytological analysis of IC1270-mediated ISR against *M. oryzae*

To gain more insight into the nature of IC1270-mediated ISR against *M. oryzae*, cytological studies were performed at sites of pathogen entry. To this purpose, we adopted the intact leaf sheath assay as described by Koga et al. (2004b). Briefly, leaf sheaths of the fifth leaf of rice plants at the 5.5 leaf stage were peeled off with leaf blades and roots. The leaf sheath was laid horizontally on a support in plastic trays containing wet filter paper, and the hollow space enclosed by the sides of the leaf sheaths above the mid vein was filled with a suspension of spores (5 x 10^4 conidia ml^{-1}) of *M. oryzae*. Inoculated leaf sheaths were then incubated at 25ºC with a 16-h photoperiod. When ready for microscopy, the sheaths were hand-trimmed to remove the sides and expose the epidermal layer above the mid vein. Lower mid vein cells were removed to produce sections three to four cell layers thick. At least five trimmed sheath tissue sections originating from different control and IC1270-treated plants were used for each sampling point.

Phenolic compounds were visualized as autofluorescence under blue light epifluorescence (Olympus U-MWB2 GPF filter set-excitation: 450 to 480 nm, dichroic beamsplitter; 500 nm, barrier filter BA515). To detect H_2O_2 accumulation, staining was performed according to the protocol of Thordal-Christensen et al. (1997) with minor modifications. Six hours before each time point, trimmed sheath segments were vacuum-infiltrated with an aqueous solution of 1 mg ml^{-1} 3,3'-diaminobenzidine(DAB)-HCL (pH = 3.8) for 30 min. Thereafter, infiltrated segments were incubated in fresh DAB solution until sampling. DAB polymerizes in the presence of H_2O_2 and endogenous peroxidase to form a brownish-red precipitate that can be easily visualized using bright-field microscopy. After staining, trimmed sheath segments were mounted in 50% glycerol. Images were acquired digitally (Olympus Color View II camera, Aartselaar, Belgium) and further processed with the Olympus analySIS cell^F software.

Artificial manipulation of the oxidative burst in detached rice leaves

For experiments in which plants were treated with the ROS-generating mixtures glucose plus glucose oxidase (G/GO) and xanthine plus xanthine oxidase (X/XO), fifth-stage leaves of four-week-old rice plants were excised and cut into 7-cm segments. *Aspergillus niger* glucose oxidase (Sigma-Aldrich, St. Louis, MO) was added to 2 mM D-glucose in 20 mM Na phosphate buffer, pH 6.5, immediately prior to plant treatment (100 units ml^{-1}). Similarly, xanthine oxidase (0.1 units ml^{-1}) was added to 1 mM xanthine in the

same buffer solution (Sigma-Aldrich, St. Louis, MO). The ROS-generating mixtures, buffer alone or buffer containing glucose (2 mM), gluconate (50 µM), glucose oxidase (100 units ml^{-1}), xanthine (1 mM), or xanthine oxidase (0.1 units ml^{-1}) were infiltrated in approximately 20 µl aliquots into five sites on the abaxial surface of the detached leaf segments using a syringe without a needle. Alternatively, detached leaf segments were infiltrated with 3-aminotriazole (10 mM) or catalase (1100 units ml^{-1}) in 10 mM MES buffer, pH 6.5. *In planta* H_2O_2 generation by G/GO, X/XO, or 3-aminotriazole was visually confirmed by means of abovementioned DAB staining procedure. Upon infiltration, detached leaf segments were immediately placed onto a glass slide in 14.5 x 14.5 cm Petri dishes lined with moist filter paper. Two hours later, 10 µl of *M. oryzae* or *C. miyabeanus* conidial suspension (5 x 10^4 sp ml^{-1} in 0.25% gelatin) was drop-inoculated in the center of the infiltrated regions. Control leaves were mock-inoculated with a 0.25% (wt vol^{-1}) gelatin suspension. After 24 h, the droplets were removed with a laboratory tissue. For challenge with *R. solani*, a 0.8-cm-diameter mycelial disc of a 7-day-old PDA culture of *R. solani* strain MAN-86 was carefully placed in the center of the infiltrated region. As a control, leaf segments were inoculated with a PDA plug without hyphae. Petri dishes with inoculated leaf segments were routinely placed on a laboratory bench and maintained at 21°C to 26°C with a 16 h photoperiod. For *M. oryzae* and *C. miyabeanus* assays, disease development was assessed 96 h post-inoculation using digital image analysis (APS assess software; Lakhdar Lamari, Winnipeg, Canada) for quantification of necrotic leaf areas. These areas were represented as the number of pixels and expressed as a percentage of the total pixel number in a fixed 1 cm^2-leaf quadrant. In case of *R. solani* inoculation, disease ratings were visually graded into five classes based on the leaf area affected; 1 = no infection, 2 = 1 to 10%, 3 = 11 to 25 %, 4 = 26 to 50%, and 5 = more than 50% of leaf area affected.

Chapter

5

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

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Accepted in Plant Physiology

Selected strains of non-pathogenic rhizobacteria can reduce disease in foliar tissues through induction of a defense state known as induced systemic resistance (ISR). Compared to the large body of information on ISR in dicotyledonous plants, little is known about the mechanisms underlying rhizobacteria-induced resistance in cereal crops. Here, we demonstrate the ability of *Pseudomonas fluorescens* WCS374r to trigger ISR in rice against the leaf blast pathogen *Magnaporthe oryzae*. Using salicylic acid-nonaccumulating NahG rice, an ethylene-insensitive *OsEIN2* antisense line and the jasmonate-deficient mutant *hebiba*, we show that this WCS374r-induced resistance is regulated by an SA-independent but JA/ET-modulated signal transduction pathway. Bacterial mutant analysis uncovered a pseudobactin-type siderophore as the crucial determinant responsible for ISR elicitation. Root application of WCS374r-derived pseudobactin (Psb374) primed naïve leaves for accelerated expression of a pronounced multifaceted defense response, comprising rapid recruitment of phenolic compounds at sites of pathogen entry, concerted expression of a diverse set of structural defenses, and a timely hyperinduction of H_2O_2 formation putatively driving cell wall fortification. Exogenous SA application alleviated this Psb374-modulated defense priming, while Psb374 pretreatment antagonized infection-induced transcription of SA-responsive *PR* genes, suggesting that the Psb374- and SA-modulated signaling pathways are mutually antagonistic. Interestingly, in sharp contrast to WCS374r-mediated ISR, chemical induction of blast resistance by the SA analog benzothiadiazole was independent of JA/ET signaling and involved potentiation of SA-responsive gene expression. Together, these results offer novel insights into the signaling circuitry governing induced resistance against *M. oryzae* and suggest that rice is endowed with multiple blast-effective resistance pathways.

Introduction

Plants have evolved a battery of sophisticated defense mechanisms to defend themselves against microbial pathogens. Apart from preformed physical and chemical barriers, plants possess an elaborate matrix of inducible defenses that become activated upon pathogen infection. These inducible responses are regulated by a network of interconnecting signal transduction pathways in which the plant hormones salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA) play central roles (Adie et al., 2007; Robert-Seilaniantz et al., 2007; Asselbergh et al., 2008b). A growing body of evidence supports the notion that these signaling pathways do not function independently, but influence each other through a complex network of synergistic and antagonistic interactions (Koornneef and Pieterse, 2008). Such crosstalk between defense pathways is thought to provide the plant with a cost-efficient regulatory potential to adaptively tailor its defense reaction to the type of attacker encountered.

Besides basal resistance responses that act at the site of pathogen infection, plants are also capable of developing a non-specific systemic resistance that is effective against future pathogen attack. This phenomenon is known as induced resistance and can be triggered by a variety of biotic and abiotic stimuli (Bostock, 2005). Over the past decade, it has become increasingly clear that the enhanced defensive capacity of induced plants does not necessarily require a direct activation of defenses, but can also result from a faster and stronger expression of basal defense responses upon pathogen attack. By analogy with a phenotypically similar phenomenon in animals and humans, this enhanced capacity to express infection-induced basal defenses is called 'sensitization', 'priming', or 'potentiation' (Conrath et al., 2002; Conrath et al., 2006). In some elegant work on the costs and benefits of priming in *Arabidopsis*, Van Hulten and associates (2006) demonstrated that the fitness costs of priming are substantially lower than those of constitutively activated defense. In addition, it was shown that the benefits of priming-mediated resistance outweigh its costs when disease occurs. Priming thus offers an elegant solution to the plant's trade-off dilemma between disease protection and the costs involved in defense activation (Conrath et al., 2006).

The classic example of an inducible plant defense response is systemic acquired resistance (SAR). SAR is triggered by a localized infection with necrotizing microbes and is manifested throughout the plant upon secondary challenge by otherwise virulent pathogens (Grant and Lamb, 2006). The onset of SAR is marked by local and systemic increases in endogenously synthesized salicylic acid (SA) and is tightly associated with the transcriptional reprogramming of a battery of defense-related genes, including those encoding pathogenesis-related (PR) proteins (Ryals et al., 1996; Maleck et al., 2000; Wang et al., 2006a). These PR proteins, of which some

possess antimicrobial activity, serve as hallmarks of SAR in several plant species and are thought to contribute to the state of resistance attained (Van Loon et al., 2006b). Transduction of the SA signal requires the function of NPR1 (also known as NIM1), a master regulatory protein that was identified in *Arabidopsis* through genetic screens for SAR-compromised mutants (Cao et al., 1994; Shah et al., 1997). Although SA is central to the induction and expression of SAR, it is not the long-distance SAR signal. Instead, exciting new data implicate methyl salicylate and a lipid-derived molecule, possibly jasmonic acid, as mobile signals for SAR in tobacco and *Arabidopsis*, respectively (Park et al., 2007; Truman et al., 2007)

Colonization of roots by selected strains of nonpathogenic plant growth-promoting rhizobacteria (PGPR) leads to a phenotypically similar form of induced resistance commonly referred to as induced systemic resistance or ISR (Van Loon et al., 1998). Although some rhizobacteria are able to trigger the SA-dependent SAR pathway (De Meyer et al., 1999b; Ryu et al., 2003; Tjamos et al., 2005), rhizobacteria-mediated ISR predominantly involves SA-independent signaling (Pieterse et al., 1996; Ahn et al., 2007; Tran et al., 2007). As for the reference strain *Pseudomonas fluorescens* WCS417r, analysis of several well-characterized *Arabidopsis* mutants revealed that ISR requires an intact response to the plant hormones jasmonic acid (JA) and ethylene (ET) and, like SAR, depends on a functional NPR1 protein (Pieterse et al., 1998; Van Wees et al., 2000). However, downstream of NPR1, the ISR and SAR signaling pathways diverge because, unlike SAR, ISR is not accompanied by the concomitant activation of *PR* genes (Pieterse et al., 1996; Van Wees et al., 1997; Van Wees et al., 1999). Instead, ISR-expressing plants are primed for enhanced expression of predominantly JA- and ET-regulated genes upon pathogen infection (Verhagen et al., 2004; Cartieaux et al., 2008).

Successful establishment of ISR depends on recognition of bacterial elicitors by the plant roots. Over the past decade, a myriad of bacterial traits operative in triggering ISR have been identified. Examples include flagella, cell envelope components such as lipopolysaccharides, and secreted metabolites, including antibiotics, quorum-sensing molecules, cyclic lipopeptides, volatiles and siderophores (Bakker et al., 2007; Ongena et al., 2007; Tran et al., 2007). However, despite the increasing amount of research devoted to the identification and characterization of bacteria-derived ISR elicitors, much remains to be discovered about how these determinants are perceived and ultimately give rise to ISR.

Compared to the vast body of information available in dicotyledonous plants, our understanding of the molecular machinery governing induced resistance responses in monocotyledonous crops is still in its infancy. Evidence demonstrating that central components of the SAR pathway, such as NPR1, are conserved in cereals has only recently been presented

(Chern et al., 2001; Chern et al., 2005b; Shimono et al., 2007; Yuan et al., 2007). Moreover, reports on SAR- or ISR-like phenomena in monocots are scarce (Kogel and Langen, 2005). Most tellingly in this regard, a 17-year-old report of systemic resistance in rice triggered by pre-inoculation with an HR-eliciting, non-pathogenic *P. syringae* strain remains one of the most compelling examples of a monocot SAR-like response to date (Smith and Metraux, 1991). Previously, we reported that root colonization of rice by *P. aeruginosa* 7NSK2 renders foliar tissues more resistant to infection by *M. oryzae* (De Vleesschauwer et al., 2006). Extensive bacterial mutant analysis and cytomolecular characterization of the defense responses activated *in planta* revealed that this 7NSK2-mediated ISR acts through secretion of the redox-active pigment pyocyanin, thereby priming systemic tissues for boosted expression of hypersensitive response-like cell death upon pathogen infection.

Aiming to further dissect the induced systemic resistance response in rice, we analyzed the bacterial determinants and host defense mechanisms underpinning ISR induced by *P. fluorescens* WCS374r. This gram-negative bacterium, originally isolated from the rhizosphere of potato, has previously been shown to suppress *Fusarium* wilt of radish (*Fusarium oxysporum* f. sp. *raphani*) and reduce disease caused by *Ralstonia solanacearum* in *Eucalyptus* (Leeman et al., 1995; Ran et al., 2005a). Remarkably, high inoculum densities of WCS374r cultivated at 28°C failed to elicit ISR in *Arabidopsis* against *P. syringae* pv. *tomato* (Van Wees et al., 1997), whereas low inoculum densities or inoculum cultivated at elevated temperatures induced resistance against a broad spectrum of pathogens with different parasitic habits (Ran et al., 2005b; Djavaheri, 2007). This wide range of effectiveness of WCS374r-elicited ISR (WCS374r-ISR) strongly suggests that multiple resistance responses are involved. Indeed, recent studies by Ran et al. (2005b) and Djavaheri (2007) demonstrated that WCS374r-ISR against Turnip crinkle virus was still functional in *Arabidopsis* genotypes impaired in JA- and ET-dependent signaling, whereas WCS374r-ISR against *P. syringae* pv. *tomato* was blocked in the latter genotypes. Hence, perception of WCS374r seems to result in the activation of multiple signal transduction pathways that all add to establishing broad-spectrum WCS374r-ISR.

In the present study, we demonstrate the ability of WCS374r to mount ISR in rice against the leaf blast pathogen *M. oryzae* and provide evidence that this WCS374r-mediated ISR is based on pseudobactin-mediated priming for a pronounced multifaceted cellular defense response. Furthermore, we show that WCS374r-triggered ISR functions independently of SA accumulation but, unlike benzothiadiazole (BTH)-inducible resistance, requires intact responsiveness to ET as well as a functional octadecanoid pathway.

Results

P. fluorescens WCS374r mounts ISR in rice to *M. oryzae*

The filamentous ascomycete *M. oryzae* is the causal agent of rice blast disease, one of the most devastating of all cereal diseases and a significant threat to food security worldwide (Talbot, 2003). To determine whether *P. fluorescens* WCS374r-mediated ISR is effective against *M. oryzae*, susceptible rice plants were grown in soil containing WCS374r bacteria and subsequently challenged with the latter pathogen. As a positive control, a subset of the plants was treated with benzothiadiazole (BTH), a functional SA analog and one of the most extensively studied plant defense activators in rice (Nakashita et al., 2003; Ahn et al., 2005b; Shimono et al., 2007). Within 4 to 5 days post-inoculation, leaves of non-induced control plants developed large, spindle-shaped lesions with a gray center (diameter > 3 mm), often surrounded by chlorotic or necrotic tissue (Fig. 5.1). In contrast, plants colonized by WCS374r exhibited a marked reduction in the number of these susceptible-type lesions, producing a resistance phenotype characterized by the appearance of many small (< 1 mm), dark-brown necrotic spots 2 to 3 days post-inoculation (Fig. 5.1). Pooled over four independent experiments, WCS374r pretreatment caused a 47% reduction in lesion number. Application of BTH (0.05 mM) induced an even higher level of protection, reducing the number of susceptible-type lesions by as much as 68% compared to non-induced controls (Fig. 5.1).

To rule out the possibility that the observed disease protection was due to direct effects of WCS374r on *M. oryzae*, possible spreading of root-inoculated bacteria to foliar tissues was assessed by plating leaf extracts from induced plants onto selective King's medium B (KB) agar plates (King et al., 1954). However, WCS374r bacteria were never detected in leaf blades or sheaths of root-treated plants, indicating that bacterial colonization remained confined to the root zone (data not shown). In conjunction with the inability of WCS374r to inhibit growth of *M. oryzae* in dual culture experiments (data not shown), these findings strongly suggest that the WCS374r-provoked disease suppression is not due to microbial antagonism but rather results from activation of the plant's own defensive repertoire.

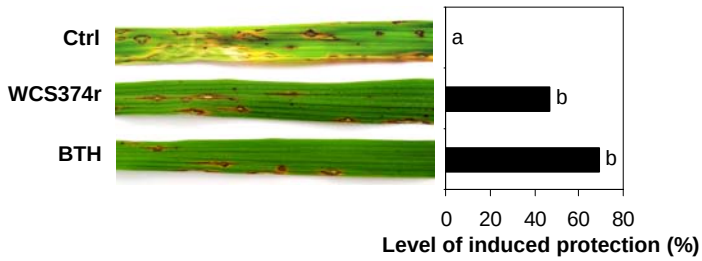

Figure 5.1. Quantification of rhizobacteria-induced systemic resistance (ISR) and BTH-triggered resistance against *M. oryzae* in rice.

ISR was induced by growing the plants in soil containing *P. fluorescens* WCS374r following application of the ISR-inducing bacteria as a seedling root dip. For chemical induction of blast resistance, plants were soil drenched with BTH (0.05 mM) 3 d before challenge. Control plants were treated with water. Plants were challenged when 4 weeks old (5-leaf stage) by spraying a spore suspension of virulent *M. oryzae* isolate VT5M1 at 1 x 104 conidia.ml-1. Six days after challenge inoculation, disease was rated by counting the number of susceptible-type lesions per leaf 4 and expressed relative to non-bacterized control plants. Statistical analysis was performed on data pooled from 4 independent experiments, because interaction between treatment and experiment was not significant at $\alpha = 0.05$ by analysis of variance. Different letters indicate statistically significant differences between treatments as analysed by non-parametric Kruskall-Wallis and Mann-Whitney comparison tests ($\alpha = 0.05$, $n > 86$). Photographs depicting representative symptoms were taken 7 d post inoculation.

WCS374r-triggered ISR to *M. oryzae* is independent of SA accumulation but requires intact responsiveness to ET as well as a functional octadecanoid pathway

To unravel the signaling circuitry governing WCS374r-mediated ISR to *M. oryzae*, bioassays were performed with transgenic and mutant rice lines impaired in various structural components of known defense pathways. As shown in Figure 5.2, SA-deficient NahG plants (Yang et al., 2004) and the corresponding wild-type line Nipponbare were equally responsive to WCS374r-mediated ISR, suggesting that WCS374r elicits ISR in rice either by activating the SA pathway downstream of SA or by functioning independently of SA. NahG plants also developed wild-type levels of protection against *M. oryzae* in response to treatment with BTH, indicating that SA accumulation is not a prerequisite for expression of BTH-inducible blast resistance. To investigate whether JA and/or ET play a role in WCS374r-mediated ISR, we tested the effectiveness of WCS374r in the ET-insensitive *OsEIN2*-suppressed transgenic line 471 (Jun et al., 2004) and the JA-deficient mutant *hebiba*, which is impaired in an as yet unidentified step of the octadecadoid pathway (Riemann et al., 2003; Sineshchekov et al., 2004). In contrast to the respective wild-type lines Dongyin and Nihonmasari, both 471 and *hebiba* were blocked in their ability to develop WCS374r-mediated ISR, whereas chemical induction of blast resistance by BTH resulted in levels of induced resistance comparable to those observed in the wild-types. The impaired ISR response of 471 and *hebiba* was not due to insufficient root colonization, since WCS374r colonized the rhizosphere of the different rice genotypes to comparable levels (5.4 ± 0.7 log cfu g^{-1}). Together, these results suggest that WCS374r-mediated ISR against *M.*

oryzae is independent of SA accumulation but, unlike BTH-inducible blast resistance, requires the operation of an ET/JA-regulated signaling pathway.

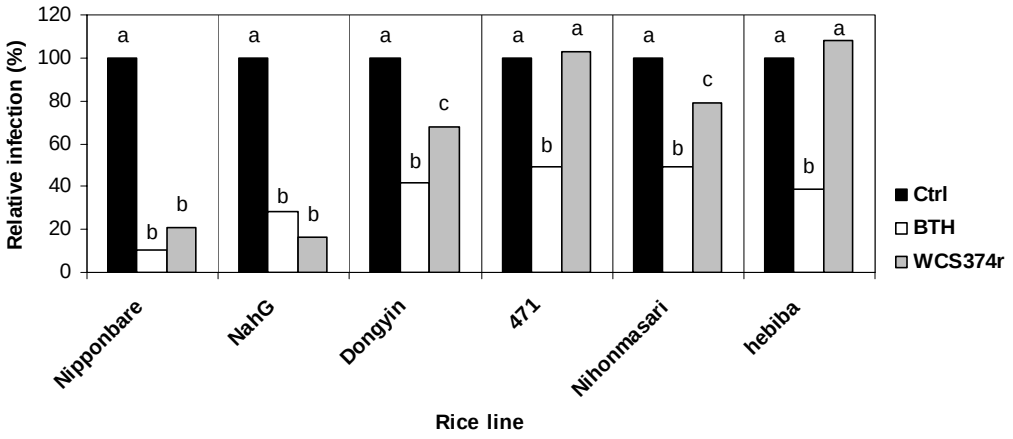

Figure 5.2. Influence of root treatment with *P. fluorescens* WCS374r or soil drench with BTH on rice blast (*M. oryzae*) severity on different signaling mutants and transgenic rice lines.

ISR was induced by growing plants in soil containing WCS374r bacteria. For chemical induction of blast resistance, plants were soil drenched with BTH (0.05 mM) 3 d prior to challenge. Control plants were treated with water. For details on *M. oryzae* bioassays, see legend to Figure 5.1. The average number of susceptible-type blast lesions on the fourth leaves of individual control plants were 60 (Nippobare), 56 (NahG), 48 (Dongyin), 59 (471), 65 (Nihonmasari) and 85 (hebiba), respectively. Within each frame, different letters indicate statistically significant differences between treatments (Kruskall-Wallis and Mann-Whitney, $a = 0.05$, $n > 23$). Data presented are from a representative experiment that was repeated twice with similar results. NahG = SA-deficient transgenic line generated in the background of cv Nipponbare; 471 = ethylene-insensitive *OsEIN2* antisense line of cultivar Dongyin; *hebiba* = jasmonate-deficient mutant of cultivar Nihonmasari.

Involvement of iron-regulated metabolites in the elicitation of ISR by WCS374r

Several lines of evidence corroborate a major role for iron-regulated bacterial metabolites in WCS374r-mediated ISR in dicotyledonous plants (Leeman et al., 1996; Ran et al., 2005a). To address whether WCS374r mounts ISR to *M. oryzae* in a similar manner, we first compared the ISR-triggering capacity of inoculum cultivated on iron-rich LB medium to that of inoculum prepared from iron-limited KB medium. Figure 5.3A shows that, in contrast to WCS374r prepared from KB, LB-grown bacteria failed to significantly reduce disease severity. Because LB- and KB-grown bacteria colonized rice to a similar extent (data not shown), the observed difference in ISR is likely due to the different iron nutritional state of both inocula. At inoculation, LB-grown inoculum had an internal iron pool visible in the red color of the bacterial pellet, whereas an internal iron pool was not observed for KB-grown WCS374r (data not shown). Although it cannot be excluded that differences in medium composition other than iron content might have contributed to the impaired ISR-triggering capacity of LB-derived inoculum, these

observations strongly suggest the involvement of iron-regulated metabolites in the elicitation of WCS374r-mediated ISR.

In order to identify such iron-regulated bacterial traits operative in triggering ISR, we compared the potential of WCS374r to induce resistance with that of a collection of mutants deficient in the production of the siderophores pseudobactin, pseudomonine and/or salicylic acid (SA). All bacterial strains were routinely grown on iron-poor KB medium. As shown in Figure 5.3B, the pseudomonine-deficient mutant 4A1 induced ISR to an extent similar to that obtained after treatment with the wild-type strain, indicating that pseudomonine is not essential for WCS374r to induce resistance (Fig. 5.3B). Conversely, treatment with either the pseudobactin-negative mutant 374-02, the pseudobactin and pseudomonine double negative mutant AT12, or the triple negative mutant BT1 no longer caused disease suppression, suggesting a pivotal role for pseudobactin in WCS374r-mediated ISR to *M. oryzae*. However, pseudobactin alone appeared to be insufficient for the onset of ISR since we failed to observe any statistically significant differences in disease severity between treatment with the pseudobactin-positive but pseudomonine- and SA-deficient mutant 4B1 and control plants. A deficiency in root colonization could be ruled out, because bacterial counts in the rhizosphere of plants inoculated with the respective mutants were similar to those of WCS374r-treated plants (data not shown). Based on these results, we initially assumed pseudobactin to act in concert with SA in the elicitation of ISR. To test this hypothesis, we next examined the effect of inoculating roots with a mix of the ISR-deficient strains 4B1 (SA^-, Psb^+) and AT12 (SA^+, Psb^-). Alternatively, plants colonized by 4B1 were complemented with a 1nM SA solution, a concentration equivalent to the in vitro SA production of 10^5 CFU of WCS374r. However, none of these combination treatments was able to restore ISR, making the involvement of SA and pseudobactin in ISR by WCS374r rather questionable (Fig. 5.3C).

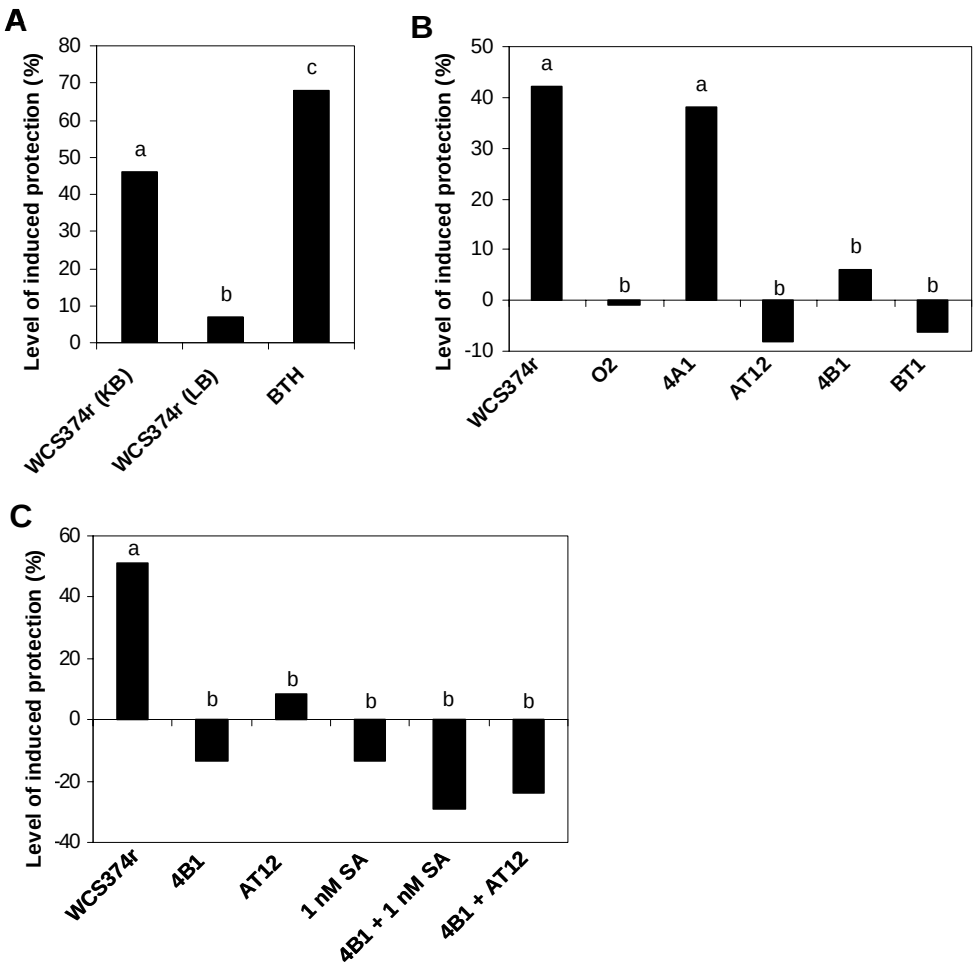

Figure 5.3. Influence of root treatment with *P. fluorescens* WCS374r and various mutants on rice blast (*M. oryzae*) severity.

Unless otherwise stated, WCS374r and derived mutants were grown on King's medium B and applied to rice roots and soil. Plants were challenge inoculated when 4 weeks old (5-leaf stage). For details on *M. oryzae* bioassays, see legend to Figure 5.1. **A**, Influence of the iron nutritional state of the bacterial inoculum on the level of induced resistance imparted by WCS374r. Bacteria were grown on iron-poor King's medium B (KB) or iron-rich Luria-Bertani medium (LB) prior to inoculation. For comparison with chemically induced blast resistance, plants were soil drenched with BTH (0.05 mM) 3 d before challenge. **B**, Quantification of ISR against *M. oryzae* triggered by WCS374r and various mutant strains. Mutants derived from WCS374r have the following characteristics: 02 (Psb−, Psm+, SA+), 4A1 (Psb+, Psm−, SA+), AT12 (Psb−, Psm−, SA+), 4B1 (Psb+, Psm−, SA−), and BT1 (Psb−, Psm−, SA−). Psb = pseudobactin, Psm = pseudomonine, SA = salicylic acid. **C**, Effect of complementing the SA-deficient mutant strain 4B1 for SA production on the level of induced protection against *M. oryzae*. SA (1 nM) was applied as a soil drench 3 days before challenge infection. Different letters indicate statistically significant differences between treatments by Kruskall-Wallis and Mann-Whitney non-parametric tests ($\alpha = 0.05$, $n > 24$). Data presented are from representative experiments that were repeated at least twice with comparable results.

Salicylic acid attenuates pseudobactin-induced resistance against *M. oryzae*

To shed more light on the role of SA and pseudobactin in WCS374r-mediated ISR, we isolated pseudobactin from stationary phase cultures of WCS374r as described before (Meziane et al., 2005), and applied the purified compound, alone or in combination with SA, to the roots of hydroponically grown rice seedlings. Consistent with a previous study (Leeman et al., 1996) and based upon the in vitro pseudobactin production of 10^5 CFU of WCS374r bacteria, we tested two different concentrations of purified pseudobactin, i.e. 12 and 70 µg per plant. As a positive control, plants were treated with BTH. As shown in Figure 5.4, purified pseudobactin applied at a concentration of 70 µg per root system increased resistance against *M. oryzae* by as much as 88%, this being similar to the level of protection induced by 0.05 mM BTH. Application of 12 µg pseudobactin per root system was slightly less effective, as evidenced by a 67% decrease in the number of susceptible-type blast lesions. Intriguingly, hydroponic feeding of a physiologically relevant 1 nM SA solution had no marked effect on disease development, whereas co-application of 1 nM SA and 70 µg pseudobactin alleviated the pseudobactin-conferred protection. While indicating that pseudobactin alone suffices for full induction of WCS374r-mediated ISR to *M. oryzae*, these findings suggest negative crosstalk in the direction of SA damping pseudobactin action.

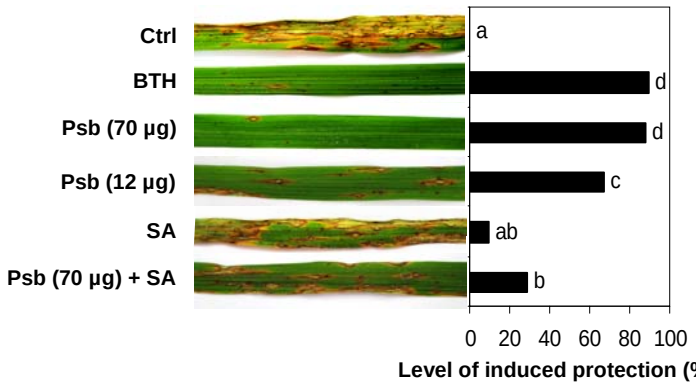

Figure 5.4. Effectiveness of benzothiadiazole (BTH), pseudobactin (Psb) or salicylic acid (SA)-induced resistance against *M. oryzae*.

To trigger resistance, rice seedlings were hydroponically fed with the various compounds by including the desired concentration in the half-strength Hoagland nutrient solution. BTH and SA were applied at a concentration of 0.05 mM and 1 nM, respectively. Pseudobactin was isolated from *P. fluorescens* WCS374r cultures and applied at a concentration of either 12 µg or 70 µg per root system. For details on *M. oryzae* bioassays see legend to Figure 5.1. Different letters indicate statistically significant differences between treatments (Kruskall-Wallis and Mann-Whitney, $\alpha = 0.05$, $n > 20$). Data presented are from a representative experiment that was repeated twice with comparable results

Histochemical analysis of pseudobactin-induced resistance against *M. oryzae*

Pseudobactin primes rice for a diverse set of HR-independent cellular responses

To further decipher the role of pseudobactin in WCS374r-conferred resistance in rice, we investigated the cytological and biochemical alterations associated with fungal restriction in pseudobactin-induced plants using the intact leaf sheath method developed by Koga et al. (2004b). Contrary to leaf blades, leaf sheath tissue is relatively flat and optically clear, which facilitates live-cell imaging, while the use of intact leaf sheaths allows the expression of numerous partial resistance responses, consistent with the continuous array of symptoms typically observed on inoculated leaf blades. Leaf sheaths of control plants and plants of which the roots were treated with either the purified pseudobactin of WCS374r (Psb374) or BTH (0.05 mM) were inoculated with a *M. oryzae* conidial suspension and sampled 18, 24, 36, and 48 h post-inoculation (hpi). Notably, microscopic assessment revealed no significant differences in the number of successful penetrations among treatments, indicating that both BTH- and Psb374-induced resistance are unlikely to impede pre-penetration development by *M. oryzae* (data not shown). Starting 36 hpi, epidermal cells were found to respond to fungal ingress through the development of various cellular reactions which we grouped into six categories, designated A-F (Fig. 5.5A). Type A represented infection sites showing successful fungal invasion in the absence of any obvious host response. Type B reactions, on the other hand, were characterized by a pale yellow or brown discoloration of the anticlinal cell walls and weakly enhanced vesicular activity. Epidermal sites in which the invasive hyphae were confined to the primary penetrated cell due to expression of the so-called 'whole plant-specific resistance' [WPSR; (Koga et al., 2004a)], a type of age-related resistance characterized by the occurrence of large, brownish granules in the cytoplasm, were scored as type C. Infection type D likewise comprised single-cell infection sites but was associated with intense browning of the anticlinal cell walls and the occurrence of round and tubular vesicles in the cytoplasm. Epidermal cells classified as type E displayed a remarkable interaction phenotype in which fungal growth was curtailed shortly after penetration by means of infection hyphae-encasing tubers, the nature of which is still elusive as staining with phloroglucinol provided no compelling evidence for the involvement of lignin-derived deposits. Finally, type F represented a hypersensitive response (HR)-like reaction as evidenced by dense granulation of the cytoplasm and a bright autofluorescence of the epidermal cell walls. An overview of the temporal changes in the frequency of the various cellular reaction types is presented in Figure 5.5B. At 36 hpi, control plants almost exclusively displayed type A reactions (up to 92% of all interactions). A decrease in type A reaction from 36 to 48 hpi was accompanied by a drastic increase in the frequency of appressorial sites exhibiting a type B reaction, reaching a

level of 55% by 48 hpi. BTH-induced resistance, on the other hand, was characterized by a high frequency of interaction sites with attacked cells expressing HR-related type F reactions (70% of all interactions), resulting in abrupt arrest of fungal proliferation. Most conspicuously, Psb374-supplied plants showed a strikingly different profile of effector responses in that the latter plants did not develop any HR-like responses, but rather mounted type D and type E reactions, accounting for 33% and 50% of all interactions by 48 hpi, respectively. Together, these observations suggest that Psb374 primes rice for a diverse set of HR-independent cellular defenses.

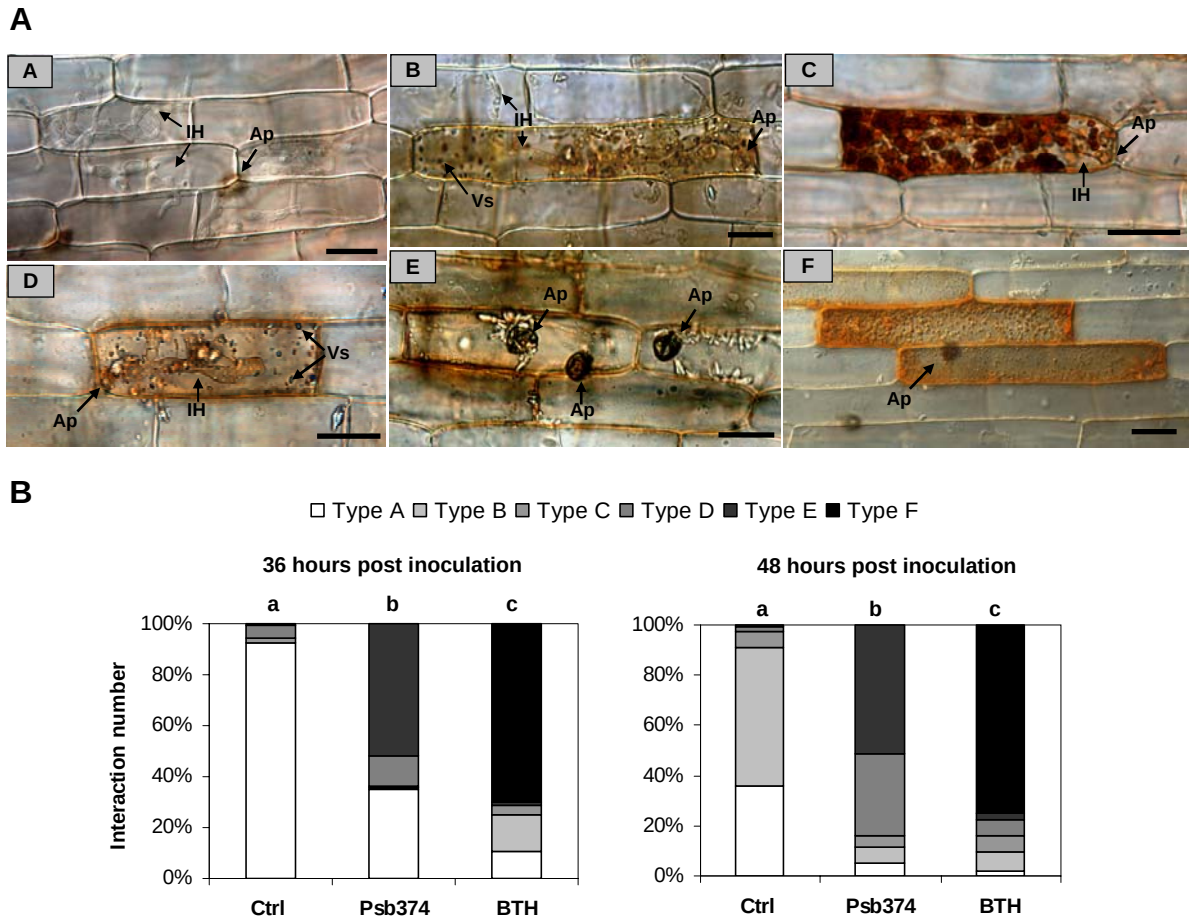

Figure 5.5. Quantitative cytological analysis of cellular responses in leaf sheath epidermal cells of control, BTH- and pseudobactin-pretreated rice plants infected with *M. oryzae*.

Roots of young hydroponically grown rice seedlings (6.5-leaf stage) were treated with either the purified pseudobactin of *P. fluorescens* WCS374r (Psb374; 70 µg/plant) or BTH (0.05 mM); 3 d later, plants were challenged by injecting the intact leaf sheaths with a conidial suspension of *M. oryzae*. **A**, Single-cell interaction phenotypes were grouped into 6 categories (A-F). Micrographs depict representative examples. (A) vigorous invasion of living tissues in the absence of visible host responses; (B) occurrence of cytoplasmic vesicles and slight browning of the anticlinal walls of the first-invaded epidermal cell following fungal invasion of neighbouring cells; (C) epidermal cells expressing so-called 'whole plant-specific resistance' (WPSR; Koga et al., 2004a) as indicated by the presence of large orange-brown granules in the cytoplasm; (D) restriction of fungal development to the first-invaded epidermal cell associated with intense browning of anticlinal epidermal cell walls and enhanced vesicular activity; (E) development of invading hyphae-embedding tubules confers prompt fungal arrest in Psb374-induced epidermal cells; (F) BTH-specified hypersensitive response-like reaction characterized by dense cytoplasmic granulation. Ap, appressorium or

appressorial site; IH, invading hyphae; Vs, vesicles. Scale bars = 20 μm. **B**, Frequency distribution of abovementioned interaction phenotypes at 36 and 48 hpi. Each bar represents means of eight replications stemming from four plants. At least 50 single-cell interaction sites originating from representative sheath sections were examined per replication. Data from one experiment are presented. Repetition of experiments led to results very similar to those shown. Bars with the same letter are not significantly different according to Kruskal-Wallis and Mann-Whitney comparison tests at $\alpha = 0.05$.

Pseudobactin primes rice for enhanced pathogenesis-related H_2O_2 formation

Production of reactive oxygen species during the oxidative burst is one of the most peculiar defense responses in plant-pathogen interactions; therefore, we next compared pathogenesis-related H_2O_2 generation using 3,3'-diaminobenzidine (DAB) staining. Consistent differences between treatments were seen from 24 hpi onward. At this time, approximately one fourth of all Psb374- or BTH-treated epidermal cells adjacent to fungal appressoria showed a local brownish staining of the anticlinal walls, whereas little staining was evident in the sheaths of control plants (data not shown). Importantly, ascorbate treatment of inoculated leaf sheaths abolished staining at the respective sites, confirming the specificity of the staining for H_2O_2 accumulation. Local DAB staining of anticlinal cell walls disappeared within 36 hpi, when the fungus had started to develop branched, bulbous invading hyphae. From this time onward, different patterns of DAB staining could be distinguished (depicted at 48 hpi in Fig. 5.6A). Interestingly, both the susceptibility-related infection type A, in which fungal hyphae vigorously invaded living tissue, and the Psb374-specified infection type D, characterized by lignituber-like structures encasing invasive hyphae, remained essentially free of DAB accumulation; reactions which we designated DAB type I and II, respectively. Conversely, in some incidences, H_2O_2 accumulated in the primary invaded cell following spread of the invading hyphae into neighboring cells (type III). Restriction of hyphal growth to the initially invaded cell was associated with variable patterns of DAB staining. In some cases, cells were filled with numerous DAB-positive vesicle-like bodies targeted to the invading hyphae (type IV), whereas in WPSR- and HR-expressing cells, H_2O_2 typically accumulated within the characteristic cytoplasmic aggregation (type V-VI). Finally, in a limited number of cases, abrupt arrest of fungal ingress coincided with massive H_2O_2 accumulation in the entire cell, beginning as early as 30 hpi (type VII). A comparative kinetic analysis of H_2O_2 formation revealed that by 36 hpi approximately 75% of all interaction sites in both control and Psb374-treated tissue lacked any DAB-detectable H_2O_2 (Fig. 5.6B). However, whereas in control cells the absence of H_2O_2 accumulation at this time point related to successful fungal colonization, lack of DAB staining in Psb374-induced tissue mainly resulted from the high ratio of appressorial sites exhibiting pathogen-blocking type E reactions. Hence, the high frequency of DAB-negative interaction sites in control and Psb374-treated plants reflects distinct cellular responses with dramatically different outcomes. By 48 hpi, the overall proportion of sites

that showed DAB staining was significantly higher in Psb374-treated plants than in control plants. In Psb374-induced tissue, a strong decline in the frequency of DAB-negative type I cells from 36 to 48 hpi corresponded to an approximately 10% increase in type III, type IV and type V reactions, whereas in control plants, the number of type I and type III reactions decreased very slowly at a rate corresponding to an increase in the number of type V reactions. Compared to the well-restricted H_2O_2 production in Psb374-supplied sheath cells, BTH-triggered HR was associated with a massive oxidative burst (type VI) beginning as early as 30 hpi, suggesting that the mechanism(s) by which BTH boosts pathogen-triggered H_2O_2 generation may be different from Psb374-conditioned priming. Starting 52 hpi, a strong accumulation of H_2O_2 was found in control mesophyll cells that appeared to collapse, whereas in Psb374- and BTH-treated plants, DAB staining in the mesophyll tissue was only rarely observed (data not shown). However, at these late infection stages, massive H_2O_2 accumulation most likely reflects deregulated cell physiology and overwhelmed anti-oxidative capacities, rather than a controlled defense response that restricts cellular accessibility for *M. oryzae*. Taken together, these results indicate that Psb374-mediated resistance against *M. oryzae* involves a timely, highly localized, and well-restricted production of H_2O_2 in the epidermis.

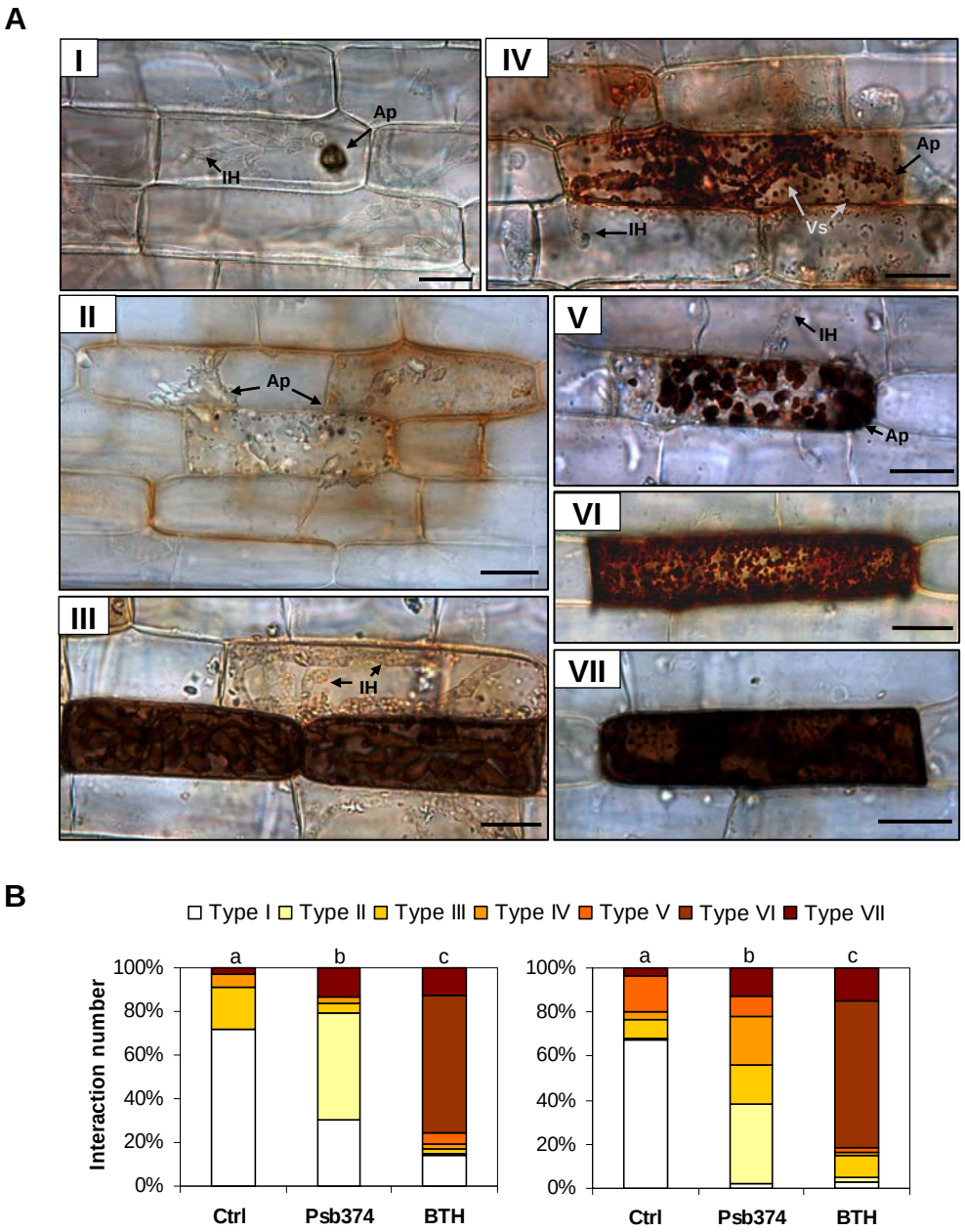

Figure 5.6. Effect of pseudobactin and BTH pretreatment on H_2O_2 accumulation in epidermal rice sheath cells inoculated with *M. oryzae*.

Roots of young hydroponically grown seedlings (6.5-leaf stage) were treated with either the purified pseudobactin of P. fluorescens WCS374r (Psb374; 70 µg/plant) or BTH (0.05 mM); 3 d later, plants were challenged by injecting the intact leaf sheaths with a conidial suspension of M. oryzae. **A**, Micrographs depicting examples of distinct H_2O_2accumulation patterns in inoculated leaf sheaths supplied with 3,3'-diaminobenzidine (DAB): (I), successful fungal colonization of living control cells, no DAB staining visible; (II) Psb374-specified fungal arrest in the primary invaded cell (see Fig. 5E) is not associated with any detectable H_2O_2accumulation. Note the difference between the natural browning of attacked cells (this picture) and the intense reddish-brown coloration due to DAB staining of H_2O_2 (pictures III-VII); (III) DAB accumulation in a primary epidermal cell following fungal invasion of adjacent cells; (IV) accumulation of DAB-positive vesicle-like bodies in the vicinity of the invasive hyphae; (V) WSPR-expressing cells (for details see legend to Fig. 5.5) filled with DAB-stained granules; (VI) DAB-positive cytoplasmic granules in BTH-treated hypersensitively reacting cells; (VII) whole-cell DAB staining. Ap, appressorium or appressorial site; IH, invading hyphae; Vs, vesicles. Scale bars = 20 µm. **B**, Frequency distribution of abovementioned DAB staining patterns at 36 and 48 hpi. Each bar represents means of eight replications stemming from four plants. At least 50 single-cell interaction sites originating from representative sheath sections were examined per replication. Data from one experiment are presented. Repetition of experiments led to results very similar to those shown. Bars with the same letter are not significantly different according to Kruskal-Wallis and Mann-Whitney comparison tests at $\alpha = 0.05$.

Pseudobactin-induced resistance against M. oryzae is associated with priming for accelerated cell wall fortification

Because H_2O_2 is often used as a substrate for peroxidase-dependent cross-linking of cell wall polymers, different staining procedures were performed to visualize changes in the cell wall. Cross-linking of cell wall proteins was detected with Coomassie blue subsequent to protein denaturation and free protein removal (Mellersh et al., 2002), whereas safranin-o was used to detect the peroxidative incorporation of phenolic compounds in the cell wall, a fortification mechanism important during lignification and suberization (Lucena et al., 2003). As shown in Figure 5.7, cell wall modification was more abundant and appeared earlier in Psb374- and BTH-treated plants than in the control treatment: starting from 24 hpi, the anticlinal walls of Psb374- or BTH-induced epidermal cells showed intense safranin staining, whereas in control plants, staining was weak and only detectable in limited zones of the anticlinal walls of a few colonized cells from 36 hpi onward (Fig. 5.7A). Likewise, protein cross-linking was seldom detected prior to 48 hpi in control plants, whereas in Psb374-supplied or BTH-induced cells, it was evident in the anticlinal and/or periclinal walls of most interaction sites 36 hpi (Fig. 5.7B). Similar results were obtained when assaying for autofluorescence, the early occurrence of which is considered a hallmark of rice defense against *M. oryzae* (Rodrigues et al., 2005). Although autofluorescence was detectable as early as 18 hpi regardless of the treatment, from this time onward, the frequency of autofluorescent appressorial sites increased much more rapidly in Psb374- or BTH-treated plants than in non-treated control plants, indicating that both inducers prime rice for augmented deposition of phenolic compounds at sites of attempted pathogen entry (Fig. 5.7C). Conceivably, enrichment of the host cell wall with phenolics contributes to the elaboration of permeability barriers preventing pathogen spread and enzymatic cell wall degradation.

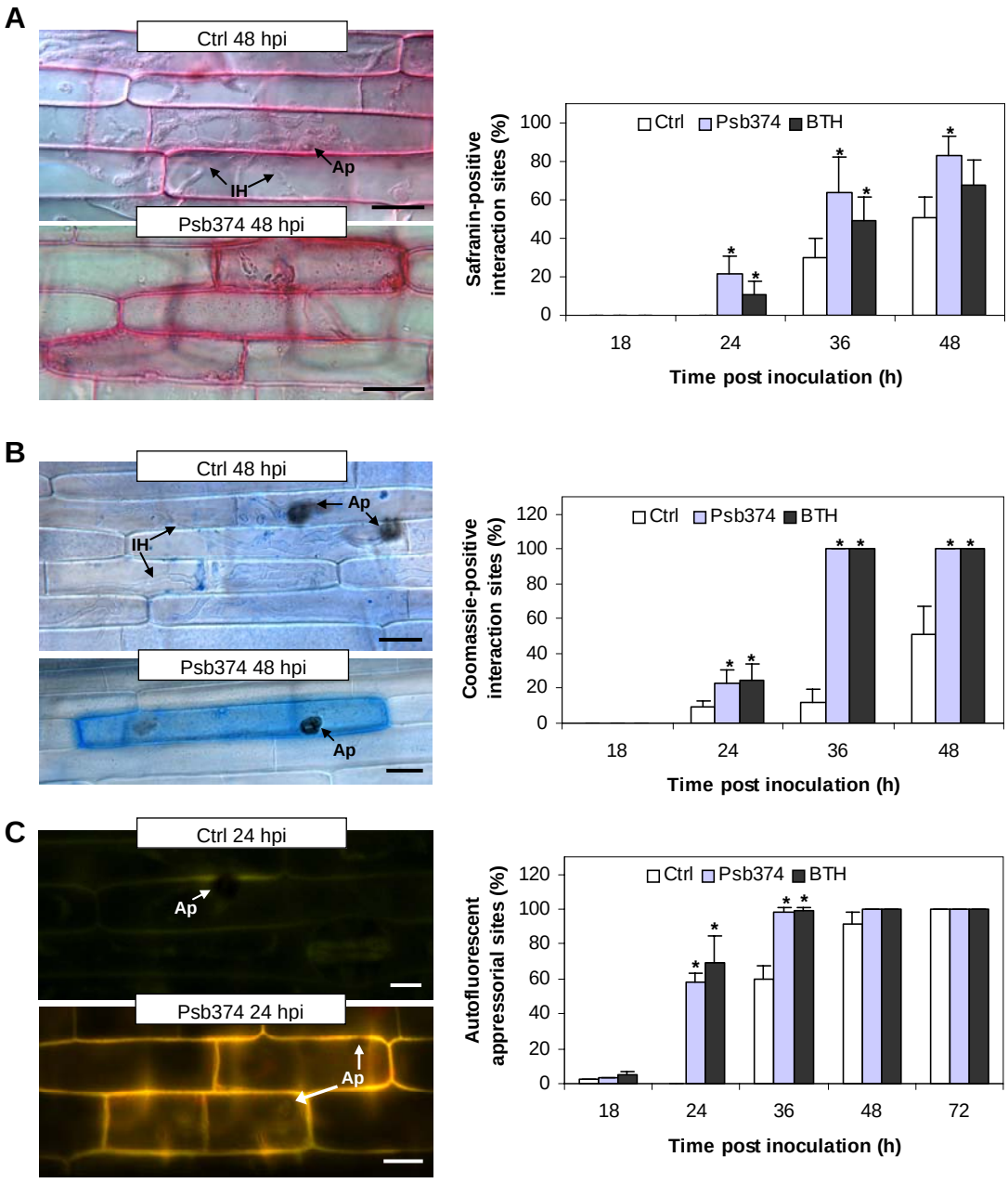

Figure 5.7. Quantitative cytological analysis of cell wall modifications in control, BTH- and pseudobactin-pretreated rice plants challenged with *M. oryzae*.

Roots of young hydroponically grown rice seedlings (6.5-leaf stage) were treated with either the purified pseudobactin of *P. fluorescens* WCS374r (Psb374; 70 µg/plant) or BTH (0.05 mM); 3 d later, plants were challenged by injecting the intact leaf sheaths with a conidial suspension of *M. oryzae*. **A** and **B**, Priming of pathogen-induced cell wall reinforcements in Psb374-treated plants. Peroxidative incorporation of phenolic compounds and protein cross-linking were visualized with safranin-o (red-pink; A) and Coomassie Blue (dark blue; B), respectively. **C**, Left, Representative epifluorescence images of control and Psb374-supplied epidermal cells at 24 hpi (blue light excitation). Right, Psb374 and BTH prime rice for accelerated deposition of autofluorogenic phenolics at sites of attempted pathogen entry. Asterisks indicate statistically significant differences compared with the non-induced control treatment. Each bar represents means and SD of six replications stemming from three plants. At least 50 single-cell interaction sites originating from representative sheath sections were examined per replication. Data from one experiment are presented. Repetition of experiments led to results very similar to those shown. Scale bars = 20 µm.

Exogenous SA abrogates Psb374-induced priming

The observation that Psb374-pretreated plants exhibited potentiated expression of multiple cellular defense responses suggested that priming for enhanced basal defense may constitute a crucial facet of the Psb374-induced resistance response. To test this hypothesis, we next examined the effect of exogenous SA application on the manifestation of Psb374-induced priming. As illustrated in Table 5.1, co-application of 1 nM SA with 70 μg Psb374 significantly decreased the frequency of Psb374-specified type E reactions, i.e. infection-blocking tubules. Adding SA to the Psb374 solution also perturbed the early occurrence of DAB staining and autofluorescence in Psb374-treated tissues and alleviated Psb374-primed protein cross-linking and cell wall fortification. Along with the suppressive effect of co-applied SA on the level of Psb374-induced protection against *M. oryzae* (Fig. 5.4), these results indicate that Psb374-triggered ISR is based on priming for enhanced expression of an attacker-induced multifaceted cellular defense program.

Table 5.1. ***Influence of SA co-application on Psb374-induced defense priming***

The data represent means and SD of 4 replicates of each 100 interaction sites per leaf sheath. Each of three independent experiments gave very similar results.

	Treatment[a]			
	Ctrl	SA	Psb374	SA + Psb374
Reaction	% of interaction sites			
Infection-blocking tubules (36 hpi)[b]	not seen	not seen	43.3 ± 18.6[b]	7.5 ± 2.4[a]
DAB staining (24 hpi)[c]	not seen	not seen	26.2 ± 8.7[b]	5.6 ± 2.1[a]
Autofluorescence (24 hpi)	2.1 ± 0.8[a]	9.4 ± 4.2[b]	66.8 ± 10.6[c]	18.6 ± 6.7[b]
Coomassie Blue staining (36 hpi)	24.6 ± 5.8[ab]	19.2 ± 4.6[a]	94.2 ± 5.2[c]	32.4 ± 9.2[b]
Safranin staining (36 hpi)	29.5 ± 10.2[a]	24.6 ± 6.8[a]	72.6 ± 8.6[b]	21.4 ± 11.3[a]

[a] SA (1 nM) and purified WCS374r pseudobactin (Psb374; 70 μg/root system) were applied either alone or in combination to the roots of hydroponically grown rice seedlings (5.5-leaf stage) 3 d prior to challenge with *M. oryzae*. [b] Interaction phenotype 'E' as described in legend to Figure 5. [c] Percentage of interaction sites associated with reddish-brown precipitates in the anticlinal cell wall. Within each row, different letters indicate statistically significant differences between treatments (Fisher's LSD test; $\alpha = 0.05$).

Psb374 antagonizes pathogen-induced activation of SA-responsive *PR* genes

The results above, together with the *PR* gene-independency of ISR in *Arabidopsis* (Pieterse et al., 1996), prompted us to investigate whether Psb374 pretreatment also affects *PR* transcript accumulation. To this end, we tested control, BTH-induced and Psb374-supplied plants for expression of the rice *PR*-like genes *OsPR1b* and *PBZ1/PR10a*. Both of these genes are known to be responsive to *M. oryzae* infection (Kim et al., 2001) and have recently been implicated in the

BTH-inducible and SA-mediated signaling branch of the rice defense network (Shimono et al., 2007). Quantitative RT-PCR analysis revealed that neither BTH application nor Psb374 treatment alone significantly altered *OsPR1b* or *PBZ1* mRNA accumulation at any of the time points investigated (Fig. 5.8A; data not shown). However, significant differences between treatments became evident when challenging with *M. oryzae*. In accordance with previous reports (Midoh and Iwata, 1996; Yang et al., 2004), *PBZ1* transcript levels responded strongly to blast infection, showing an approximately 250-fold induction relative to mock-inoculated controls by 48 hpi (Fig. 5.8A). Interestingly, application of Psb374 prior to inoculation attenuated this pathogen-induced activation of *PBZ1*, whereas pretreatment with BTH caused a faster and stronger induction of the latter gene in comparison to the expression measured in challenged, non-induced plants (Fig. 5.8A). Transcript accumulation of the *OsPR1b* gene mirrored the profile observed for *PBZ1* (Fig. 5.8B), suggesting that Psb374 antagonizes *M. oryzae*-induced transcription of SA-responsive *PR* genes.

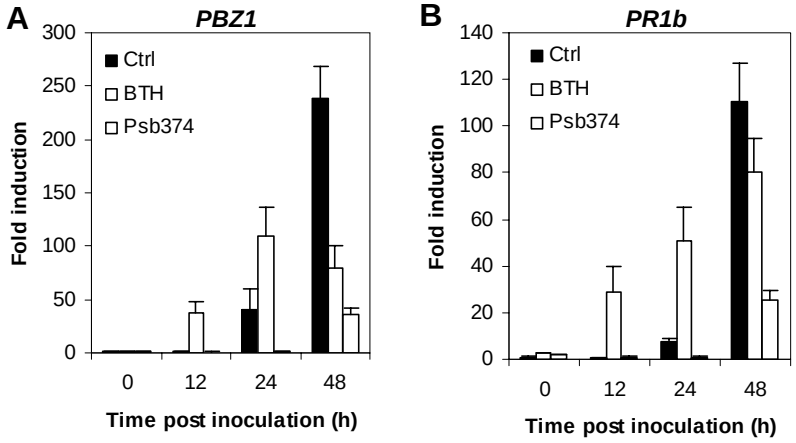

Figure 5.8. Effect of BTH and Psb374 pretreatment on *PBZ1/PR10* (A) and *PR1b* (B) transcript accumulation in *M. oryzae*-infected rice leaves.

BTH (0.05 mM) or Psb374 (70 µg/root system) were applied to the roots of four-week-old rice plants 3 d before challenge. At the indicated time points post inoculation, fully expanded fourth leaves from six plants were harvested, pooled and subjected to qRT-PCR analysis. Gene expression levels were normalized using actin (Os03g50890) as an internal reference and calculated relative to the expression in mock-treated control plants at 0 h. Data presented are means (± SD) of three replicates of a representative experiment. Two series of independent experiments were carried out giving reproducible results. Psb374 = purified pseudobactin of *P. fluorescens* WCS374r.

Discussion

Induced systemic resistance (ISR) is a phenomenon whereby disease resistance against subsequent microbial infection is induced at the whole-plant level in response to colonization of the roots by certain plant growth-promoting rhizobacteria. Compared to the relative wealth of information in experimentally tractable plant species such as *Arabidopsis*, our understanding of the molecular mechanisms underlying ISR in economically important cereal crops is still in its infancy. In the present work, we have focused on the bacterial determinants and host defense responses underlying rhizobacteria-activated ISR in rice, the most important food source worldwide and a pivotal model for molecular genetic studies of disease resistance in monocotyledonous plants. We show that colonization of the roots of rice by the well-characterized biocontrol agent *P. fluorescens* WCS374r renders foliar tissues more resistant to rice blast disease, caused by the heterothallic ascomycete *M. oryzae* (Fig. 5.1). Our data also reveal that this WCS374r-mediated ISR (WCS374r-ISR) is not based on direct activation of basal resistance mechanisms, but rather acts through pseudobactin-mediated priming for a pronounced multifaceted cellular defense program (Figs. 5.3-5.7). Moreover, we demonstrate that ISR by WCS374r requires components of the ET and JA pathways, rather then SA accumulation or enhanced *PR* gene expression, suggesting that rice might have evolved a disease-resistance pathway similar to the classic ISR pathway in *Arabidopsis* (Figs. 5.2 and 5.8).

Pseudobactin: iron-chelating protagonist in the initiation of *P. fluorescens* WCS374r-mediated ISR

To date, several bacterial traits have been implicated in the initiation of WCS374r-ISR, including the O-antigenic side chain of outer membrane lipopolysaccharides, salicylic acid, and the siderophore pseudobactin (Leeman et al., 1995, 1996; Ran et al., 2005a,b). In this study, we initially assumed pseudobactin and SA to be co-required for initiation of ISR against *M. oryzae*, based on the observation that both the pseudobactin-deficient mutant 374-02 and the pseudobactin-proficient, yet SA-negative, mutant 4B1 lost the ability to mount ISR (Fig. 5.3). However, contradictory results were obtained when testing the isolated bacterial compounds: purified WCS374r-derived pseudobactin (Psb374) triggered high levels of resistance against rice blast, whereas exogenous SA failed to cause any substantial disease reduction (Fig. 5.4), indicating that Psb374 alone suffices for induction of ISR. Moreover, co-application of SA with Psb374 attenuated the Psb374-induced resistance, prompting the question of how WCS374r bacteria, which simultaneously produce both these metabolites in vitro, are able to trigger ISR. One likely scenario originates from the observation that treatment with WCS374r does not induce systemic

resistance in *Arabidopsis* against *P. syringae* pv. *tomato*, whereas the application of SA does (Van Wees et al., 1997). This discrepancy suggests that SA produced by WCS374r is not exuded into the rhizosphere, which may be due to the fact that upon iron limitation WCS374r produces not only pseudobactin and SA, but also pseudomonine, a siderophore containing a SA moiety (Mercado-Blanco et al., 2001). Accordingly, it is plausible that in the rhizosphere, where iron-limiting conditions tend to prevail, all WCS374r-produced SA is channeled into pseudomonine that does not antagonize pseudobactin action. Nonetheless, if pseudobactin is the crucial determinant of WCS374r-ISR against *M. oryzae*, a question remains as to the ISR-minus phenotype of the pseudobactin-positive mutant 4B1. One possible explanation for these conflicting observations lies in the fact that mutant 4B1 was constructed by gene replacement of the native *psmB* gene. *PmsB* encodes a presumed isochorismate-pyruvate lyase that catalyzes the conversion of isochorismate to pyruvate and SA (Djavaheri, 2007). Interestingly, recent evidence suggests that in line with its closest homolog in *P. aeruginosa*, the catalytically promiscuous SA biosynthesis protein PchB, PmsB not only possesses isochorismate-pyruvate lyase but also chorismate mutase activity (Kunzler et al., 2005). Since chorismate mutase is located at the branch point of the shikimate pathway leading to biosynthesis of tyrosine and phenylalanine, the enzyme constitutes a key point of regulation for maintaining the correct balance of aromatic amino acids in the cell (Neuenschwander et al., 2007). Hence, it can be envisaged that a mutation in such regulatory enzyme might have a pleiotropic effect hampering the induction of ISR. Alternatively, mutant 4B1 might simply produce too little pseudobactin in the rhizosphere to be effective in inducing resistance.

Rice and *Arabidopsis* share conserved disease resistance pathways

In many dicot plants, the role of SA as a global multi-component regulator of various inducible defense responses is well established (Loake and Grant, 2007). Following pathogen infection, endogenous levels of SA and its conjugates increase dramatically, preceding the induction of *PR* genes and the onset of local and systemic acquired resistance [SAR; (Durrant and Dong, 2004)]. In rice, however, the role of SA is still a matter of debate. Rice differs from most other plants in that it contains very high basal levels of endogenous SA that are not elevated further in response to pathogen infection, making the role of the SA signaling pathway in rice disputable (Silverman et al., 1995). A number of recent reports, however, do support an active role for a BTH-inducible and WRKY45- or NPR1-regulated SA signaling pathway in the rice defense response (Chern et al., 2001; Fitzgerald et al., 2004; Chern et al., 2005b; Shimono et al., 2007; Yuan et al., 2007b). Emerging from these studies is the view that rice, in spite of its high

constitutive SA levels, has evolved an SA-mediated SAR pathway similar to that in *Arabidopsis*. In this study, we provide the first report of a similar phenomenon with regard to rhizobacteria-induced resistance signaling. ISR bioassays with SA non-accumulating NahG plants (Yang et al., 2004), the ET-insensitive *OsEIN2* antisense line 471 (Jun et al., 2004) and the JA biosynthesis mutant *hebiba* (Riemann et al., 2003) revealed that WCS374r-mediated ISR against *M. oryzae* functions independently of SA, but requires intact responsiveness to ET as well as a functional JA pathway (Fig. 5.2). In this respect, WCS374r-ISR against *M. oryzae* mirrors classic WCS417r-elicited ISR in *Arabidopsis* (Pieterse et al., 1996, 1998). Consonant with this is the finding that treatment with Psb374, which faithfully mimics WCS374r in activating ISR, does not lead to direct transcriptional activation or priming of SA-inducible *PR* genes, such as *OsPR1b* and *PBZ1* (Fig. 5.8). In contrast to WCS374r-ISR, but similar to BTH-induced resistance in dicots (Friedrich et al., 1996; Gorlach et al., 1996), chemical induction of blast resistance by exogenous application of BTH was fully retained in both 471, *hebiba* and NahG rice plants, and involved potentiation of SA-inducible gene expression (Figs. 5.2 and 5.8). Taken together, these results not only reinforce the contention that rice is endowed with a BTH-inducible SAR-like resistance pathway (Shimono et al., 2007; Yuan et al., 2007b), but also hint at a conserved mechanism for ISR signaling in rice and *Arabidopsis*. It is noteworthy, however, that unlike WCS374r-ISR, induction of systemic resistance against *M. oryzae* by *P. aeruginosa* 7NSK2 was found to be SA-dependent (De Vleesschauwer and Höfte, unpublished results), indicating that the signal transduction pathway governing rhizobacteria-mediated ISR against *M. oryzae* at least in part depends on the eliciting bacterium. Nonetheless, the apparent similarities between WCS374r- and WCS417r-activated ISR signaling in rice and *Arabidopsis* respectively, support and further extend the earlier notion of ancient plant-inducible defense pathways that are shared between monocots and dicots (Morris et al., 1998). This notion, however, does not rule out the possibility that individual plant species may differ in the fine-tune regulation of such conserved defense pathways. For instance, while ectopic expression of a rice NPR1 homolog induces constitutive activation of SA-responsive *PR* gene expression and provokes spontaneous development of a lesion mimic/cell death phenotype (Chern et al., 2005b), none of these reactions is evident in NPR1-overexpressing *Arabidopsis* until treatment with SAR inducers or pathogen infection (Cao et al., 1998). Such species-specific regulation of conserved plant defense mechanisms may also apply to ISR-associated resistance phenomena. Indeed, whereas the impaired ISR response of JA-deficient *hebiba* argues that in rice WCS374r-ISR develops coincidently with increases in endogenous JA content (Fig. 5.2), in *Arabidopsis* neither induction nor expression of WCS417r-ISR was found to be associated with substantial alterations in JA biosynthesis (Pieterse et al.,

2000). Instead, recent evidence indicates that elicitation of WCS417r-ISR sensitizes *Arabidopsis* for perception of attacker-induced JA (Pozo et al., 2008). Hence, although rice and Arabidopsis appear to share a conserved ISR pathway, the modulation of this JA-dependent resistance conduit may be quite divergent. To our interest, the significance of elevated JA levels in mediating rice disease resistance was recently highlighted by the enhanced blast resistance of transgenic rice plants over-expressing allene oxide synthase, a key enzyme in the JA biosynthetic pathway (Mei et al., 2006).

Parallels between WCS374r-mediated ISR and wound-inducible systemic resistance against *M. oryzae*

The predicted role of JA in WCS374r-ISR is reminiscent of the situation in wounded rice plants where systemic resistance against *M. oryzae* is preceded by a strong and transient accumulation of non-conjugated JA in local and systemic tissues (Schweizer et al., 1998). Wound-inducible blast resistance further resembles WCS374r-ISR in that it delivers a similar level of systemic protection without the customary *PR* gene induction and is likewise abrogated in mutant *hebiba* plants (Schweizer et al., 1998; Riemann et al., 2003; De Vleesschauwer and Höfte, unpublished results). Regarding these similarities between WCS374r-ISR and wound-induced resistance, it is tempting to speculate that both phenomena are based on similar resistance mechanisms. Such a concept would also provide a mechanistic framework for the attenuation of SA-responsive *PR* gene expression in challenged Psb374-induced plants (Fig. 5.8). In some interesting work on rice responding to mechanical wounding, Lee et al. (2004) demonstrated that JA-induced depletion of endogenous SA levels constitutes an important regulatory mechanism for JA antagonism of SA signaling. In this scenario, if the establishment of WCS374r- and Psb374-mediated ISR coincides with a JA burst, the inverse correlation between endogenous JA and SA may account for the down-regulation of at least *PR1b*, the induction of which is considered to be a reliable marker for activation of the SA-regulated defense pathway in rice (Yuan et al., 2007b). In a similar vein, antagonistic cross-talk between SA and JA signaling may also explain the inhibitory effect of exogenous SA on the Psb374-provoked resistance against *M. oryzae* (Fig. 5.4; Table 5.1). Antagonistic cross-communication between the SA and JA pathways in rice was recently shown to be orchestrated by OsWRKY13, a WRKY transcription factor functioning upstream of the rice NPR1 homolog OsNH1 (Qiu et al., 2007). Ectopic expression of *OsWRKY13* represses JA biosynthetic genes while activating a specific subset of SA-dependent genes, which suggests that OsWRKY13 antagonizes JA-dependent defenses by negative feedback

regulation of JA biosynthesis (Qiu et al., 2007). Whether *OsWRKY13* expression is altered in ISR-expressing plants is currently being investigated.

WCS374r-triggered ISR is based on pseudobactin-mediated priming for a multifaceted cellular defense response

In common with many other investigations (Benhamou et al., 1996; Ahn et al., 2002; Kim et al., 2004a; Verhagen et al., 2004; Tjamos et al., 2005; Ahn et al., 2007), our results support the view that rhizobacteria-mediated ISR is not based on direct activation of defense mechanisms, but rather results from a sensitization of the tissue to express basal defenses faster and/or more strongly upon subsequent pathogen attack. Such priming effect was borne out by the observation that challenge inoculation of Psb374-induced plants with *M. oryzae* entailed the prompt expression of a pronounced multifaceted cellular defense program, comprising rapid recruitment of phenolic compounds at sites of attempted pathogen entry, elaboration of specific sheath cell reactions, and a timely oxidative burst putatively driving cell wall fortification and protein cross-linking (Figs. 5.5-5.7). The importance of defense priming in the Psb374-activated resistance mechanism was shown by the effect of adding SA to the Psb374 feeding solution, which not only counteracted the distinct Psb374-primed cellular responses, but concurrently alleviated Psb374-provoked resistance against *M. oryzae* (Fig. 5.4; Table 5.1). In conjunction with the strict pseudobactin-dependency of WCS374r-ISR, such close correlation between the manifestation of priming and the establishment of Psb374-induced resistance infers that Psb374-mediated priming for enhanced defense may constitute the *in situ* mechanism underpinning WCS374r-ISR against *M. oryzae*. Hence, it is not inconceivable that WCS374r bacteria protect rice from *M. oryzae* by releasing pseudobactin-type siderophores into the rhizosphere, thereby inducing a pre-alerted state of defense enabling plants to respond better and more rapidly to subsequently inoculated pathogens. In line with this concept, we previously uncovered priming as a crucial facet of the resistance mechanism underlying *P. aeruginosa* 7NSK2-mediated ISR against *M. oryzae*. Feeding rice plants with the redox-active pigment pyocyanin, the crucial determinant of 7NSK2-mediated ISR, resulted in enhanced attacker-induced hypersensitive response (HR)-like cell death in naïve leaves, a phenomenon shown to be orchestrated by reiterative H_2O_2 microbursts (Chapter 3). Interestingly, similar phenocopies of hypersensitively dying epidermal cells in the vicinity of fungal hyphae were evident in challenged rice plants pretreated with BTH (Fig. 5.5), suggesting that BTH and pyocyanin might feed into a similar resistance pathway. Psb374-elicited ISR, however, was not associated with HR-like cell death, but involved the potentiation of a coordinate set of distinct cellular reactions, the fast manifestation of pathogen-blocking tubules being a prominent component (Fig. 5.5). In combination with our unpublished findings that

WCS374r- and 7NSK2-ISR differ in their requirement for SA, these results support the notion that WCS374r and 7NSK2 bacteria employ distinct strategies to mount ISR and suggest that rice is endowed with multiple, at least partly distinct, blast-effective resistance pathways. This conclusion is further supported by a large body of evidence demonstrating minimal overlap in the gene sets activated by different blast resistance inducers (Midoh and Iwata, 1996; Schweizer et al., 1997a,b; Schweizer et al., 1999; Nakashita et al., 2003; Tanabe et al., 2006).

Conclusion

In summary, we have shown that colonization of the roots of rice by pseudobactin-producing WCS374r bacteria sensitizes naïve leaves for potentiated expression of a multifaceted cellular defense response, resulting in an enhanced level of resistance against the leaf blast pathogen *M. oryzae*. Our results also provide evidence for a WCS374r-activated signaling conduit in rice similar to the classic SA-independent but JA/ET-dependent signal transduction pathway controlling rhizobacteria-mediated ISR in *Arabidopsis*. Furthermore, it is evident from the present study that WCS374r triggers a resistance that is mechanistically different from BTH-inducible blast resistance as well as systemic resistance induced by *P. aeruginosa* 7NSK2, suggesting the co-existence of multiple pathways leading to induced resistance against *M. oryzae*. Further elucidation of the bacterial traits and dynamic host responses underpinning rhizobacteria-mediated ISR in rice will not only advance our fundamental understanding of how rice plants cope with enemies in the context of induced resistance, but also may be instrumental in developing new strategies for biologically based, environmentally friendly and durable disease control in economically important cereal crops.

Materials and Methods

Plant materials

The highly susceptible rice (*Oryza sativa* spp. *indica*) cultivar CO39 was routinely used in this study. Transgenic *NahG* rice and its parental line, *japonica* cultivar Nipponbare, were a kind gift from Dr. Yinong Yang (Pennsylvania State University, USA). Seeds of cultivar Dongyin (*Oryza sativa* spp. *japonica*) and the transgenic line 471, expressing the *OsEIN2* antisense construct, were kindly provided by Dr. Gynheung An (Yonsei University, Korea), while JA-deficient *hebiba* mutant seeds and the corresponding wild-type, *japonica* cultivar Nihonmasari, were a kind gift from Dr. Peter Nick (Karlsruhe University, Germany). Unless otherwise noted, rice plants were grown on soil under greenhouse conditions (30 ± 4ºC and 16 h photoperiod). For seed multiplication, plants were propagated in the greenhouse and fertilized with 0.5% ammonium sulphate every two weeks until flowering.

Cultivation of rhizobacteria and pathogens

Bacterial strains used in this study are listed in Table 5.2. *Pseudomonas fluorescens* strain WCS374r and derived mutant strains were grown for 24 to 28 h at 28°C on King's medium B (KB; King et al., 1954) agar plates. Bacterial cells were scraped off the plates and suspended in sterile saline (0.85% NaCl). Densities of the bacterial suspensions were adjusted to the desired concentration based on their optical density at 620 nm.

Magnaporthe oryzae isolate VT5M1 (Thuan et al., 2006) was grown at 28ºC on half-strength oatmeal agar (Difco, Sparks, USA). Seven-day-old mycelium was flattened onto the medium using a sterile spoon and exposed to blue light (combination of Philips TLD 18W/08 and Philips TLD 18W/33) for seven days to induce sporulation. Conidia were harvested as described in Chapter 3 and inoculum concentration was adjusted to a final density of 1 x 10^4 spores.ml^{-1} in 0.5% gelatin (type B from Bovine skin; Sigma-Aldrich G-6650).

Induction treatments

Induced resistance assays were performed basically as described in Chapter 3. Briefly, plants were grown under greenhouse conditions (30 ± 4ºC, 16-h photoperiod) in commercial potting soil (Structural; Snebbout, Kaprijke, Belgium) that had been autoclaved twice on alternate days for 21 min. Rice seeds first were surface sterilized with 1% sodium hypochlorite for two min, rinsed three times with sterile, demineralized water and incubated on wet sterile filter paper for five days at 28ºC to germinate. Prior to sowing in perforated plastic trays (23 by 16 by 6 cm), roots of germinated seeds were dipped in bacterial suspensions (5 x 10^7 cfu.ml^{-1}) for 10 min. In addition, the bacterial inoculum was thoroughly mixed with the potting soil to a final density of 5 x 10^7 cfu. g^{-1} and, 12 days later, applied a second time as a soil drench. In control treatments, soil and rice plants were treated with equal volumes of sterilized saline.

For chemical induction of resistance, plants were treated with BTH three days prior to challenge inoculation. BTH (BION 50 WG), formulated as a water-dispersible granule containing 50% active ingredients, was dissolved in sterilized demineralized water for use and applied as a soil drench. Control plants were treated with an equal volume of water. BTH was a kind gift from Syngenta Crop Protection (Brussels, Belgium).

Pathogen inoculation and disease rating

Four-week-old rice seedlings (5-leaf stage) were challenge-inoculated with *Magnaporthe oryzae* isolate VT5M1 as described before (De Vleesschauwer et al., 2006). Six days after inoculation, disease was assessed by counting the number of elliptical to round-shaped lesions with a gray center indicative of sporulation of the fungus, and expressed relative to non-bacterized control plants.

Table 5.2. ***Bacterial strains used in this study with their relevant characteristics***

Strains	Relevant characteristics[a]	Reference or source
Pseudomonas fluorescens		
WCS374r	Psb$^+$, Psm$^+$, SA$^+$, spontaneous rifampicin-resistant mutant of WCS374; Rif^r	(Geels and Schippers, 1983)
374-02	Psb$^-$, Psm$^+$, SA$^+$, Tn5 transposon mutant of WCS374; Km^r	(Weisbeek et al., 1986)
4A1	Psb$^+$, Psm$^-$, SA$^+$, *pmsA* mutant of WCS374r obtained by site-directed mutagenesis; Rif^r, Km^r	Djavaheri, 2007
AT12	Psb$^-$, Psm$^-$, SA$^+$, Tn5 transposon mutant of 4A1; Rif^r, Km^r, Tc^r	Djavaheri, 2007
4B1	Psb$^+$, Psm$^-$, SA$^-$, pmsB mutant of WCS374r obtained by site-directed mutagenesis; Rif^r, Km^r	Djavaheri, 2007
BT1	Psb$^-$, Psm$^-$, SA$^-$, Tn5 transposon mutant of 4B1; Rif^r, Km^r, Tc^r	Djavaheri, 2007

[a] Abbreviations: Psb = pseudobactin, Psm = pseudomonine, SA = salicylic acid, Pvd = pyoverdine, Pch = pyochelin, Rif = rifampycin, Km = kanamycin, Tc = tetracycline.

Evaluation of plant colonization by *P. fluorescens* WCS374r and mutants

Bacterial colonization of the plant roots was determined by the time the bioassays were discontinued. Roots of three plants of each treatment were rinsed to remove most of the soil, weighed, and macerated in sterile demineralized water. Serial dilutions were plated on KB agar supplemented with the appropriate antibiotics: kanamycin (25 µg/ml), tetracycline (20 µg/ml) and rifampicin (200 µg/ml). Bacterial counts were made after incubation for 24 h at 28ºC. Possible spreading of root-inoculated bacteria to distal leaves was checked as stated in Chapter 3.

Purification of pseudobactin

Bacteria were grown in liquid standard succinate medium (Meyer and Abdallah, 1978) and pseudobactin was extracted and purified according to Meziane et al. (2005). To avoid contamination with salicylic acid or pseudomonine, mutant 4B1 was used.

Application of purified compounds

For experiments in which purified pseudobactin and/or SA were applied to rice seedlings, plants were grown in a hydroponic gnotobiotic system. Surface-sterilized rice seeds were germinated for 5 days at 28ºC on wet filter paper. After incubation, germinated seeds were sown in perforated plastic trays (23x16x6 cm) filled with sterilized vermiculite, and supplemented with half-strength Hoagland solution. Every three days, 0.5 L of the nutrient solution was added to each tray containing 12 seedlings. In this model, various concentrations of pseudobactin and SA were applied to the plants three days before challenge by including the desired concentration in Fe-EDTA-free nutrient solution (Acros, Geel, Belgium).

Visualisation of defense responses

To gain more insight into the cytomolecular mechanisms underlying pseudobactin- and BTH-induced resistance against *M. oryzae*, intact leaf sheath assays were performed as described by Koga et al. (2004b). Briefly, leaf sheaths of the sixth leaf of rice plants at the 6.5 leaf stage were peeled off with leaf blades and roots. The leaf sheath was laid horizontally on a support in plastic trays containing wet filter paper, and the hollow space enclosed by the sides of the leaf sheaths above the mid vein was filled with a conidial suspension (5 x 10^4 conidia. ml^{-1}) of *M. oryzae*. Inoculated leaf sheaths were then incubated at 25ºC with a 16-h photoperiod. When ready for microscopy, the sheaths were hand-trimmed to remove the sides and expose the epidermal layer above the mid vein. Lower mid vein cells were removed to produce sections three to four cell layer thick. For time-course experiments, sheath sections were generally sampled at 18, 24, 30, 36, 48 and 72 h post inoculation and at least six trimmed sheath tissue sections originating from different plants were used for each sampling time point.

Phenolic compounds were visualized as autofluorescence under blue light epifluorescence (Olympus U-MWB2 GPF filter set-excitation: 450 to 480 nm, dichroic beamsplitter; 500 nm, barrier filter BA515). To detect H_2O_2 accumulation, staining was according to the protocol of Thordal-Christensen et al. (1997) with minor modifications. Six hours before each time point, trimmed sheath segments were vacuum infiltrated with an aqueous solution of 1 mg/ml 3,3'-diaminobenzidine(DAB)-HCL (pH = 3.8) for 30 min. Infiltrated segments were then further incubated at room temperature in above mentioned DAB solution until sampling. DAB polymerizes in the presence of H_2O_2 and endogenous peroxidase to form a brownish-red precipitate that can be easily visualized using bright-field microscopy. Specificity of the DAB staining was verified by adding 10 mM ascorbic acid. For protein cross-linking, staining was performed as described by Mellersh et al. (2002). Trimmed sheath segments were submerged in 1% SDS for 24 h at 80

ºC, stained in 0.1% Coomassie blue in 40% ethanol/10% acetic acid for 15 min, and subsequently rinsed in a solution of 40% ethanol/10% acetic acid. For analysis of callose deposition, trimmed sheaths were stained for 5 min in a solution containing 0.01% (w/v) of aniline blue and 0.15M K_2HPO_4. To visualize cell wall modifications, safranin-O staining was performed according to Lucena et al. (2003) by incubating cut sheath segments in 0.01% safranin-O in 50% ethanol for 2 min. After staining, trimmed sheath segments were mounted in 50% glycerol. Images were acquired digitally (Olympus Color View II camera, Aartselaar, Belgium) and further processed with the Olympus analySIS cell^F software.

RNA extraction, cDNA synthesis and quantitative RT-PCR analysis

Total RNA was isolated from frozen leaf tissue using the Invisorb Spin Plant RNA Mini kit (Invitek, Berlin, Germany) and subsequently Turbo DNase-treated according the manufacturer's instructions (Ambion/Applied Biosystems, Lennik, Belgium). Before first-strand cDNA synthesis, the absence of genomic DNA was confirmed by PCR. RNA concentration was checked before and after Turbo DNase digestion. First-strand cDNA was synthesized from 2 μg of total RNA using Affinityscript reverse transcriptase and oligo dT primers (Stratagene/Bio-Connect, Huissen, The Netherlands), according to the manufacturer's instructions. The following primer sequences were used: for *actin* (Os03g50890, similar to AB047313.1), forward 5'- GCGTGGACAAAGTTTTCAACCG-3' and reverse 5'-TCTGGTACCCTCATCAGGCATC-3'; for *PBZ1* (Os12g36880, similar to D38170), forward 5'-CCCTGCCGAATACGCCTAA-3' and reverse 5'-CTCAAACGCCACGAGAATTTG-3'; and for *PR1b* (Os01g28450, similar to U89895), forward 5'-GGCAACTTCGTCGGACAGA-3' and reverse 5'-CCGTGGACCTGTTTACATTTT- 3'. For each primer pair, the optimal annealing temperatures were pre-determined by gradient PCR using a Thermocycler (Bio-Rad, Belgium). Furthermore, for each target, primer concentrations were optimized by performing a primer titration. Quantitative PCR amplifications were conducted in optical 96-well plates with the Mx3005P real-time PCR detection system (Stratagene, La Jolla, CA, U.S.A), using Sybr Green master mix (Stratagene/Bio-Connect, Huissen, The Netherlands) to monitor dsDNA synthesis. The expression of each gene was assayed in triplicate in a total volume of 25 μl including a passive reference dye (ROX) according to the manufacturer's instructions (Stratagene, La Jolla, CA, U.S.A). The thermal profile used consisted of an initial denaturation step at 95ºC for 10 min, followed by 40 cycles of 95ºC for 30 s, 60ºC for 60 s, and 72ºC for 60 s. Fluorescence data were collected during the annealing stage of amplification. To verify amplification of one specific target cDNA, a melting-curve analysis was included according to the thermal profile suggested by the manufacturer. The amount of plant RNA in each sample was normalized using actin (Os03g50890) as internal control and samples collected from control plants at 0 h post inoculation were selected as a calibrator. The generated data were analyzed with the Mx3005P software (Stratagene, La Jolla, CA, U.S.A). For all amplification plots, the optimal baseline range and threshold cycle values were calculated using the Mx3005P algorithm. Gene expression in control, BTH- and pseudobactin-treated samples was expressed relative to the calibrator and as a ratio to actin expression using the measured efficiency for each gene.

Chapter

6

Global switches and fine-tuning: ABA modulates plant pathogen defense

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Published in: *Molecular Plant-Microbe Interactions* **21:**709-719.

Plants are obliged to defend themselves to a wide range of biotic and abiotic stresses. Complex regulatory signaling networks mount an appropriate defense response depending on the type of stress that is perceived. In response to abiotic stresses such as drought, cold and salinity, the function of abscisic acid (ABA) is well documented: elevation of plant ABA levels and activation of ABA-responsive signaling result in regulation of stomatal aperture and expression of stress-responsive genes. In response to pathogens, the role of ABA is more obscure and is a research topic that has long been overlooked. This paper aims to evaluate and review the reported modes of ABA action on pathogen defense and highlight recent advances in deciphering the complex role of ABA in plant-pathogen interactions. The proposed mechanisms responsible for positive or negative effects of ABA on pathogen defense are discussed, as well as the regulation of ABA signaling and in planta ABA concentrations by beneficial and pathogenic microorganisms. In addition, the fast-growing number of reports that characterize antagonistic and synergistic interactions between abiotic and biotic stress responses point to ABA as an essential component in integrating and fine-tuning abiotic and biotic stress response signaling networks.

6.1. Introduction

The capacity of plants to cope with the constant threat of a variety of plant pathogens demonstrates the efficiency of their defensive machinery. Plants possess pre-formed physical and biochemical barriers. When these constitutive defenses are overcome by a pathogen, recognition leads to a complex signaling cascade of inducible defense responses. The phytohormones, salicylic acid (SA), jasmonate (JA) and ethylene (ET) were shown to modulate these signaling pathways. Instead of forming isolated hormonally controlled signaling cascades, complex regulatory signaling networks with frequent cross-talk mount an appropriate defense response depending on the type of pathogenic stimuli that is present (Glazebrook, 2005; Thomma et al., 2001, Lorenzo and Solano, 2005, van Loon et al., 2006a). Furthermore, there is frequent cross-talk between the signaling networks controlling the responses to abiotic stresses (Fujita et al., 2006; Mauch-Mani and Mauch, 2005). The use of shared components in biotic and abiotic stress responses is rationalized by economical use of biochemical resources, whereas antagonistic relationships between different stress responses result from the plants' need to activate an appropriate response to the type of stress that is encountered.

The phytohormone abscisic acid (ABA) not only regulates plant developmental processes such as seed maturation, dormancy, inhibition of germination, photoregulation, inhibition of lateral root formation, senescence and flowering inhibition, but also has a primary function in response to salt, drought, osmotic and cold stress (Finkelstein et al., 2002; Finkelstein and Rock, 2002). In addition to this well-studied function in the response to abiotic stress, a fast growing number of studies have demonstrated that ABA is also prominently involved in the response to pathogens and is implicated in the integration of different stress response signaling networks. However, to date our knowledge regarding the functions of ABA in response to pathogens is still very fragmentary. ABA was reported to play an ambivalent role in pathogen defense and several putative mechanisms were proposed (reviewed by Mauch-Mani and Mauch, 2005). This paper aims to evaluate and review the reported modes of ABA action on pathogen defense and highlight recent advances in deciphering the complex role of ABA in plant-pathogen interactions.

6.2. Mechanisms involved in the modulation of disease resistance by ABA

Mounting evidence suggests that ABA plays an ambivalent role in defense responses to pathogens, acting both as a positive and negative regulator of disease resistance by interfering at multiple levels with biotic stress signaling. In this context, a wide range of putative mechanisms underpinning the beneficial and detrimental effects of ABA on plant defense have been proposed, including the suppression of SA- and ET/JA-dependent basal defenses, synergistic cross-talk with JA signaling, suppression of ROS generation, induction of stomatal closure, and stimulation of callose deposition. A comprehensive overview of the effects of ABA in various plant-pathogen interactions is provided in Table 6.1 and the proposed modes of ABA action will be discussed in this section. Generally, the outcome of alterations in ABA content or ABA signaling seems independent of the pathogen lifestyle or mode of pathogen attack, as ABA can influence resistance against both necrotrophs and biotrophs positively and negatively. Furthermore, also the plant species can only partly attribute to the diverse effects of ABA on resistance. For example, in tomato ABA is predominantly associated with susceptibility, but in Arabidopsis both negative and positive effects on resistance were reported. Even concluding that the role of ABA is plant-pathogen interaction-specific seems insufficient to explain all of the contradictory results, as within the same interaction several modes of action of ABA with divergent effects on disease resistance might be involved at different stages of infection. For instance, in Arabidopsis, ABA-regulated stomatal closure is a key element of pre-invasion SA-regulated innate immunity to *P. syringae* (Melotto et al., 2006), whereas post-penetration virulence of the same pathogen depends on ABA-mediated suppression of several basal defense responses (Mohr and Cahill, 2003; de Torres-Zabala et al., 2007; Mohr and Cahill, 2007). These results demonstrate that also timing of infection is a crucial element in the regulatory role of ABA on pathogen defense.

6.2.1. Suppression of PAL activity, secondary metabolites and SA accumulation

A possible mechanism that can explain the negative impact of ABA on pathogen defense is the suppression of phenylalanine ammonia lyase (PAL) activity by basal or elevated ABA levels (Ward et al., 1989; McDonald and Cahill, 1999; Audenaert et al., 2002a). PAL is a key enzyme in the early steps of the phenylpropanoid biosynthetic pathway, leading to the production of secondary antimicrobial metabolites, including phytoalexins and phytoanticipins. Incompatible interactions between soybean and *Phytophthora sojae* were marked by a sharp increase in PAL activity within 4 hpi, which was not present in compatible interactions and could be suppressed by exogenous ABA application (McDonald and Cahill, 1999). Reversely, artificial reduction of ABA levels with norflurazon during inoculation with compatible isolates elevated PAL activity and led to the

formation of incompatible lesions (McDonald and Cahill, 1999). It was shown earlier in the same plant-pathosystem that ABA suppresses PAL activity at the transcriptional level (Ward et al., 1989). Furthermore, resistance in this interaction was correlated with the accumulation of the phenylpropanoid-derived compound glyceollin, which could be suppressed by ABA treatment, and was not correlated with lignin deposition nor with the expression of a hypersensitive response (HR) (Mohr and Cahill, 2001). These data show that plant ABA levels can determine the outcome of a plant-pathogen interaction by controlling the accumulation of phytoalexin production through regulation of phenylpropanoid biosynthesis.

Besides leading to the formation of antimicrobial secondary metabolites, the phenylpropanoid pathway is involved in the synthesis of the plant defense hormone SA. In Arabidopsis, gene-for-gene resistance to *Pseudomonas syringae* is SA-dependent and application of exogenous ABA prevents the accumulation of SA and suppresses resistance (Mohr and Cahill, 2007). Transcriptome analysis confirmed the ABA-mediated suppression of genes in the early steps of the phenylpropanoid pathway including PAL and 4-coumarate/CoA ligase (Mohr and Cahill, 2007). In tomato, it was shown that the ABA-deficient *sitiens* mutant is hypersensitive to the SA analogue BTH and displays a hyperinduction of PAL activity after pathogen attack (Audenaert et al., 2002a). Comparison of the transcriptome of *sitiens* and wild-type tomato confirmed transcriptional activation of the phenylpropanoid biosynthetic pathway in *sitiens*, and also showed an increased accumulation of SA-inducible defense-related transcripts such as PR1 both prior to and quickly after inoculation with *B. cinerea* (Asselbergh et al., 2007). A higher basal PR1 mRNA accumulation was also detected in other ABA-deficient tomato mutants (Thaler and Bostock, 2004). In addition, down-regulation of β-1,3-glucanase transcripts, another SA-inducible PR protein (PR2), was detected in tobacco cell cultures treated with ABA (Rezzonico et al., 1998). Together, these results indicate that exogenous ABA application can suppress SA accumulation and SA-inducible defense transcript accumulation, whereas a decrease in endogenous ABA results in constitutive activation and hyperinduction of SA-dependent defenses.

Besides the repression of phenylpropanoid biosynthesis, other mechanisms were suggested for the suppressive effect of ABA on SA-inducible gene expression. Adie et al. (2007) proposed that ABA-SA antagonism could also be explained by an indirect effect based on the ABA-mediated induction of JA biosynthesis (See below). Another conceivable justification for ABA-SA antagonism lies in the positive effect of ABA on callose formation (see below). As callose was shown to block SA-inducible defense responses (Nishimura et al., 2003), the action of ABA on SA-dependent responses could be partly due to enhancement of callose deposition.

Tabel 6.1. ***Effect of ABA on plant-pathogen interactions and proposed mode of action.***

Host plant	Pathogen	Proposed mode of ABA action on defence responses[a]	ABA decrease		inactivation of ABA signaling		ABA increase		reference
			method - effect on resistance[a,b,c]		method - effect on resistance[a,b,d]		method - effect on resistance[a,b,e]		
Arabidopsis	*Pseudomonas syringae*	suppression of basal defence responses	bm: *aao3*	+ +	im: *abi1-1*, *abi2-1*, *35S::HAB1*	+ +	ex	-	de Torres-Zabala et al.,, 2007
Arabidopsis	*Pseudomonas syringae*	suppression of SA responses and lignin accumulation	bm: *aba1-1*	=	im: *abi1-1*	=	ex, ds	- -	Mohr & Cahill, 2003, Mohr & Cahill, 2007
Arabidopsis	*Pseudomonas syringae*	stomatal closure in innate immunity pathway	bm: *aba3-1*	- -	gcsm: *coi1-20*, *ost1-2*		ex		Melotto et al.,, 2006
Arabidopsis	*Alternaria brassicicola*	stimulation of JA biosynthesis	bm: *aao3-2*, *aba2-12*	- -	im: *abi4-1*	- -	ND		Adie et al.,, 2007
Arabidopsis	*Alternaria brassicicola*	priming for callose deposition	bm: *aba1-5*	-	im: *abi4-1*	-	ex	+	Ton & Mauch-Mani, 2004
Arabidopsis	*Plectosphearella cucumerina*	priming for callose deposition	bm: *aba1-5*	=	im: *abi4-1*	=	ex	+	Ton & Mauch-Mani, 2004
Arabidopsis	*Plectosphaerella cucumerina*	ND	bm: *aba1-6*	+ +	im: *abi1-1*, *abi2-1*	+ +	ND		Hernández-Blanco et al.,, 2007
Arabidopsis	*Sclerotinia sclerotiorum*	stomatal closure	bm: *aba2-1*	- -	im: *abi1-1*(-), *abi2-1*(=), *abi3-1*(-)	-/=	ND		Guimarães & Stotz, 2004
Arabidopsis	*Ralstonia solanacearum*	signaling in irx-mediated resistance (leading to antimicrobial compounds)	bm: *aba1-6*	- -	im: *abi1-1*, *abi2-1*	- -	ND		Hernández-Blanco et al.,, 2007
Arabidopsis	*Leptosphaeria maculans*	signaling in RLM pathway leading to callose-dependent and -independent resistance	bm: *aba1-3, aba2-1*, *aba3-1*	-	im: *abi1-1*(-), *abi4-1*(- -), *abi2-1*(=), *abi3-1*(-), *abi5-1*(=)	-/- -/=	ex	=	Kaliff et al.,, 2007
Arabidopsis	*Fusarium oxysporum*	suppression of JA/ET responses	bm: *aba2-1*	+	ND		ND		Anderson et al.,, 2004
Arabidopsis	*Hyaloperonospora parasitica*	ND	bm: *aba1-1*	+ +	im: *abi1-1*	=	ex, ds	=	Mohr & Cahill, 2003
Arabidopsis	*Pythium irregulare*	stimulation of JA biosynthesis	bm: *aao3-2*, *aba2-12*	- -	im: *abi4-1*	- -	ND		Adie et al.,, 2007
Arabidopsis	*Botrytis cinerea*	ND	bm: *aao3-2*, *aba2-12*	+ +	im: *abi4-1*	+	ND		Adie et al.,, 2007

Tabel 6.1. ***Continued***

Tomato	*Botrytis cinerea*	suppression of SA responses, PAL activity, hydrogen peroxide accumulation and cell wall fortification	bm: *sitiens, notabilis, flacca;* bi: fluridone	+ +	ND	ex, ss, ds	-	Audenaert et al.,, 2002; Asselbergh et al.,, 2007; Achuo et al.,, 2006
Tomato	*Erwinia chrysanthemi*	suppression of ROS accumulation and cell wall fortification	bm: *sitiens*	+ +	ND	ex	- -	Asselbergh et al.,, 2008
Tomato	*Oidum neolycopersici*	ND	bm: *sitiens*	+	ND	ex	=	Achuo et al.,, 2006
Tomato	*Pseudomonas syringae*	suppression of SA responses	bm: *sitiens, flacca*	+	ND	ss	-	Thaler & Bostock, 2004
Tomato	*Sclerotinia sclerotiorum*	ND	bm: *sitiens*	+ +	ND	ND		Asselbergh and Höfte, unpublished results
Tobacco	*Peronospora tabacina*	ND	ND		ND	ex	- -	Salt et al.,, 1986
Tobacco	*Ralstonia solanacearum*	ND	ND		ND	ex	-	Steadman & Sequira, 1970
Tobacco	Tobacco Mosaic Virus	stimulation of callose deposition	ND		ND	ex	+	Whenham et al.,, 1986; Balazs et al.,, 1973; Rezzonico et al.,, 1998
Barley	*Erysiphe graminis* f. sp. *hordei*	ND	ND		ND	ex	- -	Edwards, 1983
Barley	*Blumeria graminis f. sp. hordei*	ND	ND		ND	ex, os, ps	+	Wiese et al.,, 2004
Potato	*Phytophthora infestans*	unknown	ND		ND	ex	- -	Henfling et al.,, 1980
Potato	*Cladosporium cucumerinum*	ND	ND		ND	ex	- -	Henfling et al.,, 1980
Rice	*Magnaporthe grisea*	suppression of 'whole plant-specific resistance'	bi: fluridone	+	ND	ex, cs	-	Koga et al.,, 2004
Rice	*Bipolaris oryzae*	priming for MPK5-mediated repression of ethylene signaling	bi: fluridone	=	ND	ex	+	De Vleesschauwer and Höfte, unpublished results
Bean	*Colletotrichum lindemuthianum*	ND	bi: fluridone	-	ND	ex	+	Dunn et al.,, 1990
Lily	*Botrytis elliptica*	stomatal closure in probenazol-induced resistance	ND		ND	ex	+	Lu et al.,, 2007
Wheat	*Erysiphe graminis* f. sp. *tritici*	ND	ND		ND	ND		Nikitina & Talieva, 2001
Soybean	*Phytophthora sojae*	suppression of PAL activity, glyceollin accumulation	bi: norflurazon	+ +	ND	ex	- -	Ward et al.,, 1989; McDonald & Cahill, 1999; Mohr & cahill, 2001

[a]: ND: not determined; [b]: =: no significant effect; +: moderate positive effects; + +: relatively strong positive effects; -: moderate negative effects; - -: relatively strong negative effects on disease resistance, based on the authors' evaluation of disease indexes in each report; [c]: bm: biosynthesis mutants; bi: biosynthesis inhibition; [d]: im: insensitive mutants; gcsm: guard cell signaling mutants; [e]: ex: exogenous application; ds: drought stress; cs: cold stress; ss: salt stress; os: osmotic stress; ps: proton stress.

6.2.2. Suppression of ROS accumulation

The importance of reactive oxygen species (ROS) accumulation during pathogen defense is well documented (Lamb and Dixon, 1997; Wojtaszek, 1997; Apel and Hirt, 2004; Torres and Dangl, 2005). Analysis of the resistance mechanisms of the ABA-deficient *sitiens* tomato mutant revealed the importance of rapid and extensive hydrogen peroxide accumulation in arresting the necrotrophic pathogens *B. cinerea* and *E. chrysanthemi* (Asselbergh et al., 2007; Asselbergh et al., 2008). Extracellular hydrogen peroxide accumulation and activation of peroxidases in *sitiens* caused rapid cell wall modification upon pathogen inoculation by protein cross-linking and incorporation of phenolic compound, causing the arrest of pathogen progression. Pathogen susceptibility could be restored by application of exogenous ABA or by pharmacologic disruption or removal of hydrogen peroxide accumulation (Asselbergh et al., 2007). Extensive ROS accumulation and increases in peroxidase activity as a result of ABA-deficiency is consistent with the hyperinduction of SA-inducible defenses in ABA mutants (Asselbergh et al., 2007, Audenaert et al., 2002a). Many studies report on the relationship between ROS and SA in biotic stress responses. It is believed that ROS and SA work together in a self-amplifying system in establishing systemic acquired resistance (Alvarez et al., 1998; Van Camp et al., 1998; Van Breusegem et al., 2001; Durrant and Dong, 2004). This model is corroborated by ABA deficiency-triggered potentiation of both ROS- and SA-mediated defense. On the other hand, ROS are important messengers in ABA-mediated stress responses. ROS are key signals in regulating stress-adaptive ABA responses (Pastori and Foyer, 2002) and ABA-signaling in guard cells requires ROS formation to interact with Ca-channels to induce stomatal closure (Kwak et al., 2006; Li et al., 2006b). Interestingly, guard cell ABA activates ROS-generating NADPH oxidases (Kwak et al., 2006), which are also necessary for ROS-production during pathogen defense (Torres and Dangl, 2005). Furthermore, ROS-production resulting in stomatal closure could be induced by application of plant cell wall degradation products (oligogalacturonides) (Lee et al., 1999) and it was shown that hydrogen peroxide-dependent defense responses in *sitiens* are most likely also elicited by oligogalacturonides (Asselbergh et al., 2008a). Together, it seems that at least some components of ROS-generation and ROS-signaling activation are common for ABA-responsive abiotic stress signaling in guard cells and for hyperactivation of pathogen defense in ABA-deficient plants. The mechanism by which ABA-deficiency in *sitiens* leads to rapid extensive ROS formation upon pathogen attack is at present conjectural.

6.2.3. ABA effect on JA/ET-responsive defenses

The antagonistic effect of ABA on JA/ET pathogen defense signaling was proposed as an alternative mechanism for ABA to negatively influence pathogen defense (Andersen et al., 2004; Mauch-Mani and Mauch, 2005). By using *PDF1.2*, *CHI* and *HEL* transcript accumulation as markers for JA/ET responsive gene expression in Arabidopsis, it was shown that both basal and JA/ET-induced defense gene expression was suppressed by exogenous ABA and was upregulated in ABA-deficient *aba1* and *aba2* mutants, the latter resulting in increased resistance to *Fusarium oxysporum* (Andersen et al., 2004).

During wound and pathogen stress, positive and negative interactions between JA and ET signaling pathways are essential for the establishment of suitable plant defense responses. During pathogen attack, ET and JA cooperate through transcriptional induction of ethylene response factor 1 (ERF1), which results in activation of pathogen response genes such as *PDF1.2*, *CHI* and *HEL*. In response to wounding, JA activates the transcription factor AtMYC2, leading to wound stress-specific gene activation (such as *VSP* and *lox*). Repression of pathogen response genes by AtMYC2 and repression of wound response genes by ERF1 constitute important points of cross-talk between the two signaling pathways (Lorenzo and Solano, 2005). AtMYC2 expression was shown to be activated by ABA (Lorenzo et al., 2004) and it was suggested that ABA precedes JA in the activation of AtMYC2-mediated wound responses (Lorenzo and Solano, 2005). Therefore, AtMYC2 functions as a mediator of ABA to repress JA/ET-induced pathogen response (Andersen et al., 2004). However, the suppression of JA/ET-induced pathogen response genes by ABA can not be solely attributed to AtMYC2, as a suppressive effect remained in an *Atmyc2*-negative mutant background (Andersen et al., 2004). Antagonistic interactions between ET and ABA signaling form an alternative mechanism of ABA to repress the JA/ET-induced pathogen response. For example, it was shown that ET treatment quickly induces activation of ABI1 and ABI2, two negative regulators of ABA signaling (De Paepe et al., 2004). Furthermore, by using ABA and ET signaling mutants, it was shown that the relationship between the two phytohormones is mutually antagonistic in vegetative tissues (Andersen et al., 2004).

Recent evidence suggests that the antagonistic ABA-ET crosstalk might be modulated by the ethylene-responsive element binding factor AtERF4 (Yang et al., 2005). AtERF4 is a transcriptional repressor whose expression is induced by ABA, ET, or JA exogenous treatment, while its overexpression leads to the inhibition of GCC box-containing defense genes, ethylene insensitivity, and decreased ABA sensitivity. In rice, exogenous ABA treatment has been shown to decrease endogenous ET levels, thereby increasing host susceptibility to *Magnaporthe oryzae* (Yang 2007; Chapter 7). Elegant research by Yang's group revealed that the suppressive effect of ABA on the

ET signaling pathway is mediated by the *OsMPK5* gene. *OsMPK5* encodes an ABA-inducible mitogen-activated protein kinase that positively regulates endogenous ABA content and abiotic stress tolerance while repressing endogenous ET levels, pathogenesis-related gene expression and resistance to *Magnaporthe oryzae* (Xiong and Yang 2003; Yang 2007).

Although antagonistic interactions between JA and ABA have been reported (Moons et al., 1997; Lorenzo and Solano, 2005), these two hormones often act as positive regulators in the same signaling pathway. In Arabidopsis guard cells both JA and ABA induce stomatal closure by activation of identical secondary messengers, such as ROS, NO, Ca^{2+} permeable cation channels and S-type anion channels (Munemasa et al., 2007). Recently, Adie et al., (2007) demonstrated that ABA is an essential signal leading to JA biosynthesis with resultant activation of defense responses against the damping-off oomycete *Pythium irregulare*. Defense signaling against this pathogen relies partly on ET and SA, but is predominantly mediated by JA, with JA-insensitive *coi1* mutants showing extreme susceptibility. Transcriptome analysis of wild type, ET-, SA- and JA-related mutants (Col0, *ein2-5*, *sid2-1* and *coi1-1* respectively) after infection with *P. irregulare* allowed the division in JA/ET/SA-dependent and JA/ET/SA-independent genes induced by *P. irregulare*. *P. irregulare*-induced JA/ET/SA-dependent genes were dominated by JA-responsive genes. Meta-analysis confirmed the dependence on JA-responsive genes as well as revealed high similarity of the *P. irregulare*-induced transcriptome with the response to ABA. Promoter analysis of the JA/ET/SA-dependent *P. irregulare*-induced genes also revealed an overrepresentation of ABA-response elements. Furthermore, the *P. irregulare*-induced transcriptome independent of JA/ET/SA clustered together with the profiles of responses to ABA and abiotic stresses. ABA-deficient (*aba2-12*) and ABA-insensitive (*abi4-1*) mutants showed impaired JA biosynthesis and increased susceptibility upon *P. irregulare* infection (Adie et al., 2007). Together, these results elegantly show the requirement for ABA signaling to activate JA-dependent resistance to *P. irregulare*. Interestingly, this study also confirmed the down-regulation by ABA of a group of JA/ET-responsive genes, such as *PDF1-2*, *HEL* and *b-CHI*, which confirmed earlier findings (Anderson et al., 2004). However, the transcriptomic view showed that the major effect of ABA is the opposite, activating many ABA-specific and ABA/JA-related defense genes (Adie et al., 2007).

Collectively, it appears that the cross-talk of ABA with JA and ET signaling pathways occurs at multiple convergence points with opposite effects on disease resistance. On the one hand, ABA acts negatively on the specific set of pathogen response genes that are controlled synergistically by ET and JA, leading to enhanced disease susceptibility. On the other hand, ABA positively affects JA biosynthesis in the activation of defense responses against the oomycete *P. irregulare*.

6.2.4. Stomatal closure

When effects of ABA on disease resistance are evaluated, special care should be taken to discriminate between direct and indirect effects on pathogen defense, especially during interactions with root rot and/or wilting pathogens. Since these types of pathogens impinge plant water balances or fluxes and cause severe dehydration stress, ABA-induced abiotic stress responses to enhance dehydration stress tolerance will be activated and can thereby reduce disease symptoms. ABA-induced stomatal closure to limit evaporation water loss and to counteract wilting symptoms is a nice example of an indirect positive effect of ABA on pathogen defenses, because it is principally an abiotic stress response and not a response to biotic stress.

In addition to these indirect effects, a biologically very relevant direct positive effect of ABA signaling on pathogen defense is by closing stomata to prevent pathogen invasion. It was recently shown that stomatal closure is integral to pre-invasion pathogen-associated molecular pattern (PAMP)-induced innate immunity to bacteria (Melotto et al., 2006; Underwood et al., 2007). Stomata close upon recognition of plant pathogens, human pathogens (plant non-pathogens) and isolated PAMP molecules, a process that requires ABA signaling in guard cells and ABA biosynthesis (Melotto et al., 2006). Moreover, it was elegantly demonstrated that *Pseudomonas syringae* pv. *tomato* needed the virulence factor coronatine in order to enter internal leaf tissue by inhibiting ABA-induced stomatal closure. Coronatine, a JA-mimic, counteracts PAMP-induced stomatal closure downstream of ABA, but requires functional COI1 signaling. Interestingly, PAMP-induced stomatal closure was compromised in SA-deficient transgenic *nahG* plants and SA-biosynthetic mutant *eds16-2* plants, indicating that defense trough stomatal closure is an integral part of the SA-regulated innate immune system (Melotto et al., 2006). These results show that counteracting ABA-dependent signaling in guard cells is a pathogenic strategy to overcome pre-invasion SA-regulated innate immunity. This is in sharp contrast to the up-regulation of ABA signaling and ABA biosynthesis needed for post-penetration virulence (de Torres-Zabala et al., 2007) and the repression of SA accumulation and SA-dependent defense gene expression by ABA during infection (Mohr and Cahill, 2007), both in the same plant-pathosystem. Interestingly, stomatal defense and bacterial suppression of stomatal defense seem common phenomena in plant-bacterium interactions, as PAMPs also induce stomatal closure in tomato, which could also be modulated by *Pseudomonas syringae* pv. *tomato* (Melotto et al., 2006). Considering that in natural environments, bacterial and many fungal pathogens rely entirely on accidental wounds or natural plant openings such as stomata to enter internal plant tissues, the impact of stomatal defense on plant-pathogen interactions in nature can hardly be overestimated. It remains to be elucidated whether PAMP-induced ABA-signaling is limited to guard cells, or if ABA-induced signaling early

upon pathogen recognition also occurs in other plant cell types.

In addition, it was shown earlier that the fungal toxin fusicoccin promotes stomatal opening and antagonizes ABA-induced stomatal closure (Marré, 1979). Also the fungal necrotrophic pathogen *Sclerotinia sclerotiorum* uses the virulence factor oxalate to prevent ABA-induced stomatal closure during infection (Guimarães and Stotz, 2004). Increased wilting and facilitation of hyphal emergence and secondary colonization were proposed to result from the prevention of stomatal closure. The mechanism by which oxalate suppresses ABA-induced stomatal closure remains unknown (Guimarães and Stotz, 2004), and the elucidation of this mechanism will be further complicated by the multiple functions of oxalate in necrotrophic virulence (Van Kan, 2006). Nevertheless, ABA-induced stomatal closure is undoubtedly an important plant defense strategy towards pathogens.

6.2.5. Stimulation of callose deposition

Another positive effect of ABA on pathogen defense is by its ability to stimulate callose deposition. Callose is a β-1,3-glucan that is deposited in cell wall appositions (papillae) that can block pathogen entry (Aist, 1976). It was reported that both ABA signaling and callose formation are prerequisites for β-amino butyric acid (BABA)-triggered induced resistance to *Plectosphaerella cucumerina* and *Alternaria brassicicola* in Arabidopsis (Ton and Mauch-Mani, 2004). Treatment with exogenous ABA could mimic the effect of BABA and resulted in priming for callose and resistance to *P. cucumerina*. In addition, Arabidopsis resistance to *Leptosphaeria maculans* through the $RLM1_{col}$ pathway and to *Pythium irregulare* was shown to be partly mediated by ABA-dependent callose formation, next to callose-independent ABA-dependent resistance mechanisms (Kaliff et al., 2007; Adie et al., 2007). ABA treatment in barley also caused papillae-mediated resistance against *Blumeria graminis* f. sp. *hordei* (Wiese et al., 2004). In the interaction of tomato with *B. cinerea*, callose deposition was low in the ABA-deficient *sitiens* mutant and was not important for its resistant response. However, ABA-dependent callose formation was involved in basal defense of wild-type tomato (Asselbergh and Höfte, 2008). It was hypothesized that basal ABA levels in tomato are sufficiently high to create a primed state for callose deposition, while in Arabidopsis, which contains about 20-fold less ABA than tomato, exogenous ABA (or BABA) is needed to provoke priming for callose deposition (Asselbergh and Höfte, 2008). Furthermore, it seems that compared to the levels of tomato resistance caused by strong SA-dependent responses (resulting from ABA-deficiency) and to the strong effects of ABA-induced priming for callose in Arabidopsis, ABA-dependent callose formation only marginally influences resistance in tomato (Asselbergh and Höfte, 2008).

In contrast to these reports, a negative effect of ABA on callose deposition was shown in Arabidopsis challenged with *Pseudomonas syringae* pv. *tomato* (de Torres-Zabala et al., 2007). ABA-hypersensitivity (in *abi1-sup7* and *abi1-sup5* mutants) and exogenous ABA treatment strongly reduced callose deposition, while ABA-insensitive mutants (*abi1-1* and *abi2-1*) show augmented callose deposition (de Torres-Zabala et al., 2007).

It is noteworthy that in some cases ABA does not directly regulate callose deposition upon pathogen attack, but rather modulates the priming of its deposition (after BABA treatment) (Flors et al., 2005). Also, the fact that BABA treatment enhances the capacity to resist abiotic stress (Ton et al., 2005), indicates that priming for callose deposition is mediated by ABA-responsive signaling components that are common for biotic and abiotic stress responses. This view is supported by a recent study, which shows that salt stress and BABA act synergistically in tomato to induce resistance to *Pseudomonas syringae* pv. *tomato* (Baysal et al., 2007).

The molecular mechanisms behind the modulation of callose by ABA remain to be elucidated. It was suggested that ABA could control callose deposition by regulating vesicle-mediated transport of callose synthase proteins. Transcriptional activation by ABA of specific N-ethyl-malmeimide-sensitive fusion protein attachment protein receptors (SNAREs) was speculated to direct callose synthase proteins to the site of pathogen attack (Flors et al., 2005). Alternatively, it was proposed earlier that ABA down-regulates β-1,3-glucanases, which use callose as a substrate (Rezzonico et al., 1998).

6.3. ABA mediates global shifts in plant stress response priority

6.3.1. ABA negatively regulates disease phenotypes in a forceful manner

Although ABA can affect disease resistance both positively and negatively, ABA seems to act as a negative regulator of defense in most plant-pathogen interactions that were studied (Table 6.1). One common trend that is observed among the different interactions in which ABA negatively influences disease resistance, are the relatively strong effects of ABA on disease phenotypes. For example, ABA-pre-treatment of potato slices altered the interaction with an incompatible isolate of *Phytophthora infestans* to obtain disease symptoms indistinguishable from a compatible interaction. Furthermore, the same ABA treatment allowed development of *Cladosporium cucumerinum*, normally a non-pathogen of potato (Henfling et al., 1980). In tomato, occurrence of maceration caused by *Erwinia chrysanthemi* was strongly reduced in the ABA-deficient *sitiens* mutant and spreading maceration symptoms were completely absent. This drastic reduction of disease symptoms is

remarkable, considering that sources of resistance to this broad-spectrum pathogen are rare (Asselbergh et al., 2008a). Fast and extensive extracellular hydrogen peroxide accumulation in *sitiens* was shown to be essential in establishing resistance to both *E. chrysanthemi* and *B. cinerea* (Asselbergh et al., 2007; Asselbergh et al., 2008a). Interestingly, production of ROS is normally not effective against necrotrophic pathogens such as *B. cinerea*, or can even facilitate necrotrophic tissue colonisation (Govrin and Levine, 2000). The effective arrest of *B. cinerea* by a timely hyperinduction of hydrogen peroxide-dependent defenses in *sitiens* illustrates the strong effect of ABA-deficiency on the defensive capacity towards pathogens (Asselbergh et al., 2007). Analysis of *sitiens* defense activation after inoculation with *E. chrysanthemi* pathogenicity mutant strains and with *E. chrysanthemi* culture filtrate, demonstrated that defenses are activated by *E. chrysanthemi* type II secreted proteins, which mainly consist of pectinases (Asselbergh et al., 2008a). *E. chrysanthemi* pectinolytic cell wall degradation causes the release of plant cell wall oligogalacturonides, which are known and potent endogenous elicitors of plant pathogen defenses (Ridley et al., 2001). Defense activation by endogenous elicitors that are pathogen non-specific is consistent with the broad spectrum of pathogens that is unsuccessful in efficiently infecting *sitiens* plants (Asselbergh et al., 2008a). These observations indicate that ABA has the capacity to negatively affect a broad range of plant pathogen interactions in an extreme and forceful manner.

6.3.2. ABA acts as a virulence factor of plant pathogens.

The potency of ABA to suppress pathogen defense responses is exemplified by the exploitation of ABA as a virulence factor by plant pathogens. A recent study elegantly demonstrated that *Pseudomonas syringae* type III-secreted effectors (T3SE) target the Arabidopsis ABA signaling pathway to cause disease (de Torres-Zabala et al., 2007). Exogenous ABA decreases resistance in this interaction, and in addition, ABA insensitivity (in *abi1.1* and *abi2.1* mutants) or hypersensitivity (in *abi1.sup7* and *abi2.sup5* mutants) led to restriction or enhanced bacterial multiplication, respectively. Comparison of the Arabidopsis transcriptome after infection with wild-type and T3SE-negative *P. syringae* mutants revealed the overrepresentation of ABA-dependent gene expression in response to T3SE. The induction of ABA signaling by T3SE was represented by the upregulation of known ABA responsive genes, by the presence of ABA-responsive elements (ABRE) in the promoter regions of T3SE-induced genes and by the similarity between the transcriptomic profiles after T3SE-induction and ABA treatment. The upregulation of the ABA biosynthetic gene *NCED3* revealed the stimulation of ABA biosynthesis by T3SE, which was confirmed by ABA measurements. Finally, transgenic expression of the conserved *P. syringae* effector AvrPtoB induced *NCED3* expression and elevated ABA levels *in planta* (de Torres-Zabala

et al., 2007). These findings demonstrate that bacterial effector-mediated elevation of plant ABA biosynthesis and signaling is a major virulence strategy, which leads to the suppression of defense responses.

In addition to regulating plant ABA biosynthesis, micro-organisms are known to synthesize ABA themselves. ABA is produced by different types of fungi, including ascomycetes, basidiomycetes and zygomycetes (Crocoll et al., 1991, Dörffling et al., 1984). To our knowledge, all fungal ABA-producing strains isolated so far are associated with plants. ABA biosynthesis in fungi differs from plant biosynthesis and is mediated through a direct pathway via farnesyl diphosphate (Hirai et al., 2000). The fungal ABA biosynthetic pathway is biochemically best characterized in *Cercospora* species (Oritani and Kiyota, 2003) and in *B. cinerea* (Siewers et al., 2006). Recently, it was found that ABA biosynthetic genes in *B. cinerea* are organized in a gene cluster consisting of at least four co-regulated genes (Siewers et al., 2006). Kettner and Dörffling (1995) demonstrated earlier that elevated tomato ABA levels during *B. cinerea* tissue colonization resulted from fungal stimulation of plant ABA biosynthesis and fungal inhibition of plant ABA catabolism, as well as from production of ABA and its precursor by the fungus. These findings, together with the capacity of phytopathogenic fungi from taxonomically unrelated groups to produce ABA, indicate that elevation of host ABA levels can function as a general pathogenic strategy to suppress host defenses. Assessment of the pathogenicity of ABA- and ABA precursor-negative fungal mutants, such as those described by Siewers et al., (2006), could further elucidate the function of ABA in pathogen virulence.

Relevant information regarding a suppressive role of ABA on plant defense can possibly also be derived from the interactions of plant roots with arbuscular mycorrhizal (AM) fungi and nitrogen-fixating bacteria, as in these symbiotic interactions plant defense responses are suppressed (García-Garrido and Ocampo, 2002) and alterations in plant hormone homeostasis were reported, including increases in ABA content (Esch et al., 1994; Meixner et al., 2005). A direct link between ABA and successful AM colonization was recently provided in the tomato – *Glomus intraradices* interaction (Herrera-Medina et al., 2007). Colonization of the ABA-deficient *sitiens* mutant was less frequent and arbuscule development was incomplete. Reversely, application of exogenous ABA increased AM colonization in wild-type and mutant plants. The authors suggested that impairment of AM development in ABA-deficient mutants was at least partly attributable to the antagonistic interaction of ABA with ET (Herrera-Medina et al., 2007).

6.3.3. ABA levels mediate a global shift in the priority of response to biotic and abiotic stress.

When responding to different stresses, integration and concordance of stress signaling networks is essential for an adequate response of appropriate amplitude and different types of stress require distinct and specific responses. The need to prioritize specific stress responses coupled to simultaneous down-regulation of others, justifies the antagonistic interplay commonly observed between different stress signaling networks. In nature, the co-occurrence of drought stress and pathogen attack is rare, as the great majority of pathogens require relatively humid conditions for infection and the establishment of disease (Agrios, 2005). Furthermore, drought or dehydration stress forms a much greater threat to plant survival than pathogen infection, which is consistent with the plant's need to be able to quickly prioritize drought stress responses at the expense of growth and the responses to other stresses. ABA-responsive signaling functions as a global switch to activate the drought stress response and represses many other plant processes, among those the response to pathogens. This is consistent with ABA repressing both JA/ET-controlled and SA-controlled pathogen defense and increasing susceptibility to both necrotrophic and biotrophic pathogens. The dominant nature of ABA action was also confirmed by Anderson et al. (2004) who showed that JA/ET-dependent defense gene suppression by ABA cannot be reversed by JA or ET application. The strong antagonistic effect between abiotic and pathogen responses is also exemplified in the ABA-deficient *sitiens* tomato mutant. When grown under conditions of high relative humidity, ABA deficiency does not result in major morphological abnormalities. Nevertheless, ABA-deficient plants are unable to cope with drought or cold stress due to the lack of ABA-mediated stomatal regulation (Nagel et al., 1994). However, the ability of ABA-deficient tomato to block the necrotrophic pathogens *B. cinerea* and *Erwinia chrysanthemi* reveals its enormous defensive capacity towards biotic stress (Asselbergh et al., 2007; Asselbergh et al., 2008a). This was also reflected at the transcriptome level, as *sitiens* exhibits higher expression of defense-related genes prior to infection and shows a further elevation quickly after *B. cinerea* inoculation (Asselbergh et al., 2007). This demonstrates that deficiency in ABA results in a global shift towards strong pathogen defense responses at the expense of reduced tolerance to abiotic stress. Taken together with the strong negative effects of ABA on disease phenotypes and the function of ABA as a virulence factor against the central role of ABA in abiotic stress responses, it seems that in general strong decreases in ABA levels lead to hyperactivation of pathogen defense together with a reduced capacity to react to abiotic stress, whereas elevation of ABA levels leads to enhanced abiotic stress responses and suppression of pathogen defense responses.

6.4. ABA integrates and fine-tunes different stress responses

6.4.1. Overlap between abiotic and biotic stress responses

In addition to the apparent role of ABA levels in mediating a global shift between abiotic and biotic stress responses, ABA-responsive signaling seems to interfere at multiple steps in various signal transduction cascades, leading to fine-tuning and integration of different stress responses. Economical use of biochemical resources implies a significant amount of overlap between the responses to different stresses and explains the use of common signaling components in the response to both biotic and abiotic stresses. Furthermore, biotic and abiotic stress responses can even show considerable overlap at the level of signal perception. For example, root rot or wilting pathogens can cause dehydration stress and thereby trigger an abiotic stress response. As a result, the influence of ABA on disease signaling is extremely divergent and disease resistance can be positively or negatively affected by ABA. An overview of ABA action on pathogen defense responses described in this paper is given in Fig. 6.1. Additional points of convergence between the signaling responses to abiotic and biotic stress have been characterized (reviewed by Fujita et al., 2006; Mauch-Mani and Mauch, 2005). However it seems that only a tiny portion of the total overlap of abiotic and biotic stress signaling networks has been described. Several signaling mechanisms of high complexity are shared between ABA abiotic stress signaling and pathogen defense and constitute means of overlap between different pathways, including Ca^{2+} and Ca-dependent protein kinase signaling (Klüsener et al., 2002; Ludwig et al., 2004), ROS- and nitric oxide-signaling (Pastori and Foyer, 2002; Apel and Hirt, 2004), mitogen-activated protein kinase (MAPK) signaling cascades (Xiong and Yang, 2003; Fujita et al., 2006) and various transcription factor families, containing functional domains such as AP2, WRKY, bZIP/HD-ZIP, MYB, MYC and several classes of zinc-fingers (Chen et al., 2002; Li et al., 2004; Zhu et al., 2005; Anderson et al., 2004; Mengiste et al., 2003). The number of reports that functionally characterize transcription factors and signaling components involved in both biotic and abiotic stresses is growing fast and originates from studies on various plant species. For example, the pepper C3-H-C4 type RING-finger protein CaRFP1 functions as an early defense regulator controlling disease susceptibility and osmotic stress tolerance, probably by influencing SA and ABA signaling, respectively (Hong et al., 2007). Also the barley ERF-type transcription factor HvRAF enhances pathogen resistance and salt tolerance (Jung et al., 2007). Ectopic expression of HvRAF in Arabidopsis confers its conserved function. Similarly, ectopic expression of the rice Osmyb4 upstream transcription factor in Arabidopsis was shown to have a positive effect on the responses to abiotic (cold, drought, salt), environmental (ozone, UV) and biotic (TNV, *B. cinerea*, *P. syringae*) stresses (Vannini et al., 2006).

However, overexpression of the same gene in tomato only improved tolerance to drought stress and virus infection, while other stress responses were not improved (Vannini et al., 2007), demonstrating that the conservation of stress response machinery in dicotyledonous plants is only partial. Further unraveling of the components regulating the signaling events between different stress stimuli and their resulting defense measures will contribute to understanding the integration of overlapping stress signaling networks and the complex role of ABA herein. Even greater challenges will presumably lie in combining information from different plant species on partially conserved stress response signaling networks and translating this knowledge into applied agricultural benefits.

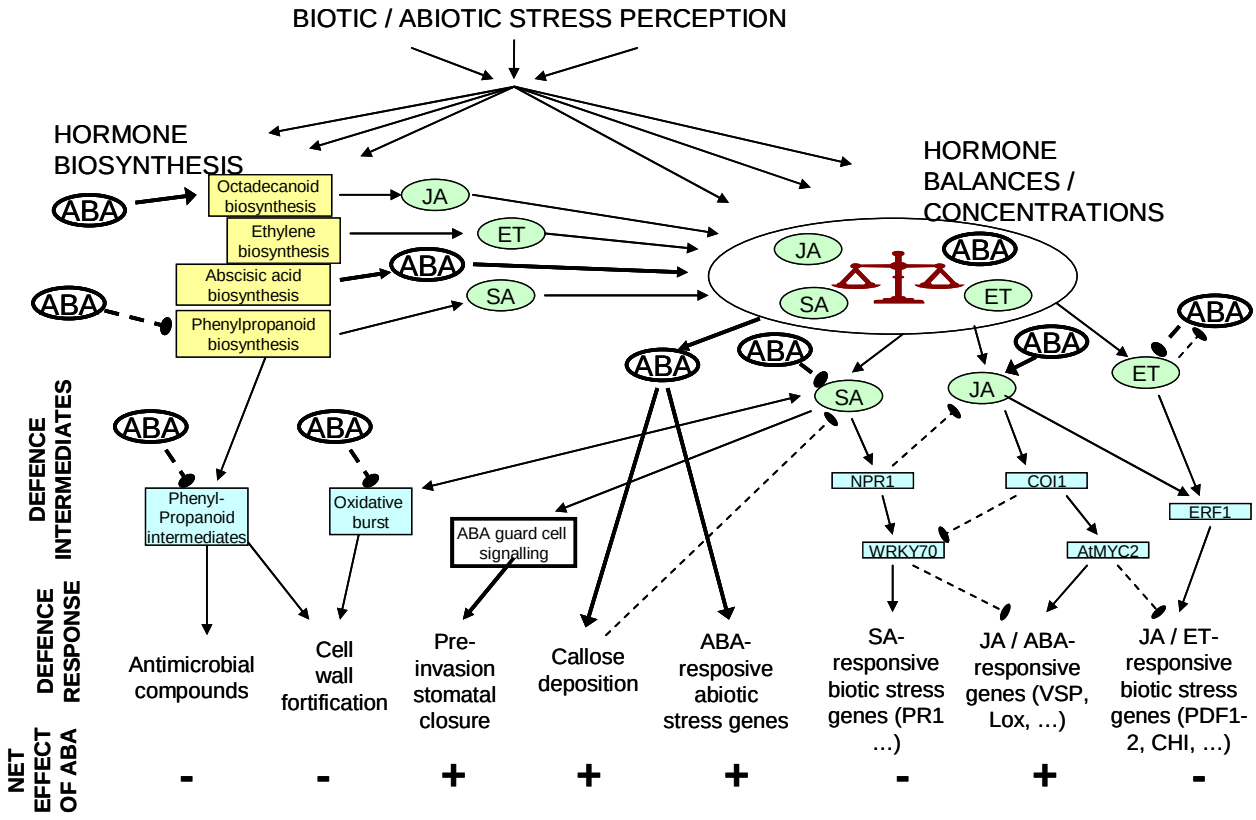

Figure 6.1. Schematic representation of ABA interfering with plant abiotic and biotic stress responses.

Emphasis is given to ABA influencing pathogen defense responses via different modes of action rather than to the functions of ABA signaling in abiotic stress responses. The representation of JA/ET/SA crosstalk involving the effectors AtMYC2, NPR1, COI1, ERF1, and WRKY70 was partly based on a model by Lorenzo and Solano (2005). Sharp full-line arrows represent stimulatory effects and blunt dotted line arrows represent repressive effects. Effects of ABA are marked in bold.

6.4.2. Complexity of ABA-mediated responses

The process of establishing and interpreting possible functions of ABA in plants is hindered by the complexity of ABA-mediated responses. ABA-dependent responses are regulated by controlling *de novo* ABA synthesis. The first steps of ABA biosynthesis occur in chloroplasts where cleavage of caretenoids by nine-cis epoxycaretenoid dioxygenases (NCEDs) is the rate-limiting step and transcriptional regulation of the NCEDs is the major control point of ABA biosynthesis. In addition, the level of ABA in plants is not only controlled by its synthesis, but also through its catabolism (Schwartz et al., 2003). However, ABA-regulated processes constitute more than a simple response to *in planta* bulk ABA concentrations (Wilkinson and Davies, 2002). During drought stress, instant stomatal closure is essential for the plants' survival and is mediated by ABA-perception at the guard cells. Whereas severe drought stress that causes water deficit in the shoot is followed by a drastic increase in levels of intracellular leaf ABA, drought stress that is only perceived at the roots specifically increases apoplastic ABA in guard cells without influencing symplastic leaf ABA contents or leaf bulk apoplastic ABA (Wilkinson and Davies, 2002). This implicates not only the existence of ABA perception sites at different locations and the co-regulation of chemical and hydraulic signals, but also implies a whole-plant modulation of the ABA-signal, including differential xylem loading in the roots, ABA sequestration into a symplastic leave reservoir and alteration of guard cell sensitivity to ABA (Wilkinson and Davies, 2002). In addition, drought stress-induced ABA can stimulate primary root elongation while shoot growth decreases, leading to increased water absorption and reduced water loss, respectively (Sharp, 2002). These and other findings indicate that, even during a single stress response, different signaling mechanisms are required that can be stage, organ or cell specific.

One mechanism that can help to explain the diversity in ABA responses is the existence of different ABA receptors. Indeed, over the last few years, different research groups have characterized three proteins that each fulfill the biochemical requirements of an ABA receptor (stereospecific and saturable high affinity binding to one binding site). The Arabidopsis nuclear protein FCA (for flowering control protein A) is an RNA-binding protein and is required for ABA-signaling in controlling flowering and lateral root formation, but not in seed germination or the stomatal response (Razem et al., 2006). The Arabidopsis protein ABAR/CHLH (putative ABA receptor/Mg-chelatase H subunit) specifically binds ABA in chloroplasts, functions at the whole plant level and controls seed germination and stomatal movement (Shen et al., 2006). Finally, ABA perception at the cell surface was reported to be mediated by a G-protein coupled receptor (Liu et al., 2007a), but its function in ABA responses still needs to be unambiguously demonstrated (Gao et al., 2007).

Downstream of ABA perception, the components of the complex signaling network include RNA-binding proteins (Hugouvieux et al., 2001), protein kinases (Osakabe et al., 2005), protein phosphatases (Leung et al., 1997) and multiple-type transcription factors (Finkelstein and Rock, 2002). In addition, a recent transcriptomic analysis that used ABA structural analogues to detect genes that are weakly induced by ABA estimated that about 14% of Arabidopsis genes are ABA-regulated (Huang et al., 2007). Furthermore, comparison of the ABA-regulated genes that were identified in other studies (Hoth et al., 2002; Seki et al., 2001; Leonardt et al., 2004) suggests that the full potential of ABA-responsive gene regulation has not yet been identified (Huang et al., 2007). Taken together, our understanding of ABA-perception, ABA-signaling networks and whole plant ABA-mediated plant responses is still very fragmentary and incomplete. Further elucidation of the particularly complex mechanisms of responses to ABA will help to clarify many important plant processes in which ABA is involved, including the response to pathogens.

6.5. Conclusions

The modulation of disease resistance by ABA is a particularly complex phenomenon and our knowledge on the diverse regulatory effects of ABA on defense responses currently fails to provide us with straightforward interpretations or clear-cut models on how ABA affects disease resistance. ABA seems to have divergent effects on defence responses, and the outcome on disease resistance seems to be plant-pathogen interaction-specific, rather than to depend on the plant species or the lifestyle of the pathogen that is involved (Table 6.1). Moreover, even within the same plant-pathosystem, ABA can have diverse effects depending on the timing of infection. In Arabidopsis inoculated with *P. syringae* for example, ABA-induced stomatal closure can prevent pathogen invasion, while ABA suppresses post-invasion disease resistance (Melotto et al., 2006; de Torres-Zabala et al., 2007; Mohr and Cahill 2007).

One function of ABA in plant pathogen interactions can be obtained from the capacity of plant ABA levels to control a global shift between the response to abiotic and biotic stress. During abiotic stresses such as drought stress, to which the plant's response is more crucial for survival compared to biotic stress, plant ABA levels rise, which results in a priority to confer abiotic stress tolerance and a decrease in capacity to resist pathogens. This view is consistent with the function of ABA as a virulence factor of plant pathogens (de Torres-Zabala et al., 2007), with the dominant nature of ABA to suppress JA/ET or SA-controlled pathogen defense responses (Andersen et al., 2004, Audenaert et al., 2002) and with the strong effects on disease phenotypes, characterized by the apparently complete abolishment of defense (Henfling et al., 1980). Conversely, ABA-

deficiency, which results in a decreased tolerance to abiotic stresses, can result in an extremely high capacity to resist pathogen infection (Asselbergh et al., 2007; Asselbergh et al., 2008a).

In addition to the ABA-controlled global switch in response priority towards biotic or abiotic stress, ABA greatly influences the large and complex overlap between abiotic and biotic stress signaling pathways at multiple levels, resulting in both positive and negative regulation of defense to pathogens. Our current knowledge only covers small fragments of these signaling pathways and unraveling of the multiplex role of ABA herein is further complicated by the complexity of ABA-mediated signaling responses in general. However, the fast-growing number of reports that deal with ABA regulating pathogen defense responses should further establish and illuminate the function of ABA as a key player in plant biotic stress responses.

Chapter

7

Abscisic acid-induced resistance against the brown spot pathogen *Cochliobolus miyabeanus* in rice involves MAPK-mediated repression of ethylene signaling

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Submitted to Plant Physiology

The plant hormone abscisic acid (ABA) is involved in an array of plant processes, including the regulation of gene expression during adaptive responses to various environmental cues. Apart from its well-established role in abiotic stress adaptation, emerging evidence indicates that ABA is also prominently involved in the regulation and integration of pathogen defense responses. Here, we demonstrate that exogenous ABA application enhances basal resistance of rice (*Oryza sativa*) to the brown spot-causing ascomycete *Cochliobolus miyabeanus*. Microscopic analysis of early infection events in control and ABA-treated plants revealed that this ABA-inducible resistance (ABA-IR) is based on restriction of fungal progression in the mesophyll. We also show that ABA-IR does not rely on boosted expression of SA-, JA-, or callose-dependent resistance mechanisms but, instead, requires a functional Gα-protein. In addition, we present several lines of evidence suggesting that ABA steers its positive effects on brown spot resistance through antagonistic cross-talk with the ET signaling pathway. Exogenous Ethephon application enhances susceptibility, whereas genetic or pharmacological disruption of ET signaling renders plants less vulnerable to *C. miyabeanus* attack, thereby inducing a level of resistance similar to that observed in ABA-supplied plants. Additionally, ABA treatment alleviates *C. miyabeanus*-induced activation of the ET-reporter gene *EPB89*, while de-repression of pathogen-triggered *EBP89* transcription via RNAi-mediated knockdown of *OsMPK5*, a MAP kinase gene showing a potentiated expression pattern in ABA-induced leaves, compromises ABA-IR. Collectively, these data favor a model in which exogenous ABA induces resistance against *C. miyabeanus* by suppressing pathogen-induced ET action in an *OsMPK5*-dependent manner.

Introduction

To effectively combat invasion by microbial pathogens, plants have evolved sophisticated mechanisms providing several strategic layers of constitutive and induced defenses. Pre-formed physical and biochemical barriers constitute the first line of defense and fend off the majority of pathogens. However, should the pathogen overcome or evade these constitutive defenses, recognition of pathogen-derived molecules by plant receptors leads to the activation of a concerted battery of defenses designed to impair further pathogen spread. These inducible defenses are regulated by the coordinated activity of an elaborate matrix of signal transduction pathways in which the plant hormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) act as key signaling molecules (Lorenzo and Solano, 2005; Grant and Lamb, 2006; Adie et al., 2007). In response to pathogen attack, plants produce a highly specific blend of SA, JA, and ET, resulting in the activation of distinct sets of defense-related genes (Glazebrook et al., 2003). It is thought that this so-called signal signature, which varies greatly in quantity, timing and composition according to the type of attacker encountered, plays a primary role in the orchestration of the plant's defense 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).

Over the past decade, it has become increasingly clear that a plant's resistance to attack is not brought about by the isolated activation of parallel, linear signaling conduits, but rather is the consequence of a complex network of synergistic and antagonistic interactions (Kunkel and Brooks, 2002; Koornneef and Pieterse, 2008). In addition to differential signal signatures, such pathway crosstalk provides the plant with a powerful regulatory potential to fine-tune its defense response to best suit a specific threat. Thus, despite some exceptions (Thaler et al., 2004; Stout et al., 2006; Asselbergh et al., 2007), it is generally accepted that SA promotes resistance against pathogens with a biotrophic lifestyle, whereas JA and ET act as positive signals in the activation of defenses against necrotrophic pathogens and herbivorous insects (Thomma et al., 2001; Rojo et al., 2003; Glazebrook, 2005). Additionally, the primary mode of interaction between the SA and JA/ET signaling pathways appears to be mutual antagonism with corresponding trade-offs between biotroph resistance, on the one hand, and resistance to necrotrophic pathogens and insect herbivores, on the other hand (Bostock, 2005; Stout et al., 2006; Spoel et al., 2007). However, this is likely an oversimplified model as synergistic actions of SA and JA/ET have been reported as well (Van Wees et al., 2000; Mur et al., 2006; Adie et al., 2007; Truman et al., 2007).

Although mechanistic explanations of antagonistic and cooperative crosstalk are scarce, a number of transcription factors and effector proteins have been characterized that are critical in the circuitry controlling signal sensitivity and transduction in induced defense. For instance, SA repression of JA signaling requires the activation of proteins such as NPR1 and WRKY70 that activate expression of SA-responsive genes while repressing JA-dependent genes (Spoel et al., 2003; Li et al., 2004; Li et al., 2006a). Besides transcription factors, crosstalk between the SA and JA signaling pathways may also be mediated by fatty acid-derived signals and/or glutaredoxin genes (Kachroo et al., 2003b; Ndamukong et al., 2007). Other important effectors that contribute to differential response activation include mitogen-activated protein kinases (MAPKs). *Arabidopsis* MPK4 is one such kinase and has been shown to regulate SA/JA crosstalk by simultaneously repressing SA biosynthesis and promoting the perception of or response to JA, thereby functioning as a molecular switch between these mutually antagonistic pathways (Brodersen et al., 2006). On the other hand, fine-tune regulation of the antagonism and cooperation between JA and ET depends on the balance of activation by both hormones of ERF1 and MYC2, two opposing transcription factors that differentially regulate divergent branches of the JA signaling pathway involved in the response to necrotrophic pathogen attack and wounding, respectively (Berrocal-Lobo et al., 2002; Lorenzo et al., 2003; Lorenzo et al., 2004).

In contrast to the overwhelming amount of information with respect to SA, JA, and ET serving as important regulators of induced disease resistance, the role of abscisic acid (ABA) in plant defense is less well understood, and even controversial. Most comprehensively studied as a global regulator of abiotic stress adaptation, ABA has only recently emerged as a key determinant in the outcome of plant-pathogen interactions. In most cases, ABA behaves as a negative regulator of disease resistance. Exogenous application of ABA increases the susceptibility of various plant species to bacterial and fungal pathogens (Mohr and Cahill, 2003; Thaler et al., 2004; Achuo et al., 2006; Asselbergh et al., 2007; Mohr and Cahill, 2007), while disruption of ABA biosynthesis was shown to confer resistance to, amongst others, the necrotroph *Botrytis cinerea* (Audenaert et al., 2002a) and virulent isolates of the bacterial speck pathogen *Pseudomonas syringae* pv *tomato* DC3000 in tomato (Thaler and Bostock, 2004), and the oomycete *Hyaloperonospora parasitica* in *Arabidopsis* (Mohr and Cahill, 2003). Moreover, an intriguing study by de Torres-Zabala and coworkers (2007) revealed that *P. syringae* hijacks the ABA biosynthetic and response machinery to cause disease in *Arabidopsis*, suggesting that ABA is a susceptibility factor for this bacterium. This detrimental effect of ABA on pathogen resistance is likely explained by its well-documented ability to counteract SA- and JA/ET-dependent basal defenses (Asselbergh et al., 2008b).

In contrast, some studies describe a positive role of ABA in activation of defense responses and pathogen resistance. For instance, ABA primes for callose accumulation and thereby enhances basal resistance in response to *Blumeria graminis* f. sp. *hordei* and activates induced resistance in response to the necrotrophic fungi *Alternaria brassicicola* and *Plectosphaerella cucumerina* (Ton and Mauch-Mani, 2004; Wiese et al., 2004; Flors et al., 2008). In the case of bacterial leaf pathogens, ABA plays a crucial role in the activation of stomatal closure that, as part of the SA-regulated innate immune system, represents a major barrier to bacterial infection (Melotto et al., 2006). Furthermore, a recent study in *Arabidopsis* uncovered a new role for ABA in defense against insects (Bodenhausen and Reymond, 2007). ABA thus appears to play a complex and ambivalent role in the plant's defense response, acting as either a positive or negative regulator of disease and pest resistance by interfering at multiple levels with biotic stress signaling cascades.

Rice is the most important staple food crop in the world, only rivaled in importance by maize and wheat. However, despite its emergence as a pivotal model for monocotyledonous plants, surprisingly little is known about the effector responses and hormonal signal transduction pathways underlying rice disease resistance. This is particularly true for rice brown spot disease, caused by the ascomycete *Cochliobolus miyabeanus* (anamorph: *Bipolaris oryzae*). One of the most devastating rice diseases in rainfed ecosystems, brown spot adversely affects the yield and milling quality of the grain (Dela Paz et al., 2006). In 1942, an epidemic of the disease was one of the major factors contributing to the great Bengal famine, which reportedly claimed the lives of no less than 2 million Indians (Stuthman, 2002). Nowadays, brown spot is as prevalent as ever with recent studies by Savary et al. (2000a,b) showing that among the many diseases occurring in rice fields, brown spot, along with sheath blight, accounts for the highest yield loss across all production situations in South and Southeast Asia. Although the genetic and molecular basis of the rice-*C. miyabeanus* interaction is still poorly understood, like other *Cochliobolus* species, the fungus appears to employ a varied arsenal of phytotoxins to trigger host cell death (Xiao et al., 1991).

Here, we show that pretreatment of rice with ABA renders leaves more resistant to *C. miyabeanus* attack and present results supporting ABA-mediated repression of pathogen-induced ET action as the causal resistance mechanism. In addition, we provide novel evidence regarding the role of the ABA-inducible MAP kinase gene *OsMPK5* as a pivotal regulator of this ABA/ET crosstalk, and describe how ABA might interfere with the postulated fungal manipulation of the plant.

Results

Exogenous ABA treatment induces resistance against *Cochiobolus miyabeanus* in rice

Six rice cultivars, including four *indica* and two *japonica* lines, were screened with two *C. miyabeanus* strains, both of which were isolated from diseased rice in field plots at the International Rice Research Institute in the Philippines (Fig. 7.1A). With the exception of *japonica* cultivar CR203, isolate Cm988 was highly virulent on all cultivars tested, causing typical ellipsoidal light- or dark-brown lesions with a grey sporulating center, often surrounded by chlorotic tissue. On most cultivars, these susceptible-type lesions coalesced within 96 h postinoculation (hpi), killing large areas of affected leaves (Fig. 7.1C, no ABA treatment). By contrast, in case of infection by strain Cm963, fungal development was restricted to a few dark-brown necrotic spots, representing a genetically resistant reaction (Ou, 1985). Owing to its differential response to Cm988 and Cm963 and its widespread use as a pathogen-susceptible control in numerous other studies, *indica* cultivar CO39 was chosen for further analysis.

In a first attempt to unravel the signaling network(s) orchestrating rice defense against *C. miyabeanus*, we examined the effect of various signaling molecules and so-called plant defense activators on brown spot disease development. To this end, five-week-old CO39 seedlings were sprayed until runoff with the respective compounds and, three days later, inoculated with the virulent strain Cm988. Consistent with previous reports (Ahn et al., 2005b), treatment with 0.1 mM JA yielded no significant protection against *C. miyabeanus* (Fig. 7.1B), even though this concentration is high enough to induce JA-responsive *JIOsPR10* transcription (Jwa et al., 2001). Higher concentrations of JA also failed to trigger induced resistance, suggesting that JA is not a major signal for activation of defenses against *C. miyabeanus*. Intriguingly, pretreatment with 0.5 mM Ethephon, an ET-releasing plant growth regulator, rendered plants more vulnerable to brown spot disease compared to non-induced controls. The disease-promoting effect of Ethephon strikingly contrasted with the enhanced resistance observed in response to exogenously administered ABA. Supplying plants with 0.1 mM ABA 3 d prior to inoculation induced high levels of protection, as shown by a dramatic decrease in size, type and number of brown spot lesions in ABA-supplied leaves (Fig. 7.1C). On the other hand, foliar application of the synthetic SA analog BTH (0.5 mM) or soil drench treatment with 150 µM BABA, a non-protein amino acid and potent elicitor of broad-spectrum disease resistance in dicot plants (Ton et al., 2005; Flors et al., 2008), resulted in a rather weak and statistically not significant reduction in disease severity compared with control plants. Collectively, these data uncover ABA as a powerful activator of induced resistance against *C. miyabeanus* and suggest that ET acts as a negative signal in the signaling circuitry underlying rice defense against this ascomycete.

A

Table I. *Virulence pattern of two* C. miyabeanus *isolates on six rice cultivars*

	Host response to individual *C. miyabeanus* isolates[a]	
Rice cultivar	Cm988	Cm963
Chiembac	S	R
CO39	S	R
C101PKT	S	R
CR203	I	R
Shin2	S	R
Pi-N4	S	I

[a] R = resistant (score 0-3), S = susceptible (score 4-6), and I = intermediate (score 7-9) interaction according to the standard brown spot evaluation scale of the International Rice Research Institute (1996).

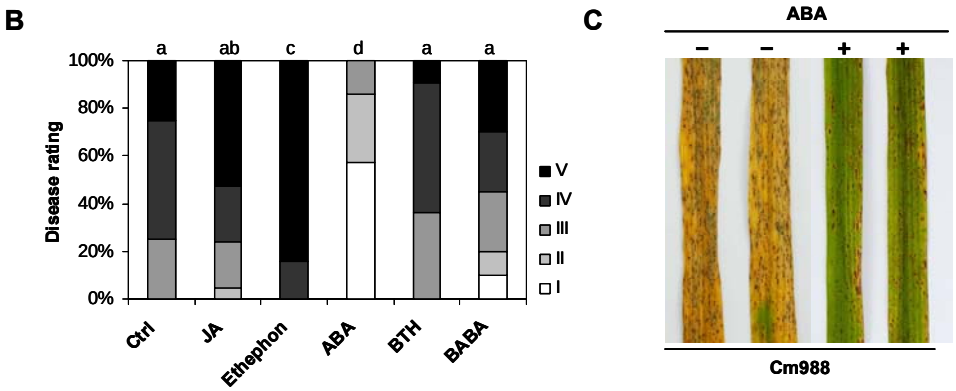

Figure 7.1. Exogenous application of abscisic acid induces resistance against *C. miyabeanus* in rice.

A, Virulence pattern of two *C. miyabeanus* isolates on six rice cultivars. All cultivars were inoculated when five weeks old by spraying a conidial suspension of *C. miyabeanus* at 1 x 10^4 sp ml^{-1}. Four days later, cultivars were scored as resistant (R), intermediate (I), or susceptible (S) according to the standard brown spot evaluation scale of the International Rice Research Institute (Standard evaluation system for rice, 1996). **B**, Effect of pretreatment with various plant defense activators and signaling molecules on subsequent infection with *C. miyabeanus*. Five-week-old CO39 plants were sprayed until runoff with 0.1 mM JA, 0.5 mM Ethephon, 0.1 mM ABA , 0.5 mM BTH or soil-drenched with 0.15 mM BABA. Control plants were treated with water. Three days after chemical treatment, plants were challenged with virulent *C. miyabeanus* Cm988. Disease evaluation was performed 4 d postinoculation, using a 1-to-5 disease severity scale as described in Materials and Methods. Different letters indicate statistically significant differences (Mann-Whitney test; $\alpha = 0.05$). **C**, Photographs depicting representative symptoms were taken 5 days postinoculation. Data represent one of three experiments with similar results.

ABA-induced resistance (ABA-IR) against *C. miyabeanus* is based on restriction of fungal progression in the mesophyll

To gain more insight into the nature of ABA-inducible brown spot resistance, we next analyzed fungal development and cellular defense reactions in mock- and ABA-treated CO39 leaf sheaths following challenge with virulent Cm988. Regardless of ABA treatment, conidial attachment and germination occurred within 6 hpi, followed by normal hyphal growth and appressorium-mediated penetration attempts (Fig. 7.2A). Interestingly, at some interaction sites, invading hyphae differentiated into subcuticular finger-shaped multicell complexes (Fig. 7.2B), resembling the extracellular infection structures, so-called stroma, frequently formed by *Venturia inaequalis* and *Bipolaris sorokiniana* (Ortega et al., 1998; Schafer et al., 2004). Further ramification of

hyphal tissue occurred predominantly but not exclusively intercellular (Figs. 7.2C, D), giving rise to a dense network that eventually penetrated all host tissue types. Epidermal and mesophyll tissue necrotization was closely associated with successful fungal infestation, whereby necrotization usually preceded fungal growth, suggesting the involvement of *C. miyabeanus*-secreted phytotoxins. Comparing control inoculated and ABA-treated plants, we found no marked differences in abovementioned infection events, except for a drastic reduction of fungal spreading in the mesophyll tissue of ABA-supplied leaf sheaths. By 36 hpi, fungal spreading in control inoculated leaves amounted to approximately 1,400 μm, corresponding to 20-25 mesophyll cells spanned by the fungus, as compared with 300 μm in ABA-pretreated sheath cells. Together, these observations suggest that restriction of fungal proliferation during the mesophyll-based growth phase, rather than a preinfectional, epidermis-based resistance reaction, is the cause for the reduced disease susceptibility in ABA-treated plants.

ABA-IR against *C. miyabeanus* acts through a callose-independent mechanism

Recent evidence has implicated ABA as a positive signal in priming of callose biosynthesis upon pathogen recognition, which suggests a putative mechanism explaining the role of ABA in defense activation (Ton and Mauch-Mani, 2004; Flors et al., 2008). Callose deposition is a hallmark of basal defense to attempted fungal and bacterial penetration and may serve to fortify cell walls in order to inhibit pathogen penetration of the cell. To ascertain the role of callose in the case of *C. miyabeanus*, we studied the deposition of this compound and its effect on resistance in mock- and ABA-treated leaves stained with aniline blue. Deposition of callose, as visualized by an intense yellow-green fluorescence under UV light, was detectable as early as 8 hpi in epidermal control cells in close contact to the invading hyphae. This fluorescence was infrequently only present in appositions or papillae around the site of penetration, more normally being seen to encompass large multi-spot deposits located in the close vicinity of the periclinal and anticlinal cell walls of both infected and neighboring epidermal cells (Fig. 7.2E). Although no differences were evident between ABA- and control-treated plants in the onset of callose formation, ABA-induced plants tended to accumulate less callose-associated fluorescence following Cm988 challenge than inoculated controls (data not shown). To determine whether this altered callose formation contributed to the ABA-induced resistance, fifth and sixth stage leaves from 5-week-old CO39 plants were detached and supplied from the cut base with a solution containing 0.1 mM ABA and different concentrations of the callose inhibitor 2-deoxy-D-glucose (2-DDG; Ton and Mauch-Mani, 2004; Asselbergh et al., 2008b). Twenty-four hours later, the leaves were drop-inoculated with a Cm988 conidial suspension, and the level of induced resistance was quantified

by determining average lesion diameters 60 hpi. However, as demonstrated by the results presented in Figure 7.3, removal of callose formation with 2-DDG had no marked impact on the resistance response of ABA-treated plants, indicating that callose is not a critical factor in the establishment of ABA-IR to *C. miyabeanus*.

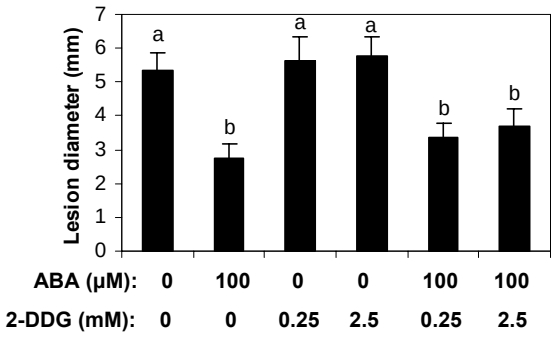

Figure 7.3. Effect of the callose-inhibitor 2-deoxy-D-glucose (2-DDG) on the level of basal and ABA-induced resistance against *C. miyabeanus* in rice.

Leaves of 5-week-old CO39 plants were detached and supplied from the cut base with different concentrations of ABA and 2-DDG. Twenty-four hours later, treated leaves were inoculated with five 10-µl droplets of a *C. miyabeanus* Cm988 conidial suspension (5 x 10^4 sp ml^{-1}). Resistance was quantified by measuring lesion diameters 60 hpi. Data shown are means ± SE of at least 19 infection sites from 4 different leaves. Different letters indicate statistically significant differences (Duncan; $\alpha = 0.05$). The experiment was repeated twice with similar results.

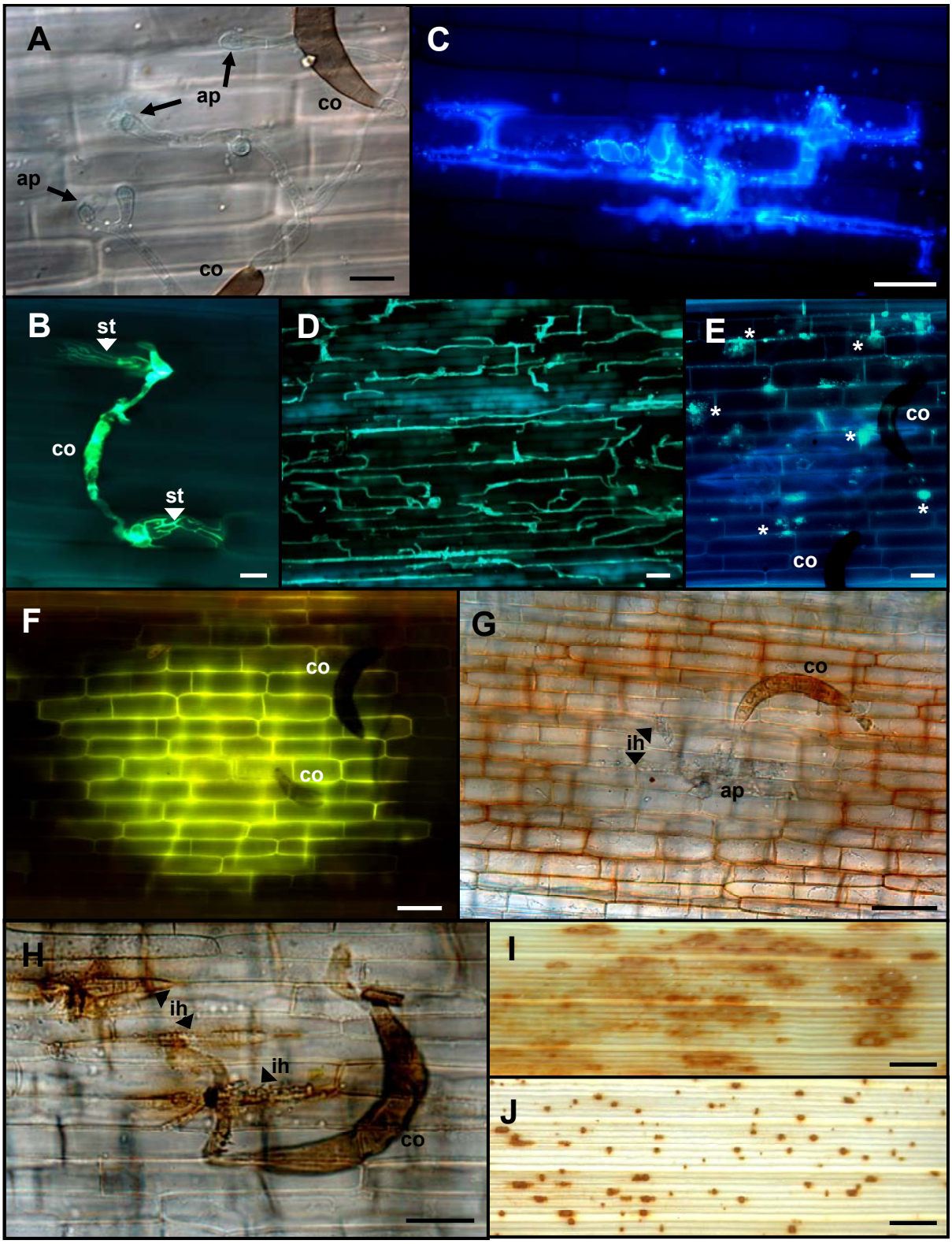

Figure 7.2. Microscopic analysis of the early infection events in ABA-supplemented rice sheaths inoculated with *C. miyabeanus*.

Five-week-old CO39 plants were sprayed until runoff with water or 0.1 mM ABA and, 3 d later, challenged with a conidial suspension of virulent Cm988 containing 1 x 10^4 spores ml^{-1}. co = conidium. **A** through **F**, micrographs of typical infection sites in control-treated plants. Similar phenomena were observed in ABA-induced tissues save for a drastic reduction of fungal spreading in the mesophyll. **A**, Appressorial (ap) formation on leaf sheath epidermis (6 hpi). Bar = 20 μm. **B**, Following appressorium-mediated penetration, invading hyphae frequently differentiate into

subcuticular stroma-like complexes (st). Fungal hyphae were stained with KOH-aniline blue and visualized under UV excitation. Bar = 20 μm. **C**, Epifluorescence image of a representative epidermal cell illustrating intercellular fungal progression. Hyphae were stained with calcofluor. Bar = 50 μm. **D**, Extreme fungal spreading in control plants 36 hpi. Extracellular mycelium was stained using KOH-aniline blue and analyzed under UV excitation. Bar = 50 μm. **E**, Callose formation (white asterisks) at and around sites of attempted pathogen entry at 12 hpi (aniline blue stain). The dispersed pattern of callose accumulation strongly suggests the involvement of fungal toxins. Bar = 20 μm. **F**, Massive autofluorescence of both invaded and surrounding epidermal cells under blue light excitation (8 hpi). Bar = 50 μm. **G** through **J**, H_2O_2 accumulation in control and ABA-treated leaf sheaths inoculated with *C. miyabeanus*. In control plants at 12 hpi (**G**), strong DAB staining developed in the anticlinal walls of epidermal and mesophyll cells surrounding the site of infection, whereas penetrated, hyphae (hy)-containing cells remained essentially free of DAB accumulation. Reversely, in ABA-treated tissue (**H**), DAB staining was exclusively detectable at sites of penetration. Bars = 50 μm. **I** and **J**, Overview pictures of DAB staining on infected leaf blades of control- (**I**) and ABA-treated (**J**) plants 36 hpi. Bars = 2 mm.

Influence of exogenous ABA treatment on pathogenesis-related H_2O_2 generation

The callose-independency of ABA-IR prompted us to assay for other biochemical defense responses. Tissue autofluorescence, due to accumulation of phenolics compounds, is a key event in R-protein-mediated resistance against the rice blast-causing ascomycete *Magnaporthe oryzae* (Koga, 1994). In case of *C. miyabeanus*, however, rapid recruitment of phenolics does not appear to constitute an effective defense mechanism as all interaction sites exhibited a strong blue light-induced autofluorescence as early as 8 hpi (Fig. 7.2F), irrespective of ABA treatment or the inherent level of resistance of the cultivars used (data not shown). In contrast, striking differences were observed when staining leaves with diaminobenzidine (DAB), a histochemical reagent for H_2O_2 (Thordal-Christensen et al., 1997). In control plants challenged with virulent Cm988 at 12 hpi, strong DAB staining developed in the anticlinal walls of non-penetrated epidermal and mesophyll cells surrounding the site of infection, whereas little staining was evident in infected, hyphae-containing cells (Fig. 7.2G). Reversely, in ABA-induced tissues, DAB accumulation was tightly restricted to the site of penetration with adjacent non-penetrated cells being void of DAB-detectable H_2O_2 (Fig. 7.2H). Supplementing the DAB solution with ascorbate markedly reduced staining at the respective sites, indicating that the staining was due to H_2O_2 accumulation (data not shown). At later time points, fungal progression in the mesophyll layer resulted in an intense DAB staining dispersed throughout the inoculation site in both control and ABA-induced plants. However, while control plants developed large, dark-brown patches comprising about 30 DAB-stained mesophyll cells, in ABA-induced sheaths, DAB accumulated in discrete, small clusters with between 4 and 8 mesophyll cells per interaction site. On the macroscopic level, this was reflected by large, DAB-soaked lesions occurring on leaves of challenged control plants (Fig. 7.2I; 36 hpi), as opposed to the small, pinpoint-size spots visible on ABA-treated plants (Fig. 7.2J; 36 hpi). Collectively, these data suggest a dual role of H_2O_2 in the rice-*C. miyabeanus* interaction and argue that one mechanism of ABA action is to modulate pathogenesis-related ROS formation.

The alpha subunit of heterotrimeric G protein but not SA accumulation is required for ABA-IR against C*. miyabeanus*

Mounting evidence indicates that defense signaling is not a linear single-response event, but a complex network involving a number of different signals and effectors (Koornneef and Pieterse, 2008). Therefore, to further elucidate how ABA-induced plants counteract hyphal invasion, we used several mutant and transgenic rice lines affected in hormonal and nonhormonal resistance pathways to dissect the involvement of known plant defense mechanisms. Given the recent identification of a heterotrimeric G protein-coupled protein as a pivotal ABA receptor controlling all major ABA responses in *Arabidopsis* (Liu et al., 2007a) and the well-described role of the G-protein alpha subunit in rice pathogen defense (Suharsono et al., 2002; Komatsu et al., 2004), we first tested the effectiveness of ABA in mutant *Daikoku dwarf* plants. These so-called *d1* mutant plants, which are in the background of *japonica* cultivar Nipponbare, are defective in the sole alpha subunit of heterotrimeric G-proteins in rice (Ashikari et al., 1999; Fujisawa et al., 1999). Consistent with the role of G-alpha in basal resistance against the bacterial leaf blight pathogen, *Xanthomonas oryzae* pv. *oryzae* (Komatsu et al., 2004), *d1* mutants were highly susceptible to infection by virulent *C. miyabeanus* Cm988 (Fig. 7.4A). Furthermore, in contrast to wild-type Nipponbare plants, mutant *d1* plants failed to develop ABA-IR, indicating that G-alpha controls both basal and ABA-inducible resistance against *C. miyabeanus*. Although it cannot be completely excluded that the ABA-IR-minus phenotype of *d1* is due to the excessive fungal colonization in this mutant, the latter hypothesis is rather unlikely as the use of lower inoculum densities, resulting in less severe disease symptoms, yielded comparable results (data not shown).

In several plant-pathosystems, ABA has been shown to influence disease outcome through its effect on SA-regulated defense (Robert-Seilaniantz et al., 2007; Asselbergh et al., 2008b). To investigate the SA-dependence of ABA-inducible resistance against *C. miyabeanus*, wild-type Nipponbare and SA-deficient NahG transgenic plants (Yang et al., 2004) were routinely sprayed with 0.1 mM ABA and subsequently tested for expression of IR. As shown in Figure 7.4A, NahG plants retained the strong level of ABA-inducible resistance characteristic for wild-type plants, indicating that SA accumulation is not an essential prerequisite for ABA-induced resistance against *C. miyabeanus*. To further probe whether ABA elicits an SA-independent defense mechanism, we examined the expression of the SA-responsive genes *OsPR1b* and *PBZ1* in CO39 plants following challenge infection. Both of these *PR*-like genes are responsive to BTH treatment and have recently been shown to function in the NPR1-dependent branch of the rice SA pathway (Shimono et al., 2007). Quantitative RT-PCR analysis revealed that treatment with 0.1 mM ABA alone did not induce transcription of either gene (Fig. 7.4B). Moreover, at all time

points investigated, pathogen-induced *OsPR1b* and *PBZ1* transcription was considerably lower in ABA-supplied plants compared to non-induced controls. Collectively, these data suggest that ABA-inducible resistance against *C. miyabeanus* does not rely on boosted expression of SA-inducible defense responses.

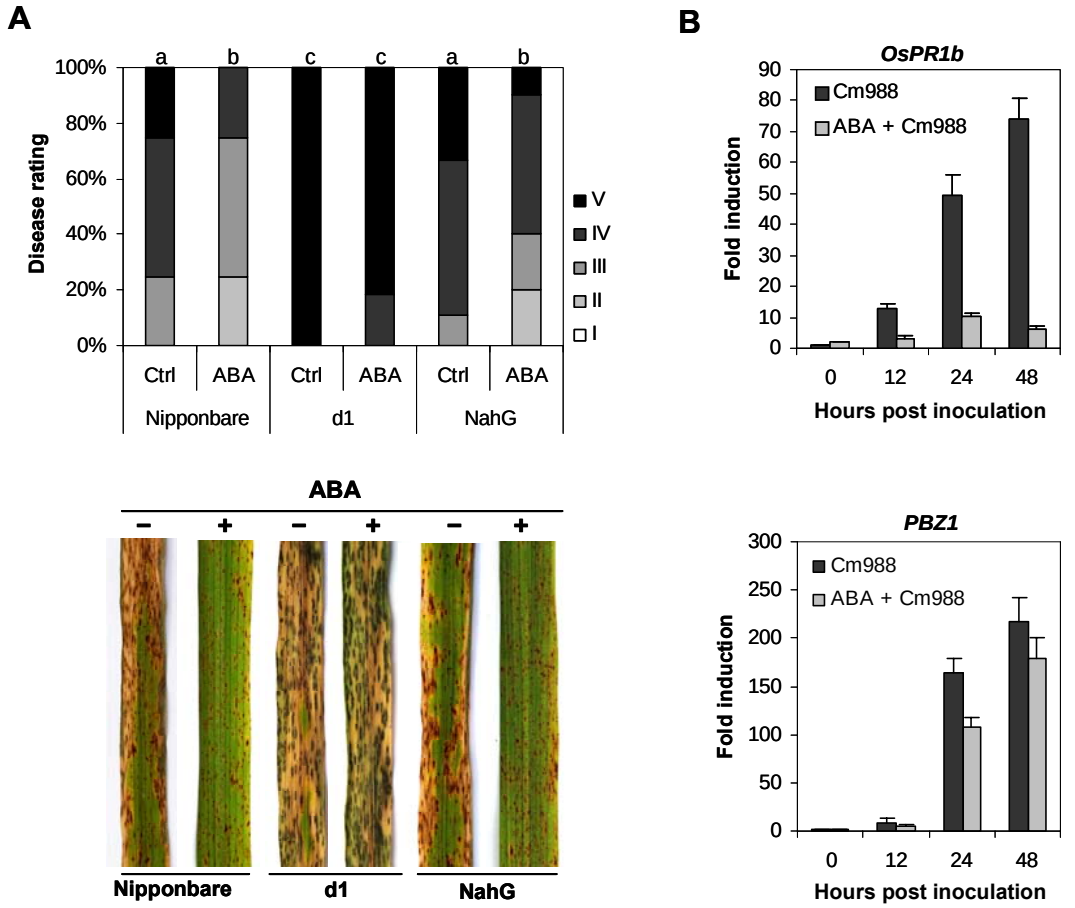

Figure 7.4. ABA-induced resistance against *C. miyabeanus* is independent of SA accumulation but requires the heterotrimeric G-protein alpha subunit.

A, Wild-type Nipponbare, mutant *d1*, and transgenic NahG plants were sprayed until run-off with water or ABA (0.1 mM) and, 3 d later, challenged with virulent *C. miyabeanus* Cm988. Disease evaluation was performed 4 d postinoculation, using a 1-to-5 disease severity scale as described in Materials and Methods. Different letters indicate statistically significant differences (Mann-Whitney test; $n \geq 12$; $\alpha = 0.05$). Photographs depicting representative symptoms were taken 5 days postinoculation. Repetition of experiments led to results very similar to those shown. **B**, Effect of ABA pretreatment on transcript accumulation of *OsPR1b* and *PBZ1* in leaves of CO39 inoculated with Cm988. At the indicated time points postinoculation, fully expanded fifth and sixth leaves from five plants were harvested, converted to cDNA and subjected to quantitative RT-PCR analysis. Gene expression levels were normalized using actin as an internal reference and expressed relative to the normalized expression levels in mock-treated control plants at 0 h. Data presented are means (± SD) of three replicates from a representative experiment. Plants were treated and inoculated as described in Figure 7.1.

The role of JA-dependent defenses in ABA-IR against *C. miyabeanus*

Besides crosstalk with the SA pathway, ABA has also been found to modulate JA-regulated resistance mechanisms (Anderson et al., 2004; Adie et al., 2007). To decipher the role of the JA-mediated defense pathway in ABA-IR against *C. miyabeanus*, we tested the effectiveness of ABA in the JA biosynthesis mutant *hebiba* (Riemann et al., 2003). By analogy with the results obtained in the Nipponbare and CO39 lines, treatment of wild-type Nihonmasari plants with 0.1 mM ABA resulted in a statistically significant reduction in disease severity compared to non-induced controls (Fig. 7.5A). Mutant *hebiba* plants, however, failed to develop resistance when induced by ABA, which could point to JA-regulated defenses being an integral part of the ABA-induced resistance machinery. However, as non-induced *hebiba* plants were much more sensitive to *C. miyabeanus* infection than wild-type, it is equally possible that the failure of ABA to induce resistance in *hebiba* is due to a lower efficacy of ABA in face of the high infection pressure in this mutant. To discriminate between these possibilities, we examined the effect of exogenous ABA application on the activity of lipoxygenase (LOX; EC 1.13.11.12), a key JA biosynthetic enzyme, in wild-type Nihonmasari leaves. Interestingly, whereas ABA pretreatment had no significant impact on the steady-state kinetics of LOX in mock-inoculated controls, it severely attenuated pathogen-induced LOX activation in Cm988-challenged leaves (Fig. 7.5B). Similar results were obtained when monitoring the expression of the JA-inducible defense gene *JIOsPR10* (Jwa et al., 2001) in leaves of CO39 plants upon infection with Cm988. As expected, *JIOsPR10* mRNAs accumulated to high levels in inoculated control plants, resulting in an approximately 9-fold induction relative to mock-treated plants by 48 hpi (Fig. 7.5C). However, *JIOsPR10* expression was induced only slightly, if at all, in CO39 leaves pretreated with 0.1 mM ABA. Together with the inability of exogenously administered JA to cause substantial disease reduction (Fig. 7.1B), these results suggest that JA-dependent defense mechanisms do not contribute significantly to ABA-inducible brown spot resistance.

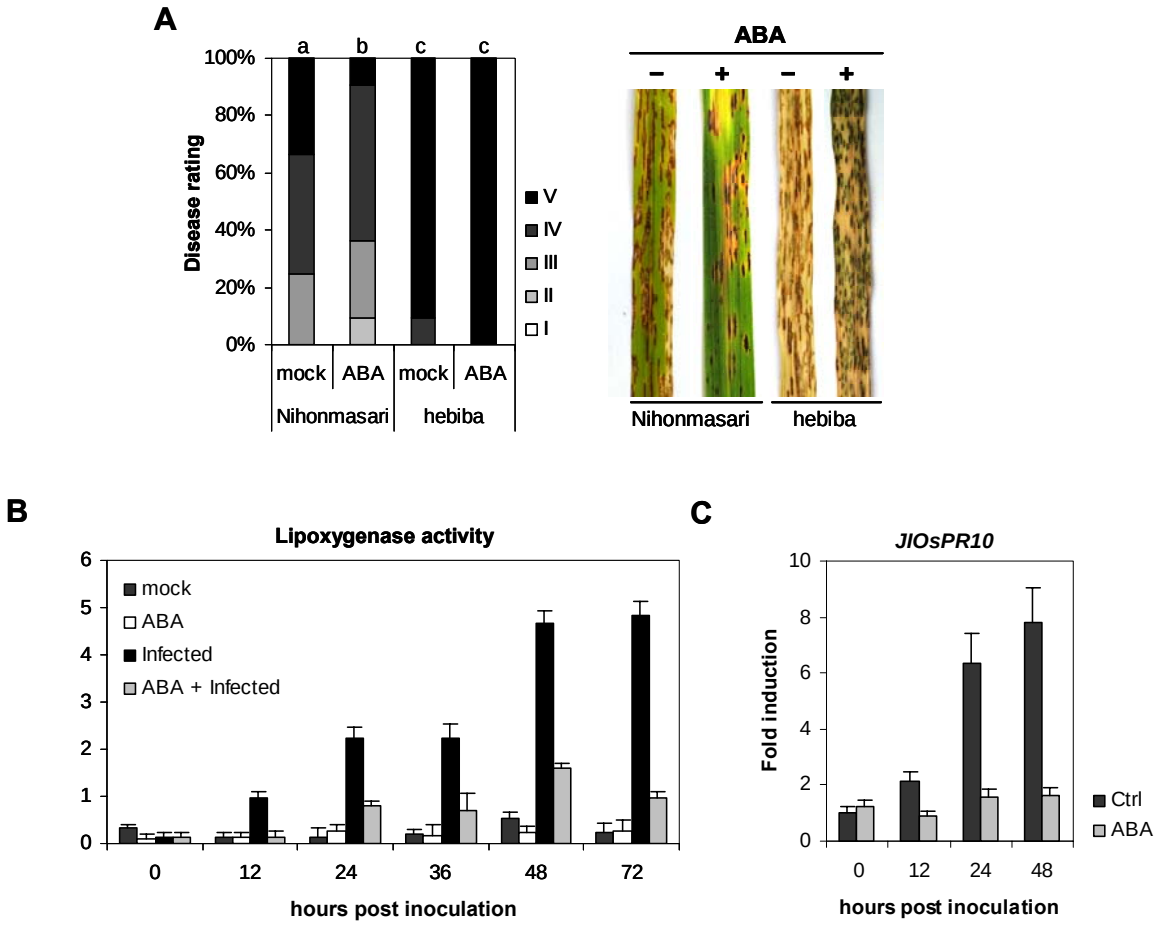

Figure 7.5. Role of the JA pathway in ABA-inducible resistance (ABA-IR) against *C. miyabeanus* in rice.

A, ABA-IR is blocked in the JA-deficient mutant *hebiba*. Wild-type Nihonmasari and mutant *hebiba* plants were sprayed with water or ABA (0.1 mM) and, 3 d later, challenged with the virulent *C. miyabeanus* strain Cm988. Disease evaluation was performed 4 d postinoculation, using a 1-to-5 disease severity scale as described in Materials and Methods. Different letters indicate statistically significant differences (Mann-Whitney test; $n \geq 12$; $\alpha = 0.05$). Photographs depicting representative symptoms were taken 5 days postinoculation. Repetition of experiments led to results very similar to those shown. **B**, Effect of ABA pretreatment on *C. miyabeanus*-induced lipoxygenase activity. Lipoxygenase activity was measured at 234 nm in samples taken from the fifth and sixth stage leaves of Nihonmasari plants at different time points after inoculation. Each bar represents average data and SD from two independent experiments. **C**, Effect of ABA pretreatment on expression of the JA-responsive PR gene *OsPR4* in leaves of CO39 plants inoculated with *C. miyabeanus* Cm988. At the indicated time points postinoculation, fully expanded fifth and sixth leaves from five plants were harvested, converted to cDNA and subjected to quantitative RT-PCR analysis. Gene expression levels were normalized using actin as an internal reference and expressed relative to the normalized expression levels in mock-treated control plants at 0 h. Data presented are means (± SD) of three replicates from a representative experiment.

Repression of ET signaling confers enhanced resistance against *C. miyabeanus*

The observation that ABA-IR against *C. miyabeanus* is not brought about by hyperactivation of SA- or JA-mediated defense responses prompted us to assess the involvement of the ET pathway. To this end, we quantified ABA-IR in wild-type Dongyin and *OsEIN2* antisense transgenic plants (Jun et al., 2004). Similar to its counterpart in Arabidopsis, *OsEIN2* is predicted to encode a positive regulator of the rice ET response. Accordingly, the *OsEIN2* antisense transgenics, which display a somewhat stunted phenotype, exhibit ET-insensitivity and show a decreased expression of ET-responsive defense genes (Jun et al., 2004). Intriguingly, in our assays, non-induced *OsEIN2* antisense seedlings were significantly more resistant to infection by *C. miyabeanus* compared with the wild-type background, indicating that ET action interferes with basal resistance to *C. miyabeanus* (Fig. 7.6A). Moreover, while the level of resistance of non-induced *OsEIN2* transgenic plants mirrored that of ABA-induced wild-type plants, treatment with ABA failed to cause an additional reduction in disease severity on the *OsEIN2* transgenic plants, suggesting that exogenous ABA may induce resistance against *C. miyabeanus* through repression of ET signaling. Consistent with this hypothesis, infiltration of detached wild-type leaves with silver thiosulfate (STS), an inhibitor of ethylene action (Navarre and Wolpert, 1999), reduced subsequent symptom development to the same extent as treatment with ABA (Fig. 7.6B). Furthermore, co-application of ABA with STS resulted in a similar reduction in lesion size relative to treatment with either compound alone. On the other hand, infiltration of the ET biosynthesis inhibitor aminooxyacetic acid (AOA) yielded significantly lower protection levels, suggesting that ET action, rather than *de novo* ET synthesis, is the crucial factor modulating *C. miyabeanus* pathogenicity. To further test whether ABA-IR against *C. miyabeanus* is associated with a down-regulation of ET-dependent defenses, we analyzed the expression of the ethylene-responsive element-binding protein gene *EBP89* (Yang et al., 2002) in non-induced control and ABA-IR-expressing CO39 plants. Figure 7.6C shows that in control, non-induced leaves, *EBP89* transcript levels accumulated rapidly, reaching a maximum 12 h after Cm988 challenge. In ABA-treated samples, *EBP89* expression likewise peaked at 12 hpi, albeit to a significantly lower extent. Furthermore, whereas in control samples *EBP89* mRNA levels were still high at 24 and 48 hpi, showing an approximately 10-fold induction over the mock control, they had decayed to near basal levels in ABA-treated samples. In conjunction with analyses of the *OsEIN2*-suppressed transgenic plants and the disease-promoting effect of exogenous Ethephon application (Fig. 7.1B), these results support the notion that the ET pathway contributes to *C. miyabeanus* pathogenicity and strengthen the hypothesis that ABA-IR involves repression of *C. miyabeanus*-induced ET action.

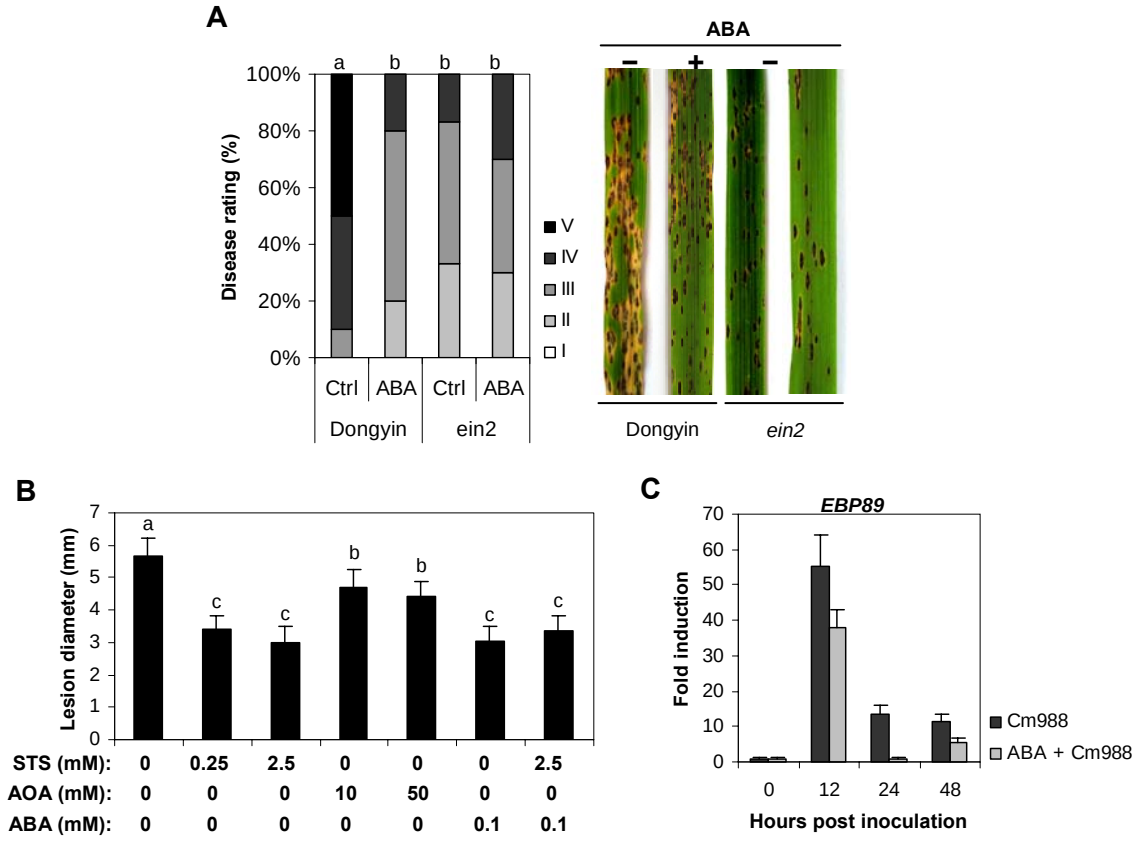

Figure 7.6. Involvement of the ET pathway in ABA-inducible resistance against *C. miyabeanus* in rice.

A, Antisense suppression of *OsEIN2* increases brown spot resistance. Wild-type Dongyin and *OsEIN2* antisense plants were sprayed with water or ABA (0.1 mM) and, 3 d later, challenged with virulent Cm988. Disease evaluation was performed 4 d postinoculation, using a 1-to-5 disease severity scale as described in Materials and Methods. Different letters indicate statistically significant differences (Mann-Whitney test; $n \geq 12$; $\alpha = 0.05$). Photographs depicting representative symptoms were taken 5 days postinoculation. Repetition of experiments led to results very similar to those shown. **B**, Effect of AOA and STS, inhibitors of ET biosynthesis and action, respectively, on *C. miyabeanus* resistance. Leaves of 5-week-old Dongyin plants were detached and pressure-infiltrated at five sites with different concentrations of AOA and STS. ABA treatment, fungal inoculation and disease evaluation was performed exactly as described in legend to Figure 7.3. Values presented are means and SE of at least 19 infection sites stemming from 4 different leaf segments. Different letters indicate statistically significant differences (Duncan; $\alpha = 0.05$). **C**, Effect of ABA pretreatment on expression of the ET-responsive transcription factor gene *EBP89* in leaves of CO39 plants inoculated with virulent Cm988. At the indicated time points postinoculation, fully expanded fifth and sixth leaves from five plants were harvested, converted to cDNA and subjected to quantitative RT-PCR analysis. Gene expression levels were normalized using actin as an internal reference and expressed relative to the normalized expression levels in mock-treated control plants at 0 h. Data presented are means (± SD) of three replicates from a representative experiment.

ABA-induced resistance against *C. miyabeanus* depends on the MAP kinase gene *OsMPK5*

Protein kinases operate at the core of signal transduction networks, channeling information from upstream effectors to downstream cellular responses. One such kinase is the ABA-inducible mitogen-activated protein kinase OsMPK5, the role of which as a critical regulator of pathogen defense and abiotic stress tolerance in rice is well-documented (Xiong and Yang, 2003). In order to examine whether *OsMPK5* is also involved in ABA-IR against *C. miyabeanus*, we initially analyzed *OsMPK5* transcript levels in control non-induced and ABA-IR-expressing CO39 plants. As shown in Figure 7.7A, *OsMPK5* showed a potentiated expression pattern in ABA-treated plants following Cm988 challenge, indicating that ABA primes rice for enhanced *OsMPK5* transcription. To determine whether this primed *OsMPK5* response is required for ABA-IR against *C. miyabeanus*, we determined the level of ABA-inducible brown spot resistance in the *OsMPK5*-suppressed transgenic line RI7 (Xiong and Yang, 2003). This transgenic line, which exhibits constitutive expression of several *PR* genes, was generated by introducing a double-stranded RNA interference (dsRNAi) construct in the background of cultivar Nipponbare (Xiong and Yang, 2003). Although symptom development was slightly accelerated in the dsRNAi transgenics, we were unable to detect any reproducible or significant differences in overall disease severity between non-induced wild-type and similarly treated *OsMPK5*-silenced plants, suggesting that *OsMPK5* only plays a minor role in basal resistance against *C. miyabeanus* (Figs. 7.7B and C). However, *OsMPK5* does appear to be an integral component of ABA-IR against *C. miyabeanus* as treatment with 0.1 mM ABA resulted in a substantial reduction of disease in wild-type Nipponbare but not in *OsMPK5*-silenced plants.

The observation that *OsMPK5* is necessary for ABA to induce resistance to *C. miyabeanus* prompted us to assess whether this MAP kinase gene also is implicated in orchestrating ABA/ET crosstalk in rice. To this end, we tested wild-type Nipponbare and RNAi *OsMPK5* plants for expression of the ET-reporter gene *EBP89*. In accordance with the results obtained in the Dongyin background, *EBP89* expression responded strongly to pathogen infection in both wild-type and transgenic Nipponbare plants, resulting in an approximately 50-fold induction by 12 hpi. However, suppression of Cm988-induced *EBP89* expression resulting from ABA pretreatment, a typical reaction in wild-type plants, was severely attenuated in *OsMPK5*-silenced plants (Fig. 7.7D). In light of the results presented in Figure 7.6, we interpret these data to suggest that RNAi-mediated suppression of *OsMPK5* affects ABA-induced resistance against *C. miyabeanus* by blocking the antagonistic action of ABA on ET signaling.

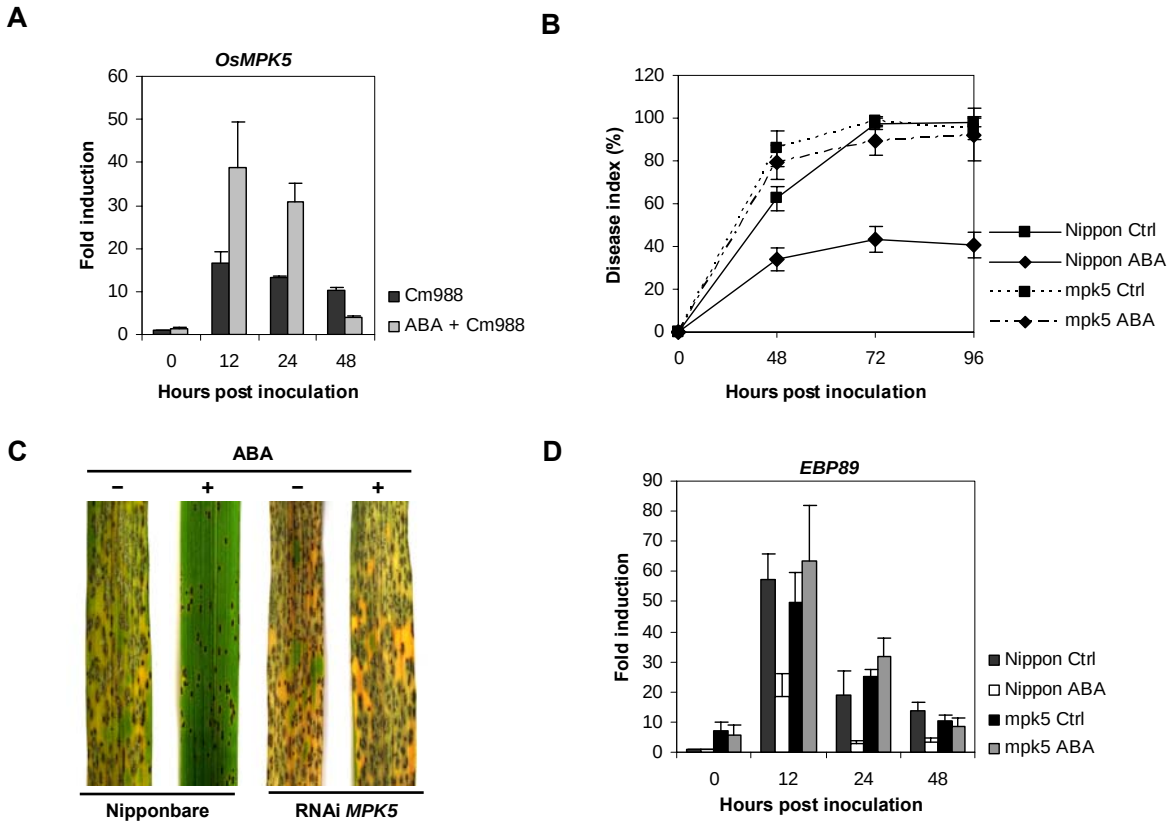

Figure 7.7. ABA induces resistance to *C. miyabeanus* in an *OsMPK5*-dependent manner.

A, Effect of ABA pretreatment on *OsMPK5* transcript accumulation in leaves of CO39 plants inoculated with virulent *C. miyabeanus* Cm988. At the indicated time points postinoculation, fully expanded fifth and sixth leaves from five plants were harvested, converted to cDNA and subjected to quantitative RT-PCR analysis. Gene expression levels were normalized using actin as an internal reference and expressed relative to the normalized expression levels in mock-treated control plants at 0 h. Data presented are means (± SD) of three replicates from a representative experiment. **B** and **C**, RNAi-mediated silencing of *OsMPK5* attenuates ABA-IR. Photographs depicting representative symptoms were taken 5 days postinoculation. Plants were treated and inoculated as described in Figure 7.1. **D**, ABA-induced repression of *C. miyabeanus*-activated ET signaling is blocked in RNAi *OsMPK5* plants. qRT-PCR analysis of *EBP89* transcription in wild-type Nipponbare and *OsMPK5*-suppressed plants was performed exactly as described in Figure 7.6.

Discussion

With its relatively compact and fully sequenced genome, ease of transformation, well developed genetics, and the availability of a dense physical map, rice is considered a model monocot system (Hsing et al., 2007; Miyao et al., 2007; Jung et al., 2008). However, although significant progress has been made in cloning rice disease resistance genes and functional genomics in general (Jung et al., 2008; Leung, 2008), very little is known about the effector responses and hormonal signaling pathways operative in determining rice resistance. In this study, we have analyzed the cellular and molecular basis of rice brown spot disease, caused by the fungal pathogen *Cochliobolus miyabeanus*. The data presented here offer a first insight into the myriad cellular responses that enable rice to fend off *C. miyabeanus* infection and have revealed

several heretofore unknown aspects of pathway crosstalk in the rice signaling circuitry. In particular, we have shown that exogenous application of ABA induces resistance against *C. miyabeanus* by suppressing pathogen-induced ET action. Furthermore, our data indicate that this ABA/ET antagonism is orchestrated by the ABA-inducible MAP kinase gene *OsMPK5*.

In contrast to the well-established role of ABA in abiotic stress adaptation (Fujita et al., 2006), its contribution to disease resistance is less well understood, and even contentious. Whereas the majority of reports have shown an inverse correlation between endogenous ABA levels and resistance to pathogens with diverse parasitic habits in several plant species (Audenaert et al., 2002a; Mohr and Cahill, 2003; Asselbergh et al., 2007; de Torres-Zabala et al., 2007), others have pinpointed a positive role for this hormone in plant defense activation (Ton et al., 2005; Adie et al., 2007; Hernandez-Blanco et al., 2007). This ambivalent ABA response is also reflected in rice-pathogen interactions. While our data uncover ABA as a powerful activator of resistance against *C. miyabeanus*, Koga et al. (2004a) previously reported that exogenous ABA treatment enhances basal susceptibility to the rice blast pathogen *Magnaporthe oryzae*. Even within the same plant-pathosystem, ABA can have divergent effects depending on the timing of infection. This was unambiguously shown in the Arabidopsis-*P. syringae* interaction, where ABA appears to have a role in both pre-invasion innate immunity and post-invasion virulence (Melotto et al., 2006; de Torres-Zabala et al., 2007). Hence, a complex picture is emerging in which ABA functions as a global multi-component regulator of biotic stress-response pathways.

In line with the multiplex role of ABA in regulating pathogen defense, a wide range of putative mechanisms underpinning ABA action have been proposed (Asselbergh et al., 2008b). One of the most comprehensively studied defense responses in relation to ABA-provoked fungal resistance is the enhanced deposition of callose at sites of attempted pathogen entry (Ton and Mauch-Mani, 2004; Kaliff et al., 2007; Flors et al., 2008). In our system, however, we found no compelling evidence for the involvement of callose, as pharmacological disruption of callose deposition with the callose synthesis inhibitor 2-DDG did not significantly interfere with the resistance response of ABA-treated plants (Fig. 7.3). Another important connection between ABA signaling and pathogen defense responses is the generation of reactive oxygen species, including the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (Torres et al., 2006). Tellingly, several lines of evidence indicate that the same NADPH-dependent respiratory burst oxidase homologs are involved in ROS formation leading to ABA-induced stomatal closure and elicitation of hypersensitive cell death in response to avirulent pathogen attack (Kwak et al., 2003; Torres et al., 2005). Under our experimental conditions, H_2O_2 in control plants started to accumulate from 12 hpi in the anticlinal walls of epidermal and mesophyll cells surrounding the

site of infection, where it was intimately associated with spreading cell death (Fig. 7.2F). In ABA-treated plants, however, H_2O_2 was often present from 8 hpi specifically in the anticlinal walls of infected epidermal cells, with neighboring non-penetrated cells remaining essentially free of H_2O_2 (Fig. 7.2G). Such bimodal H_2O_2 pattern suggests that *C. miyabeanus*, in spite of its generally assumed purely necrotrophic lifestyle, might have a short biotrophic phase in the epidermis during which it is sensitive to H_2O_2-dependent defenses. This is supported by the frequent observation of living though hyphae-containing epidermal cells in the early stages of fungal infection, i.e. prior to 12 hpi (Fig. 7.2F; data not shown). Accordingly, although not sufficient to block *C. miyabeanus* ingress, the prompt formation of H_2O_2 in ABA-treated plants may slow down fungal invasion, allowing the plant to adequately mobilize the available biochemical and structural defenses to effectively halt the invading pathogen in the mesophyll. On the other hand, one might speculate that H_2O_2 accumulation in non-penetrated control cells may support cell death to pave the way for *C. miyabeanus* in its necrotrophic growth stage.

One particularly interesting finding in this study was the hypersusceptibility of so-called *d1* mutant plants, which are defective in the sole heterotrimeric G-protein α-subunit gene present in rice (Fujisawa et al., 1999). Interestingly, the *d1* mutation not only affected basal resistance to *C. miyabeanus*, but also blocked the expression of ABA-IR, which points to a mechanistic connection between G-protein signaling and ABA-inducible pathogen resistance. This view is consistent with the recent discovery of a G protein-coupled receptor protein as a crucial ABA receptor mediating the large majority of ABA-responses in *Arabidopsis* (Liu et al., 2007a). Moreover, there is ample evidence indicating that ABA signaling processes in both seeds and guard cells involve components of the heterotrimeric G-protein complex, further supporting our hypothesis (Pandey and Assmann, 2004; Fan et al., 2008; Wang et al., 2008a). According to the G-protein signaling paradigm, the lack of Gα not only abolishes Gα-mediated signaling but also results in free Gßγ, thereby possibly enhancing Gßγ signal output (Pandey et al., 2006). The increased disease susceptibility and lack of ABA-IR observed in *d1* could therefore be accounted for by either loss of the corresponding Gα-mediated signaling or by the constitutive activation of the Gßγ subunit.

The involvement of Gα in basal and ABA-inducible defense to *C. miyabeanus* is consistent with a number of other reports implicating a role for this G-protein subunit in rice pathogen defense. For instance, upon infection with a virulent strain of bacterial blight (*Xanthomonas oryzae* pv. *oryzae*), symptom development in *d1* mutants is more severe than that in wild-type plants (Komatsu et al., 2004). Moreover, despite not being affected in basal resistance, *d1* mutant lines exhibit a highly reduced response upon inoculation with avirulent *M. oryzae* (Suharsono et al., 2002). Apparently contradictory results, however, were found in *Arabidopsis* where the Gα-

deficient mutant *gpa1-4* showed enhanced disease resistance to several necrotrophic pathogens, including *Plectosphaerella cucumerina* (Llorente et al., 2005; Trusov et al., 2006). In this context, it may be noteworthy that rice and *Arabidopsis* Gα-deficient mutants display remarkably different phenotypes. Most conspicuously, the *d1* mutation causes severe dwarfism, while in *Arabidopsis* a similar mutation results in rather the opposite effect, with mutants slightly larger than wild-type (Fujisawa et al., 1999; Ullah et al., 2003).

Similar to *d1*, the JA-deficient mutant *hebiba* failed to express resistance when induced by ABA, suggesting that ABA-IR might develop coincidently with increases in endogenous JA levels (Fig. 7.5A). Although such a concept would be consistent with recent results in *Arabidopsis* supporting a model for ABA inducing JA biosynthesis in the activation of defenses against the soil-borne oomycete *Pythium irregulare* (Adie et al., 2007), it is hard to reconcile with our findings that exogenous ABA treatment alleviated *C. miyabeanus*-induced activity of the key JA biosynthetic enzyme lipoxygenase and that ABA-IR was not affected upon infiltration of the LOX inhibitors ETYA and SHAM (Fig. 7.5B; data not shown). Moreover, in accordance with the failure of exogenous JA to induce brown spot resistance, transcriptional analysis of the JA-inducible *JIOsPR10* gene (Jwa et al., 2001) did not reveal any primed activity of the JA-defense pathway in ABA-treated plants (Figs. 7.1 and 7.5B). It can therefore be concluded that ABA-mediated protection against *C. miyabeanus* does not rely on potentiation of JA-inducible defenses. In a similar vein, the failure of BTH to reduce disease severity as well as the ability of ABA to trigger resistance in SA-deficient NahG rice also rule out a major involvement of the SA pathway.

Besides interactions with SA and JA, there is overwhelming evidence that ABA modulates ET-signaling (Beaudoin et al., 2000; Ghassemian et al., 2000; Tanaka et al., 2005). Although most examples of ABA/ET interactions have been described in sugar signaling (Leon and Sheen, 2003), Anderson et al. (2004) recently demonstrated the existence in *Arabidopsis* of an antagonistic ABA/ET connection that interferes with defense gene expression and disease resistance against the necrotroph *Fusarium oxysporum*. Interestingly, several lines of evidence suggest that such negative ABA/ET crosstalk also underlies the beneficial protective effect of ABA treatment on brown spot resistance. First, lesions caused by *C. miyabeanus* infection were more severe on wild-type seedlings pretreated with the ET-releasing chemical Ethephon, implying a negative role for ET in rice defense against this pathogen (Fig. 7.1C). Second, disruption of the ET pathway, either by antisense suppression of *OsEIN2*, a central signal transducer in the rice ET pathway (Jung et al., 2004), or infiltration of STS, a well-known inhibitor of ET action, yielded levels of protection similar to those observed in ABA-treated wild-type plants (Figs. 7.6A and B). Moreover, exogenous ABA treatment of the *OsEIN2* transgenics or co-application of ABA with STS did

not cause a further increase in resistance, suggesting that ABA specifically targets the ET pathway to condition brown spot resistance. Third, transcript levels of the ethylene-responsive transcription factor gene *EBP89* were markedly lower in ABA-treated plants than in wild-type after *C. miyabeanus* attack (Fig. 7.6C). Fourth, RNAi suppression of the ABA-inducible MAP kinase gene *OsMPK5* de-repressed *C. miyabeanus*-activated *EBP89* transcription, a phenomenon which coincided with a loss of ABA-IR (Fig. 7.7). Taken together, these results favor a model whereby ABA protects rice from *C. miyabeanus* attack by antagonizing pathogen-induced ET signaling in an *OsMPK5*-dependent manner. Implicit here is the view that *C. miyabeanus* hijacks the rice ET signaling pathway as a decoy strategy to suppress other, possibly ABA-dependent, defenses that normally serve to limit pathogen growth. In this respect, it is significant that several plant pathogens can produce ET themselves. Indeed, some *P. syringae* pathovars have shown an ability to synthesize ET both *in vitro* and *in planta* from methionine through the 2-keto-4-methylthiobutyric acid pathway (Weingart et al., 2001). This ability, together with the production of the JA mimic coronatine and auxins by the same microorganisms is assumed to contribute to hormonal saturation and consequent circumvention of effectual defenses (Cui et al., 2005; Sreedharan et al., 2006). More recently, *Ralstonia solanacearum* has been seen to produce ET by means of the HrpG regulon (Valls et al., 2006). Sufficient to affect the plant ET-response pathway, bacterial ET production is simultaneous with *TTSS* (type three secretion system) gene expression and contributes to the plant defense imbalance that favors pathogen infection. In light of these findings, it is not unlikely that *C. miyabeanus* may likewise synthesize ET in order to tap into the rice signaling infrastructure to interfere with host defense. Alternatively or in addition, *C. miyabeanus* may impact ET-responsive rice defenses via secretion of hereto-specified effector proteins. Whichever mechanism operative, manipulating plant hormone signaling and hijacking host hormonal cross-talk mechanisms represents an extremely powerful virulence strategy considering the global impact of hormone homeostasis on multiple cellular responses (de Torres-Zabala et al., 2007; Spoel and Dong, 2008).

Intriguingly, the concept that ABA-IR is based on *OsMPK5*-mediated repression of *C. miyabeanus*-induced ET action may also provide a mechanistic explanation for the aforementioned Gα-dependency of this resistance. In some interesting work using various *d1* mutant lines, Lieberherr et al. (2006) previously uncovered a pivotal role of Gα in modulating the stability and sphingolipid elicitor-induced activation of the MAP kinase OsMPK1, thus linking rice Gα to MAPK regulation. To our interest, these authors also demonstrated that RNAi-mediated knockdown of *OsMPK1* results in constitutive expression of *OsMPK5*, which is suggestive of potential crosstalk and possible functional redundancy between these evolutionary-related

MAPKs. Hence, taking these facts into account, it is not inconceivable that the inability of *d1* to develop ABA-IR against *C. miyabeanus* might result, at least in part, from a defect in OsMPK5 functioning. Analysis of the possible link between Gα, OsMPK5 and ABA-IR against *C. miyabeanus* will shed new light on ABA-induced resistance signaling and represents a major challenge for future research.

Previously, several other studies have assessed the effectiveness of ABA and ET signaling in the rice defense response. An interesting picture emerges when comparing our results with *C. miyabeanus* to those obtained with the leaf blast pathogen *M. oryzae*. For instance, whereas our data clearly indicate that ET action negatively interferes with resistance to *C. miyabeanus*, Iwai et al. (2006) recently proposed ET biosynthesis to be an integral component of *R*-gene-mediated resistance to blast. Taken together with the opposite effects of Ethephon application on blast and brown spot development (Singh et al., 2004; Fig. 7.1B), these findings argue that ET plays a dual role in the regulation of rice pathogen defense by alleviating stress caused by *M. oryzae* and promoting infection by *C. miyabeanus*. In support of this assumption, preliminary experiments revealed that antisense suppression of *OsEIN2* not only enhances resistance to *C. miyabeanus* but also renders plants more vulnerable to *M. oryzae* infection (De Vleesschauwer and Höfte, unpublished data). Intriguingly, the same *OsEIN2* antisense plants were also found to be more tolerant to cold and drought treatments (De Vleesschauwer and Höfte, unpublished data), suggesting that *OsEIN2* positively regulates ET signaling and *M. oryzae* resistance while repressing abiotic stress-adaptive ABA responses and resistance to *C. miyabeanus*. These data are particularly interesting in light of previous results showing that the RNAi *OsMPK5* transgenic line RI7, albeit deficient in ABA-IR against *C. miyabeanus* (Fig. 7.7B), exhibits constitutive expression of *PR* genes, increased levels of ET, enhanced resistance to both *M. oryzae* and the bacterial pathogen *Burkholderia glumae*, and reduced tolerance to cold, drought and salinity treatments (Xiong and Yang, 2003; Yang, 2007). Although further proof is needed, these transgene-conferred phenotypes suggest that *OsEIN2* and *OsMPK5* function as molecular switches between the ET and ABA signaling pathways, thereby differentially regulating *C. miyabeanus* defense and abiotic stress tolerance on the one hand, and resistance to *M. oryzae* as well as *B. glumae* on the other hand.

Conclusion

In summary, we have found that exogenous ABA recruits the mitogen-activated protein kinase *OsMPK5* to trigger resistance against *C. miyabeanus* by repressing pathogen-induced ET signaling. Moreover, the results presented here and those published previously (Xiong and Yang, 2003; Singh et al., 2004; Iwai et al., 2006) highlight the fine control of rice defenses to *C. miyabeanus* and *M. oryzae* through the differential engagement and balance of the ABA and ET response systems. Whether or not *C. miyabeanus* disease resistance shares substantial overlap with the signaling cascade(s) governing abiotic stress tolerance, and how upregulation of *OsMPK5* attenuates ET signaling remains to be elucidated.

Materials and Methods

Plant materials and growth conditions

Rice (*Oryza sativa*) lines used in this work included the *japonica* cultivar Nipponbare, the corresponding NahG (Yang et al., 2004) and RNAi *OsMPK5* transgenics (Xiong and Yang, 2003), and the *indica* cultivar CO39, the latter being a kind gift from the International Rice Research Institute (Manila, The Philippines). The Gα-deficient mutant *d1* (line DK-22), the *OsEIN2* antisense transgenic line 471 (wild-type: *japonica* cv. Dongyin), the JA-deficient mutant *hebiba* and the corresponding wild-type lines Nipponbare, Dongyin, and Nihonmasari, all *japonica*, were kindly provided by M. Matsuoka (Nagoya University, Japan), G. An (Yonsei University, Korea), and P. Nick (Karlsruhe University, Germany), respectively.

Seeds were surface sterilized with 2% sodium hypochlorite solution for 2 min, rinsed three times in sterile distilled water, and germinated on a wet sterile filter paper in sealed Petri dishes (≥ 92% relative humidity) at 28ºC. Five days later, germinated seeds were grown in commercial potting soil (Universal; Snebbout, Kaprijke, Belgium) under non-sterile greenhouse conditions (30 ± 4 ºC; 16 h light/8 h dark regime), as previously described (Chapter 3). Plants were watered daily and fertilized with 5g/m^2 $(NH_4)_2SO_4$ and 10g/m^2 $FeSO_4.7H_2O$ on day 8, 15, 22 and 29 after sowing. Five-week-old plants (6-7 leaf stage) were used for infection with *Cochliobolus miyabeanus*. For seed multiplication, plants were propagated in the greenhouse and fertilized with 0.5% ammonium sulphate every two weeks until flowering.

Pathogen inoculation and disease rating

Cochliobolus miyabeanus strains Cm988 and Cm963, obtained from the International Rice Research Institute (Manila, The Philippines), were grown for sporulation on PDA at 28ºC. Seven-day-old mycelium was flattened onto the medium using a sterile spoon and exposed to blue light (combination of Philips TLD 18W/08 and Philips TLD 18W/33) for 3 days to induce sporulation. Upon sporulation, conidia were harvested as described in Thuan et al. (2006) and re-suspended in 0.5% gelatin (type B from Bovine skin; Sigma-Aldrich G-6650) to a final density of 1 x 10^4 condia mL^{-1}. For inoculation, five-week-old seedlings (6.5-leaf stage) were misted with conidial suspension (1 mL per plant) using an artist airbrush powered by an air compressor. Immediately following inoculation, plants were moved into a dew chamber (30 ± 4ºC, ≥ 92% relative humidity) to facilitate fungal penetration, and, 18 h later, transferred to greenhouse conditions (28 ± 4 ºC; 16 h light, 8 h dark) for disease development. Disease symptoms were scored at four days after inoculation and disease ratings were expressed on the basis of diseased leaf area and lesion type using a 1-5 disease severity scale: I, no infection or less than 2% of leaf area infected with small brown specs less than 1 mm in diameter; II, less than 10% of leaf area infected with brown spot lesions with gray to white center, about 1-3 mm in diameter; III, average of about 25% of leaf area infected with brown spot lesions with gray to white center, about 1-3 mm in diameter; IV, average of about 50% of leaf area infected with typical spindle-shaped lesions, 3 mm or longer with necrotic gray center and water-soaked or reddish brown margins, little or no coalescence of lesions; V, more than 75% of leaf area infected with coalescing spindle-shaped lesions. All infection trials were repeated at least twice with similar results.

Chemical treatments

JA, ABA, Ethephon (2-chloroethyl phosphonic acid) and BABA were purchased from Sigma (Bornem, Belgium). BTH (BION 50 WG), formulated as a water-dispersible granule containing 50% active ingredients, was a gift from Syngenta Crop Protection (Brussels, Belgium). BTH, SA and BABA were directly dissolved in water containing 0.02% (v/v) Tween 20, whereas ABA and JA were first dissolved in a few drops of ethanol and methanol, respectively. Equivalent volumes of both solvents were added to separate control treatments to ensure that they did not interfere with the experiments. For chemical treatment of plants, intact seedlings (6.5-leaf stage) were sprayed until near runoff with a fine mist of either compound at the indicated concentrations. Control plants were sprayed evenly with a 0.02% (v/v) Tween 20 solution only. Three days post-application, chemical-treated plants were challenged with *C. miyabeanus* as described above. ß-aminobutyric acid (BABA) was applied as a soil drench (16 or 30 mg L^{-1}) 1 or 2 days prior to challenge inoculation. Only soil-drench treatments were used to avoid formation of necroses observed after spraying because such necroses might induce a SAR-like resistance pathway and mask the primary effect of BABA (Zimmerli et al., 2000).

Visualisation of defense responses

To gain more insight into the cytomolecular nature of ABA-inducible resistance against *C. miyabeanus*, intact leaf sheath assays were performed as stated in Koga et al. (2004b). Briefly, leaf sheaths of the sixth leaves of rice plants at the 6.5 leaf stage were peeled off with leaf blades and roots. The leaf sheath was laid horizontally on a support in plastic trays containing wet filter paper, and the hollow space enclosed by the sides of the leaf sheaths above the mid vein was filled with a conidial suspension of *C. miyabeanus* (1 x 10^4 conidia.mL^{-1}). Inoculated leaf sheaths were then incubated at 25ºC with a 16-h photoperiod. When ready for microscopy, the sheaths were hand-trimmed to remove the sides and expose the epidermal layer above the mid vein. Lower mid vein cells were removed to produce sections three to four cell layer thick. For time-course experiments, sheath sections were generally sampled at 6, 8, 10, 12, 18, 24, 36, and 72 h post inoculation and at least six trimmed sheath tissue sections originating from three plants were used for each sampling point. Intracellular hyphae were visualized using a modified KOH-aniline blue technique (Hood and Shew, 1996). Fresh specimens were autoclaved for 10 min at 121ºC in 1 M KOH, followed by three rinses in demineralized water. From demineralized water, specimens were mounted on glass slides in several drops of the stain solution and examined under UV excitation. The stain solution was prepared at last 2 h prior to use as 0.05% aniline blue dye in 0.067 M K_2HPO_4 at pH 9.0. Alternatively, hyphae were stained with 0.1% calcofluor M2R for 1 min and rinsed with demineralized water before microscopic observation. Phenolic compounds, on the other hand, were visualized as autofluorescence under blue light epifluorescence (Olympus U-MWB2 GPF filter set-excitation: 450 to 480 nm, dichroic beamsplitter; 500 nm, barrier filter BA515). To detect H_2O_2 accumulation, staining was according to the protocol of Thordal-Christensen et al. (1997) with minor modifications. Six hours before each time point, trimmed sheath segments were vacuum-infiltrated with an aqueous solution of 3,3'-diaminobenzidine(DAB)-HCL (1 mg mL^{-1}; pH = 3.8) for 30 min. Infiltrated segments were then further incubated at room temperature in abovementioned DAB solution until sampling. DAB polymerizes in the presence of H_2O_2 and endogenous peroxidase to form a brownish-red precipitate that can be easily visualized using bright-field microscopy. Specificity of the DAB staining was verified by adding 10 mM ascorbic acid to the DAB solution. For analysis of callose deposition, trimmed sheaths were stained for 5 min in a solution containing 0.01% (w/v) of aniline blue and 0.15 M K_2HPO_4. Callose-stained segments were examined using epifluorescence microscopy with UV filter (Olympus U-MWU2 filter set-excitation: 330-385 nm, DM 400 dichroic beam splitter and BA420 long-pass filter). After staining, trimmed sheath segments were mounted in 50% glycerol. Images were acquired digitally (Olympus Color View II camera, Aartselaar, Belgium) and further processed with the Olympus analySIS cell^F software.

Pharmacological experiments

Aminooxyacetic acid (AOA), a potent inhibitor of ET biosynthesis, the LOX inhibitors ETYA, and the callose synthase inhibitor 2-DDG were purchased from Sigma (Bornem, Belgium). Silver thiosulfate (STS), an inhibitor of ethylene action, was prepared by mixing solutions of 0.1 M sodium thiosulfate with 0.1 M of silver nitrate in a 4:1 ratio (Shoresh et al., 2005). All chemicals were dissolved in water at the indicated concentrations, with exception of ETYA, which was solubilised in a few drops of ethanol prior to diluting in water (0.2% ethanol).

For all experiments, fifth and sixth stage leaves of five-week-old rice plants were excised and cut into 7-cm segments. To evaluate the contribution of callose formation to the ABA-induced resistance, freshly detached leaf blades were fed from the cut base with a solution containing 0.1 mM ABA and 0.25 or 2.5 mM 2-DDG. After 24 h incubation, the treated segments were placed onto a glass slide in 14.5 by 14.5 cm Petri dishes lined with moist filter paper, and drop-inoculated with five 10 µl droplets of *C. miyabeanus* conidial suspension (5 x 10^4 conidia mL^{-1} in 0.25% gelatin). Control leaves were mock-inoculated with a 0.25% (wt/vol) gelatin suspension. After 24 h, the droplets were removed with a laboratory tissue. Resistance was quantified by measuring lesion diameters 60 hpi. In the case of ETYA, AOA and STS, a slightly different application method was used in that the respective chemicals were infiltrated in approximately 20 µl aliquots into five sites on the abaxial surface of detached leaf segments using a syringe without a needle. Approximately 8 h later, 10 µl of *C. miyabeanus* conidial suspension (5 x 10^4 conidia mL^{-1} in 0.25% gelatin) was drop-inoculated onto the centre of the infiltrated regions.

Enzyme extraction and LOX activity assay

Leaf samples taken from the fifth and sixth stage leaves of ~6 to 10 plants at different time points after inoculation were crushed to a fine powder under liquid nitrogen. Soluble proteins were extracted by re-suspending the powder (100 mg fresh weight) in 0.9 mL of 50 mM Na_3PO_4 buffer, pH 6.5, containing 2% polyvinylpyrrolidone, 5 mM 2-mercaptoethanol, and 0.25% Tween 20. The extracts were then incubated on ice for 30 min and centrifuged at 14,000 rpm for 10 min. The resulting supernatant was divided into aliquots, frozen in liquid nitrogen, and stored at -80ºC for further analysis. LOX activity was determined at 30ºC in 1 mL (final volume) of 50 mM Na_3PO_4 buffer, pH 6.5, containing 0.25% Tween 20 and 30 µL of extract supernatant. The reaction was started by adding 100 µL of 10 mM linoleic acid and the increase in absorbance at 234 nm was recorded for 10 min. LOX enzyme activity was calculated based on the slope of the linear part of the plot and expressed as ΔExt_{234} per min per mg protein. Controls without the addition of plant extracts were recorded as described above and subtracted from the values obtained with the plant extracts. Protein levels in enzyme extracts were determined by the Bradford method (Bradford, 1976) with bovine serum albumen as a standard.

RNA extraction, cDNA synthesis and quantitative RT-PCR analysis

Total RNA was isolated from frozen leaf tissue using the Invisorb Spin Plant RNA Mini kit (Invitek, Berlin,Germany) and subsequently Turbo DNase-treated according to the provided protocol (Ambion/Applied Biosystems, Lennik, Belgium). Before first-strand cDNA synthesis, the absence of genomic DNA was confirmed by PCR. RNA concentration was checked before and after Turbo DNase digestion. First-strand cDNA was synthesized from 2 μg of total RNA using Affinityscript reverse transcriptase and oligo dT primers (Stratagene/Bio-Connect, Huissen, The Netherlands), according to the manufacturer's instructions. Nucleotide sequences of all primers are given in Table 7.2. For each primer pair, the optimal annealing temperatures were predetermined by gradient PCR using a Thermocycler (Bio-Rad). Only primer pairs, for which PCR efficiency varied between 90 and 110%, as determined by standard amplification curves constructed from 5-fold dilutions of cDNAs, were used for expression studies. Specific amplification was checked using melting curves of qPCR products. For each target, primer concentrations were optimized by performing a primer titration. Quantitative PCR amplifications were conducted in optical 96-well plates with the Mx3005P real-time PCR detection system (Stratagene, Amsterdam, Holland), using Sybr Green master mix (Stratagene/Bio-Connect, Huissen, The Netherlands) to monitor dsDNA synthesis. The expression of each gene was assayed in triplicate in a total volume of 25 μl including a passive reference dye (ROX) according to the manufacturer's instructions (Stratagene). The thermal profile used consisted of an initial denaturation step at 95ºC for 10 min, followed by 40 cycles of 95ºC for 30 s, 58-62ºC for 60 s, and 72ºC for 60 s. To verify amplification of one specific target cDNA, a melting-curve analysis was included according to the thermal profile suggested by the manufacturer. The amount of plant RNA in each sample was normalized using actin (Os03g50890) as internal control and samples collected from control plants at 0 h post inoculation were selected as a calibrator. The generated data were analyzed with the Mx3005P software (Stratagene). For all amplification plots, the optimal baseline range and threshold cycle values were calculated using the Mx3005P algorithm. Gene expression in control and ABA-treated samples was expressed relative to the calibrator and as a ratio to actin expression using the measured efficiency for each gene.

Table 7.2. *Gene-specific primers for quantitative real-time PCR*

Genes	Genbank accession number	Forward (5'-3')	Reverse (5'-3')
Actin	X15865	GCGTGGACAAAGTTTTCAACCG	TCTGGTACCCTCATCAGGCATC
OsPR1b	U89895	GGCAACTTCGTCGGACAGA	CCGTGGACCTGTTTACATTTT
PR10/PBZ1	D38170	CCCTGCCGAATACGCCTAA	CTCAAACGCCACGAGAATTTG
JIOsPR10	AF395880	CGGACGCTTACAACTAAATCG	AAACAAAACCATTCTCCGACAG
EBP89	AJ304840	TGACGATCTTGCTGAACTGAA	CAATCCCACAAACTTTACACA
OsMPK5	AY026332	TCGATCATCCTTACCTAGAGA	TCATTTGGTCCTCGTTTAGAG

Chapter

8

Conclusions and Further Perspectives

In the absence of adaptive immunity displayed by animals, plants respond locally to biotic challenges via inducible basal defenses activated through recognition and response to conserved pathogen-associated molecular patterns. In addition to this attacker-specific primary immune response, plants can activate an additional layer of defense, thereby enhancing their defensive capacity against future attack. This so-called induced resistance often spreads systemically throughout the plant and is typically characterized by a broad spectrum of effectiveness. Depending on the organism interacting with the plant, different types of induced resistance are activated, such as systemic acquired resistance (SAR), which is initiated after gene-for-gene recognition between plant resistance proteins and microbial effector molecules (Murray and Grant, 2007), and induced systemic resistance (ISR), which is triggered upon colonization of roots by selected strains of benign rhizobacteria (Van Loon et al., 1988). Although both pathogen-induced SAR and rhizobacteria-governed ISR could provide an ecologically sound and economically viable mean of disease control, research aimed at exploring the underlying molecular mechanisms has been conducted almost exclusively in experimentally tractable model plants such as Arabidopsis, rather than in economically important cereal crops. In view of this knowledge gap, the primary objectives of this work, as stated in Chapter 1, were:

- (i) to assess whether rhizobacteria able to trigger ISR in dicots are also capable of inducing resistance in rice, a monocot model system, against a number of pathogens with distinct parasitic habits, and if so, to gain insight into the various aspects of ISR-associated resistance, ranging from the perception of bacterial resistance determinants to long-distance signal transmission and the manifestation of plant effector responses.
- (ii) to advance our understanding of the role of hormonal resistance pathways in inducible pathogen defense of rice and to elucidate how crosstalk between such signaling cascades affects the interaction of rice with the fungal pathogen *Cochliobolus miyabeanus*, causal agent of the devastating brown spot disease.

8.2. Major research findings and practical implications

8.2.1. Rice defense against *M. oryzae*, *R. solani*, and *C. miyabeanus*: differences and similarities

A potent elicitor of resistance in bean, tobacco, tomato and *Arabidopsis* against a fairly broad range of bacterial, viral and fungal pathogens (Audenaert et al., 2002b; Bigirimana and Höfte 2002; De Meyer and Höfte, 1997; De Meyer et al., 1999a,b; Ran et al., 2005b), the root-colonizing *P. aeruginosa* strain 7NSK2 was found to induce ISR against the rice blast-causing ascomycete *M. oryzae* (Chapter 3). Extensive mutant analysis and testing of purified bacterial metabolites uncovered the blue phenazine pigment pyocyanin as an essential determinant of this 7NSK2-mediated ISR. Yet, a different picture emerged when challenging with the necrotrophic sheath blight pathogen *R. solani*. While the wild-type strain 7NSK2 proved unable to significantly reduce sheath blight severity, root colonization with the pyocyanin-negative mutant 7NSK2-phzM triggered substantial levels of ISR. Hence, pyocyanin appears to act as a two-faced ISR elicitor that positively modulates 7NSK2-induced protection against *M. oryzae* while 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 phenazine antibiotic in 7NSK2-ISR as exogenous application of H_2O_2-quenching sodium ascorbate alleviated the contrasting effects of pyocyanin on *R. solani* and *M. oryzae* pathogenesis. Interestingly, similar results were obtained in response to root treatment with the *Serratia plymuthica* strain IC1270. Although highly effective against *M. oryzae*, arresting the pathogen in its biotrophic phase by boosting infection-induced H_2O_2 accumulation in the epidermis, IC1270 colonization resulted in enhanced tissue colonization by *R. solani*, an effect possibly due to stimulation of fungal toxin-triggered host cell death. Overall these results not only strengthen the notion that pathogenesis-related ROS formation can cascade either to the benefit or detriment of the plant, but also disclose that rice requires distinct resistance mechanisms to fend off *R. solani* and *M. oryzae*. From a practical point of view, this notion may have important ramifications with respect to molecular resistance breeding. Indeed, considering that the effect of ROS-fueled HR-like cell death varies dramatically according to the mode of infection and parasitic habits of the invading pathogen, the widespread cultivation 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, our observation that pre-inoculation with an avirulent HR-triggering *M. oryzae* strain favors subsequent infection with *R. solani* is of particular interest and may explain why there are no HR-

associated qualitative resistance phenomena known against this pathogen (De Vleesschauwer and Höfte, unpublished results).

Like *R. solani*, *C. miyabeanus* is generally considered a necrotrophic pathogen that depends exclusively on dead host tissue for nutrition and reproduction (Ou, 1985; Xiao et al., 1991). Consistent with this view, plant treatment with HR-eliciting pyocyanin or ROS-boosting IC1270 bacteria facilitated rapid growth and spread of *C. miyabeanus* in a manner similar to what was observed for *R. solani*, indicating a common pathogenicity mechanism for both these necrotrophs (Chapter 3). However, these apparent similarities notwithstanding, accumulating evidence suggests that also against the latter pathogens distinct resistance mechanisms are operative. Support for this notion is provided by the observations that exogenous ABA treatment, albeit highly effective against *C. miyabeanus* (Chapter 7), failed to reduce sheath blight severity (De Vleesschauwer and Höfte, unpublished results), whereas topical application of riboflavin, a water-soluble B vitamin thought to function via activation of JA-dependent defenses (Taheri, 2007), was found to induce resistance to *R. solani* while increasing susceptibility to *C. miyabeanus* (Taheri, 2007; De Vleesschauwer and Höfte, unpublished results). Taken together, a complex picture is emerging in which resistance against the major fungal rice pathogens, *M. oryzae*, *R. solani*, and *C. miyabeanus* is mediated by distinct, at least partly antagonistic, defense mechanisms.

8.2.2. Rice is endowed with multiple blast-effective resistance pathways.

Induced resistance often involves the activation of a large spectrum of inducible defense mechanisms, including the accumulation of defensive compounds with antimicrobial activity, enhanced strengthening of plant cell walls and the concerted expression of a battery of defense-related genes. However, in many cases the enhanced defensive capacity in induced plants is not associated with a direct activation of immune responses, but with priming for enhanced defense (Conrath et al., 2006; Frost et al., 2008). 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 of priming 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 broad-spectrum resistance (Van der Ent et al., 2008a).

In keeping with an extensive body of work in dicot plants, this dissertation revealed priming to be a common defense strategy that is implicated in various types of microbially and chemically induced pathogen resistance in rice. For instance, in chapter 4, evidence is reported that root

colonization by *S. plymuthica* IC1270 primes rice for boosted generation of H_2O_2, leading to an accelerated expression of HR-like cell death at sites of attempted pathogen entry and significantly enhanced resistance to *M. oryzae*. Priming was also shown to constitute a crucial facet of the resistance mechanism underpinning blast resistance induced by either 7NSK2-derived pyocyanin or the synthetic SA analog benzothiadiazole (BTH). Interestingly, histochemical analysis of the early infection events in pyocyanin- and BTH-supplemented blast resistant plants revealed that the latter compounds partially mimic IC1270 bacteria in that they were found to activate a similar set of defense reactions, characterized by hypersensitively dying cells in the vicinity of fungal hyphae (Chapter 3; Chapter 5; Chen et al., 2007). Although it does not follow that the signal transduction cascade(s) operative in IC1270-mediated ISR is (are) necessarily the same as that (those) leading to pyocyanin- or BTH-inducible blast resistance, such commonalities apparent at the level of defense mobilization suggest that these elicitors may feed into related, if not identical, resistance pathways. Further supporting this hypothesis is the overlap manifest at the level of resistance to attackers, with IC1270, BTH and 7NSK2 all being ineffective or even increasing vulnerability to *C. miyabeanus* and *R. solani* (Ahn et al., 2005b; Chapter 3; Chapter 4; Taheri, 2007). Induction of ISR by the *P. fluorescens* strain WCS374r, however, appears to rely on a different resistance mechanism and was associated with pseudobactin-mediated priming of naïve leaves for a wide array of HR-independent cellular defenses, the most prevalent component being the prompt attacker-induced manifestation of invading hyphae-embedding tubules (Chapter 5). Although further proof is needed, this apparent plasticity in the molecular processes leading to induced resistance against *M. oryzae* strongly suggests that rice possesses multiple blast-effective resistance pathways. Depending on the outcome of possible cross-talk between the latter pathways, concomitant activation of distinct systemic defense mechanisms may provide an attractive tool for the improvement of blast control. Support for this hypothesis can be deduced from the work of Van Wees et al. (2000). In this intriguing study, the authors demonstrated using various mutant and transgenic Arabidopsis lines that SA-dependent SAR and JA/ET-dependent ISR act independently and additively to increase resistance against the bacterial speck pathogen *P. syringae* pv. *tomato*. In a similar vein, simultaneous treatment of tomato seedlings with BTH and ISR-inducing PGPRs was found to be compatible in greenhouse experiments and reduced incidence of bacterial wilt caused by *Ralstonia solanacearum* (Anith et al., 2004). Taking these facts into account, it will be particularly interesting to assess whether such positive interplay also holds for a combination of WCS374r-mediated ISR and IC1270-, 7NSK2- or BTH-inducible blast resistance, to analyze the spectrum of effectiveness of the resistance

conferred and, most importantly, to evaluate the impact of such extensive defense elicitation on growth rate, plant development and crop yield.

8.2.3. Rice and Arabidopsis share evolutionary conserved defense signaling networks

Apart from their roles in plant development, plant hormones have repeatedly been implicated in the regulation of primary and induced defense responses. Upon pathogen infection, plants respond by producing a specific blend of these signaling molecules, resulting in the activation of disparate sets of defense-related genes (Maleck et al., 2000; De Vos et al., 2005; Wise et al., 2007). Historically, research aimed toward deciphering the role of hormones in plant pathogen defense has tended to focus on the involvement of SA. However, in contrast to the well-established role of SA as a global multicomponent regulator of resistance to mainly biotrophic pathogens in dicots, its contribution to disease resistance in rice is less well understood, and even contentious. Unlike most other plants, rice is endowed with very high endogenous SA levels that do not increase upon pathogen attack, which has led to the suggestion that SA is not an effective defense signal in rice (Silverman et al., 1995; Yang et al., 2004). Yet, this interpretation is hard to reconcile with the often reported ability of benzothiadiazole (BTH), a SA analog, to trigger resistance against, amongst others, *M. oryzae* and the bacterial leaf blight pathogen, *Xanthomonas oryzae* pv. *oryzae* (Schweizer et al., 1999; Nakashita et al., 2003; Shimono et al., 2007; Chapter 2; Chapter 5). A series of elegant papers, however, seem to have put a hold on this controversy and suggest that rice, in spite of its high endogenous SA content, has evolved a BTH-inducible SA-dependent resistance pathway that shares downstream components with the archetypal SAR pathway described in Arabidopsis and tobacco (Chern et al., 2001; Fitzgerald et al., 2004; Chern et al., 2005b; Shimono et al., 2007; Yuan et al., 2007). In Chapter 5, we document for the first time a similar phenomenon with respect to ISR-induced resistance signaling. Using various mutant and transgenic lines affected in either the biosynthesis or perception of SA, JA and ET, it was shown that WCS374r-ISR to *M. oryzae* is independent of SA but reliant on a functional JA pathway as well as on intact responsiveness to ET. In this respect, WCS374r-ISR mirrors classic ISR induced in Arabidopsis by the reference strain *P. fluorescens* WCS417r (Pieterse et al., 1996; Pieterse et al., 1998). Further support for this hypothesis came from the finding that, unlike BTH, hydroponic feeding of pseudobactin, a crucial determinant of WCS374r-ISR, did not result in direct transcriptional activation or priming of SA-inducible *PR* gene expression (Chapter 5). When considered together, these findings not only consolidate the earlier contention that rice is endowed with a SAR-like resistance route but also infer that rice and Arabidopsis share a conserved ISR pathway, implying that fundamental modes of resistance

elicitation and induced defense signaling have been conserved through plant evolution and diversification. It should be noted, however, that this concept does not exclude that the fine-tune regulation of such conserved resistance pathways may differ across individual plant species. Indeed, whereas the observation that WCS374r-ISR is attenuated in the JA-deficient mutant *hebiba* argues that in rice de novo JA biosynthesis is indispensable for ISR, in Arabidopsis ISR seems to be based on an enhanced sensitivity to JA, rather than on an increase in its production (Pieterse et al., 2000; Pozo et al., 2008). In conjunction with the divergent effects of NPR1-overexpression on plant growth and defense responses in both plant species (Cao et al., 1998; Chern et al., 2001; Fitzgerald et al., 2004; Chern et al., 2005b), it therefore appears that although rice and Arabidopsis share ancient plant-inducible defense pathways, the modulation of these resistance conduits and the links to other plant pathways may be quite divergent.

8.2.4. Decoy of plant defense: pathogens hijack hormonal crosstalk mechanisms as a virulence strategy

During the evolutionary arms race between plants and their intruders, the latter class of organisms has evolved a wide array of sophisticated mechanisms to circumvent, or even attenuate plant defense. Thus, many plant-pathogenic bacteria as well as fungi and oomycetes subdue host responses by injecting a large repertoire of effector proteins into host cells, thereby enabling successful infection and reproduction *in planta* (Jones and Dangl, 2006). While a number of these effector molecules disable PTI and ETI by targeting various defense-associated proteins, others induce specific host genes to enhance plant susceptibility (Rooney et al., 2005; Tian et al., 2005; Abramovitch et al., 2006; Yang et al., 2006; Bittel and Robatzek, 2007; Kramer et al., 2007; Xiang et al., 2007; Sugio et al., 2007; Zhou and Chai, 2008). Alternatively, microbial pathogens may disarm the plant's weaponry by manipulating plant hormone signaling pathways. For instance, in current research evidence was brought forward to demonstrate that the brown spot pathogen *C. miyabeanus* co-opts the ET response pathway as a virulence strategy (Chapter 7). This notion was borne out by the observation that *C. miyabeanus* infection entails a strong upregulation of ET-responsive gene expression, and was further supported by various experiments demonstrating that genetic, pharmacological or ABA-mediated repression of ET signaling renders plants considerably more resistant. Activation of the MAP kinase gene *OsMPK5* was shown to be essential in establishing resistance in ABA-induced plants, because *OsMPK5* RNAi plants were specifically blocked in aforementioned ABA-mediated repression of ET signaling and consequently failed to express ABA-IR. Besides implicating a role for the ET pathway in the pathogenicity of *C. miyabeanus*, these data favor a model whereby ABA protects rice from brown

spot infection by antagonizing pathogen-induced ET action in an *OsMPK5*-dependent manner. Considering the inhibitory effects of ET on, amongst others, the ABA signaling pathway (Chapter 2; Anderson et al., 2004; Zhou et al., 2007), one may envision that *C. miyabeanus* activates the ET pathway as a decoy to suppress other, possibly ABA-dependent, effectual host defense responses. In line with this concept, there is substantial evidence indicating that the ability to modify phytohormone signaling is indispensable for a pathogen to be successful. For example, several fungi and bacteria, including the bacterial speck pathogen *P. syringae* and the necrotrophic fungus *Botrytis cinerea*, have been shown to manipulate components of the ABA biosynthetic and response machinery as an essential strategy to cause disease (Audenaert et al., 2002a; Asselbergh et al., 2007; de Torres-Zabala et al., 2007). In addition to modifying plant hormone synthesis, microbial pathogens can also manipulate the plant's signaling infrastructure by producing phytohormones or functional mimics thereof to 'trick' the plant into activating inappropriate defenses (Robert-Seillaniantz et al., 2007; Koornneef and Pieterse, 2008; Angel-Lopez et al., 2008). An archetypical example reflecting this situation is the production by some virulent *P. syringae* strains of a phytotoxin called coronatine that structurally resembles JA derivatives, including JA-Isoleucine (Staswick, 2008). Working with COR-deficient *P. syringae* mutants and plants impaired in SA or JA signaling, several groups have demonstrated that *P. syringae* employs COR to hyperactivate JA signaling, resulting in suppression of SA-mediated defense through antagonistic cross-talk (Brooks et al., 2005; Cui et al., 2005; Laurie-Berry et al., 2006; Koornneef and Pieterse, 2008). Recently, COR was also shown to facilitate bacterial invasion by repressing ABA-mediated stomatal closure (Melotto et al., 2006). Production of gibberellic acid, cytokinin and auxin has also been described for multiple plant pathogens (Valls et al., 2006; Robert-Seillaniantz et al., 2007). Like ABA and COR, most of these hormones are known to stimulate pathogen virulence through various crosstalk mechanisms, many of which involve negative interactions with effectual SA- or JA-dependent defense pathways (Navarro et al., 2007; Wang et al., 2007a; Angel-Lopez et al., 2008; Spoel and Dong, 2008). Together these findings suggest that manipulating the plant's defensive machinery by exploiting hormonal cross-talk mechanisms is a common virulence strategy amongst plant attackers, controlling the outcome of numerous plant-microbe interactions. Fresh insights into hormone cross-talk and the various strategies used by pathogenic microorganisms to manipulate these specialized host processes is central to our understanding of plant immune responses and will undoubtedly contribute to designing effective novel strategies for engineering durable, broad-spectrum disease resistance in crop species.

8.3. Future perspectives

Although this research has significantly advanced our mechanistic understanding of induced pathogen resistance in rice, several aspects still deserve further experimental investigation. In addition, this work has opened up several perspectives for future research.

- Histochemical, biochemical and molecular analysis of rhizobacteria- or ABA-induced rice seedlings has uncovered a role for reactive oxygen species, cell wall fortification, hypersensitive response-like cell death, and defense gene expression in the establishment and/or maintenance of the induced defense state (Chapters 3, 4, 5, and 7). However, this does not exclude the involvement of other types of biochemical defense responses in the resistant reaction. Further analysis of IR-associated defense mobilisation could employ a combined transcriptome, proteome, and metabolome profiling to derive a holistic picture of the various IR phenomena observed in this study.

- In Chapter 3, we demonstrate that low-level systemic generation of pyocyanin-induced reiterative H_2O_2-microbursts in naïve leaves is a prerequisite for the successful establishment of *P. aeruginosa* 7NSK2-mediated ISR against *M. oryzae*. Several ROS-generating mechanisms have been identified, of which cell wall- and apoplast-localized peroxidases and plasmalemma-bound NADPH oxidases have received a great deal of attention (Apel and Hirt, 2004; Van Breuseghem et al., 2008). The use of inhibitor compounds blocking specific ROS-producing enzymes, organelle-specific activity measurements of enzymes involved in controlling ROS homeostasis and monitoring the expression of redox-associated genes can reveal the mechanisms that allow 7NSK2-colonized plants to hyperinduce HR-like cell death and should provide insights on the action of 7NSK2 upstream of ROS generation. A similar approach could be employed to unequivocally delineate the involvement of oxidative events in the IC1270-induced ISR response.

- Necrotrophic pathogens have long been thought of as aggressive, indiscriminate pathogens that simply ramify through defenseless plant tissue, thereby killing host cells by means of a varied arsenal of lytic enzymes and toxic molecules without having much of a real 'interaction' with their host (van Kan, 2006). Emerging evidence, however, adds some nuances to this view and suggests that necrotrophs, like their biotrophic counterparts, develop a more sophisticated relationship with their host by co-opting the

plant's apoptotic pathways. This notion was borne out by the finding that ectopic expression of metazoan cytoprotective PCD inhibitor proteins in transgenic tobacco and tomato conferred heritable resistance to a wide range of necrotrophic pathogens and toxins derived thereof (Dickman et al., 2001; Lincoln et al, 2002; El Oirdi and Bouarab, 2007). Consistent with animal antiapoptotic proteins protecting plants from necrotrophic pathogen attack is the large body of evidence indicating that host cell death induced by archetypal necrotrophs such as *Sclerotinia sclerotiorum*, *B. cinerea* or *Cochliobolus victoriae* exhibits several biochemical and morphological hallmarks of PCD, including DNA laddering, nuclear condensation and enhanced caspase-like activity (Dickman et al., 2001; Govrin and Levine, 2001; Hoeberichts et al., 2003; Coffeen and Wolpert, 2004). Considering the ambivalent role of HR-eliciting pyocyanin with respect to 7NSK2-mediated ISR (Chapter 3) and the opposite effects of IC1270 bacterization on blast, sheath blight and brown spot development (Chapter 4), it is not inconceivable that *R. solani* and *C. miyabeanus* may likewise cause disease by hijacking the rice PCD machinery. Support for this hypothesis can be inferred from the specific down-regulation of a 14-3-3 protein in a *R. solani*-resistant rice mutant (Lee et al., 2006). A role for 14-3-3 proteins in the elicitation of PCD is implied by experiments in powdery mildew-infected barley, where 14-3-3 proteins were found to activate the plasma membrane H^+-ATPase of inoculated epidermal cells, a process proposed to switch on a signaling cascade leading to HR manifestation (Zhou et al., 2000; Finnie et al., 2002). Furthermore, suppression subtractive hybridization (SSH) analysis of *R. solani*-infected rice revealed that sheath blight development correlates with down-regulation of *OsGPX1* (Zhao et al., 2008). Interestingly, this phospholipid hydroperoxide glutathione peroxidase is the closest rice homolog of tomato LePHGPx, a potent antagonist of mammalian BAX-induced cell death known to confer resistance to *B. cinerea* in tobacco (Chen et al., 2004). Further support for the idea that *R. solani* and *C. miyabeanus* may actively engage rice PCD as a pathogenicity strategy might come from the study of rice BAX-INHIBITOR 1 (Matsumura et al., 2003). Coding for a structurally and functionally conserved ancient cell death suppressor protein (Hückelhoven et al., 2003), *BI-1* has been reported to be suppressed upon exposure of rice suspension cultures to a *M. oryzae*-derived cerebroside elicitor, a phenomenon culminating in the prompt execution of HR-like cell death. Since cerebroside elicitors isolated from *R. solani* and *C. miyabeanus* are as effective in inducing HR-like cell death as those derived from *M. oryzae* (Umemura et al., 2002), it is not unlikely that the former pathogens likewise antagonize *BI-1* expression to promote their

virulence. Given the recent emergence of PCD suppressor genes such as *BI-1* and baculovirus *p35* as workable targets for genetic engineering of heritable necrotroph resistance in plants (Dickman et al., 2001; Lincoln et al., 2002; Imani et al., 2006; El Oirdi and Bouarab, 2007), the potential role of PCD regulation in rice defense against necrotrophic pathogen attack is worthy of further investigation.

- Induced resistance by WCS374r against *M. oryzae* depends exclusively on bacterial production of a pseudobactin-type siderophore (Chapter 5). Despite the widespread interest, it is still unknown how pseudobactins are perceived and ultimately give rise to ISR. An alternative to direct recognition of pseudobactin elicitors by the plant is the perception of microbially induced alterations in the plant's immediate environment, i.e. the rhizosphere (Van der Ent et al., 2008b). Given the scarcity of bioavailable iron [Fe(III)] in the rhizosphere, 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 (Vansuyt et al., 2007). In this respect, our recent observation that WCS374r aggravates chlorosis symptoms of young rice plants grown under iron-limiting conditions is of particular interest (De Vleesschauwer and Höfte, unpublished results). Strikingly, enhanced iron deficiency chlorosis was not observed in response to root colonization with rhizobacteria producing ISR-deficient pseudobactins, such as *P. aeruginosa* 7NSK2 or *P. putida* WCS358 (De Vleesschauwer and Höfte, unpublished results). Furthermore, hydroponic feeding of Psb374 was found to trigger intracellular iron depletion in systemic leaves as evidenced by the down-regulated expression of the iron homeostasis marker gene *OsFer1* (De Vleesschauwer and Höfte, unpublished results). These findings suggest that the ability of a given pseudobactin to increase blast resistance is related to its potential to deprive rice from iron. Interestingly, in a recent microarray study on iron-deficient rice, Kobayashi et al. (2005) found that iron deficiency in roots strongly induces the expression of genes involved in every predicted step of the methionine cycle, both in root and leaf tissue. Furthermore, several studies point to a role for the methionine cycle and its main intermediate, the universal substrate S-adenosyl-L-methionine (SAM), in rice defense responses to *M. oryzae*. Most tellingly in this regard, Seguchi et al. (1992) reported that the activity of the SAM utilising enzyme S-adenosyl-L-methionine decarboxylase was suppressed by as much as 50% in *M. oryzae*-inoculated rice plants , whereas such suppression was not observed in plants pretreated with the blast resistance-inducing chemical N-cyanomethyl-2-

chloroisonicotinamide. Likewise, SAM synthetase, a SAM biosynthesis gene, was found to be upregulated in probenazole-induced rice plants (Shimono et al., 2003). The link between the methionine cycle, SAM metabolism and resistance to *M. oryzae* is further strengthened by the rapid and specific expression of *OsBISAMT1*, encoding a putative SAM methyl transferase, in incompatible rice-*M. oryzae* interactions (Xu et al., 2006), and the observation that topical application of methionine not only induces production of the rice phytoalexins sakuranetin and momilactone A, but also increases resistance to subsequent blast attack (Nakazato et al., 2000). In this perspective, it is tempting to speculate that pseudobactin-type siderophores may induce resistance to *M. oryzae* by depriving rice roots from iron, leading to cytosolic iron depletion and resultant activation of the methionine cycle. This concept has received further support recently following work by Liu et al. (2007). In line with disease-related alterations in iron homeostasis in animals, these authors convincingly demonstrated that targeted redistribution of redox-active Fe inflicted by powdery mildew attack acts as an underlying factor associated with the oxidative burst and regulating cereal disease resistance. Interestingly, a model implying pseudobactin-mediated iron stress on the roots as a primary event in the elicitation of ISR might also hold for WCS417r-mediated ISR in Arabidopsis as *MYB72*, a transcription factor gene required for the onset of ISR (Van der Ent et al., 2008b), was reported to be activated exclusively in response to low iron conditions (Colangelo and Guerinot, 2004; Van de Mortel et al., 2006). Deciphering the putative role of iron homeostasis perturbation and SAM metabolism in the onset of the systemic immune response in rice may provide novel insights into the function of the methionine pathway in plant pathogen defense and could shed new light on the mode of action of ISR-eliciting pseudobactin siderophores.

- Recent studies demonstrated that, in addition to known defense pathways modulated by SA, JA, ET, or ABA, oxylipins other than JA, and hormones such as brassinosteroids, auxins, and gibberellins play important roles in plant responses to pathogen assault (Chapter 2; Robert-Seillaniantz et al., 2007; Angel-Lopez et al., 2008). Simultaneous measurements of these signaling compounds and monitoring the expression of known defense-related marker genes should expand our knowledge on rice hormone interactions in the context of biotic stress response signaling. The availability of numerous mutant and transgenic rice lines impaired in the perception or biosynthesis of this 'novel' class of defense regulators (Wang and Nick, 1998; Sharma et al., 2001; Chhun

et al., 2003; Hong et al., 2003; Sakamoto et al., 2004; Yamamoto et al., 2007) will be of particular value in deciphering the tapestry of signaling networks governing rice defense against various pathogens.

- The results denoted in Chapter 7 argue that exogenously administered ABA conditions resistance to *C. miyabeanus* in an *OsMPK5*-dependent manner by preventing the fungus from hijacking the ET signal transduction pathway. Further work should be focused on exploring whether these molecular observations (i.e. ET signaling promotes *C. miyabeanus* virulence) can be related to environmental conditions that predispose rice to brown spot. It is known that abnormal or poor soil conditions, inadequate water management and poor soil nutrition, especially potassium deficiency, favor brown spot incidence (Dela Paz et al., 2006). Taking advantage of our well-established hydroponic rice-growing system (Chapter 3), plant experiments could be set up under controlled conditions to assess how these unfavorable conditions influence brown spot incidence and affect the *in planta* hormone balances. Revelations about the basic mechanisms underlying such abiotic stress-induced plant susceptibility will not only reveal whether other resistance mechanisms against brown spot are operational besides the *OsMPK5*-mediated resistance identified in this work, but may also open new doors to design strategies for improving brown spot control.

- Modulation of pathogen resistance by ABA is a particularly complex phenomenon, involving multiple ABA perception sites, asymmetric multicomponent signaling cascades and ABA-concentration dependent processes (see Chapter 6). In-depth analysis of the spatial and temporal fluxes in endogenous ABA content by organ- and cell-specific ABA measurements should aid to further clarify and illuminate the mode of ABA action during rice-pathogen interactions. Similarly, evaluation of disease resistance and defense response activation in mutant or transgenic rice lines impaired in either ABA synthesis or perception may help to decipher the multicomponent role of ABA in the rice defense response. Potential candidate lines could include the ABA biosynthesis mutants recently developed by Fang et al. (2008).

- A long-standing goal in rice disease control is to identify and incorporate broad-spectrum durable pathogen resistance (BSDR). In addition to approaches focusing on natural germplasm (Leung et al., 2003), rice lines with induced mutations have been identified that show BSDR to multiple diseases. Because quantitative resistance phenotypes exhibited by these mutant lines could vary with genetic background, a precise analysis of the phenotypes and their genetic interactions must be done in a common genetic background. Although this problem can be addressed by incorporating individual mutations into near-isogenic lines via backcrossing and breeding, this process is time and labor intensive. In this context, the International Rice Research Institute has produced an extensive collection of chemical- and irradiation-induced mutants in the single genetic background of IR64, the most widely grown *indica* rice variety in South and Southeast Asia. About 60,000 mutants have been generated and 38,000 have been advanced to M_4 generation enabling evaluation of quantitative traits by replicated trials. Several of these mutants show enhanced resistance to both blast and bacterial blight, while others are rendered either more susceptible or more resistant to blast or blight (Hirochika et al., 2004; Wang et al., 2004; Wu et al., 2005; Wu et al., 2008). As these mutants are ideal vehicles for the identification of genes contributing to BSDR, it will be particularly interesting to test these mutants for their resistance to brown spot and sheath blight. To shed light on the type of defense responses involved, mutants with an altered resistance phenotype could be subjected to a flurry of biochemical, microscopic, molecular and genetic analyses. Alternatively, time-resolved hormone measurements coupled to genome-wide expression profiling could provide insight into the regulation of the signaling circuitry governing the gain- or loss-of-resistance phenotype. In addition, the mutants are suitable for reverse genetics through PCR detection of deletions or TILLING (targeting induced local lesions in genomes).

Addendum

Using *Serratia plymuthica* to control fungal pathogens of plants

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Published in: *CAB Reviews : Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources* 2007(2), 046. doi: 10.1079/PAVSNNR20072046.

Interest in biological control of plant pathogens has increased in recent years fuelled by trends in agriculture towards greater sustainability and public concerns over the use of hazardous pesticides in the environment. Most studies on biological control of fungal plant pathogens have tended to focus on the use of antagonistic rhizobacterial strains belonging to the genus *Pseudomonas* or *Bacillus*. However, the development of biocontrol products based on isolates belonging to the gram-negative genus *Serratia* is now gaining momentum. *S. plymuthica* is a ubiquitous bacterium that has been preferentially recovered from rhizospheres all over the world, both as a free-living and endophytic organism. Specific strains of *S. plymuthica* produce a broad pallet of antimicrobial compounds and might hold great potential as broad-spectrum biocontrol agents. This review surveys the advances of biocontrol research with respect to plant-associated *S. plymuthica* strains focusing on the principles and mechanisms of action of *S. plymuthica* and their use or potential use for the biological control of fungal plant diseases. A cursory overview of the taxonomy and ecology of *S. plymuthica* is provided as well. We highlight recent progress in the identification of antifungal secondary metabolites produced by *S. plymuthica* and pay special attention to the regulatory mechanisms underpinning the production of the latter metabolites. Finally, we discuss several strategies that may provide a basis to improve the efficacy of *S. plymuthica*-mediated biocontrol.

A.1. Taxonomy and ecology

The genus *Serratia* is named after the Italian physicist Serafino Serrati and belongs to the family Enterobacteriaceae within the Gammaproteobacteria. The only *Serratia* species recognized in the 8th edition of Bergey's Manual was *Serratia marcescens* (Buchanan and Gibbons, 1974). In their paper about the taxonomy of *Serratia*, Grimont and collaborators (1977) described four species within the genus *Serratia*: *Serratia marcescens*, *S. liquefaciens*, *S. plymuthica* and *S. marinorubra* (now called *S. rubidaea*). Nowadays, recognized species within the genus *Serratia* are *S. marcescens*, the *S. liquefaciens* complex (*S. liquefaciens*, *S. proteamaculans*, *S. grimesii*) (Grimont et al., 1981); the so-called 'unusual *Serratia* species (Stock et al., 2003): *S. ficaria* (Grimont et al., 1979), *S. fonticola* (Gavini et al., 1979), *S. odorifera* (Grimont et al., 1978), *S. plymuthica*, *S. rubidaea* (Ewing et al., 1973) and *S. entomophila* (Grimont et al., 1988); and *S. quinivorans* (Ashelford et al., 2002).

The *Serratia* species are ubiquitous and can be found in water, soil, plants and animals (including humans). *Serratia* is an opportunist that has been recognized as a human pathogen only since the 1960s. *S. marcescens* and the *S. liquefaciens* complex are routinely associated with human infections, but also the 'unusual' *Serratia* spp. (except *S. entomophila*) have been described as causing human disease.

Serratia plymuthica [Lehmann and Neumann (1896)] Breed et al. (1948) has been found in soil (Galland and Paul, 2001; Grant et al., 2002), water (Grimont and Grimont, 1978; Vivas et al., 2000) the air of poultry fattening houses (Vucemilo et al., 2005), insects (Grimont and Grimont, 1978; Kobayashi and Ichikawa, 1990; Stojek and Dutkiewicz, 2004; Tothprestia and Hirshfield, 1988) as an opportunistic pathogen in fish (Austin and Stobie, 1992; Nieto et al., 1990) and on cold smoked rainbow trout (Lyhs et al., 1998) and fresh tunafish (López-Sabater et al., 1993). In addition, a variety of clinical infections have been attributed to this microorganism. *S. plymuthica* has been isolated from blood cultures, surgical wound exudates, the peritoneal fluid, infections of bone marrow, central venous catheters and a human burn site (Carrero et al., 1995; Clark and Janda, 1985; Domingo et al., 1994; Horowitz et al., 1987; Reina et al., 1992; Zbinden and Blass, 1988). In general, *S. plymuthica* is considered to cause nosocomial infections, which means infections as a result of treatment in the hospital. However, we would like to insert a word of caution on the interpretation of these data. Although *S. plymuthica* has frequently been recovered from the human body, it was rarely isolated as the sole bacterial species present. Based on an international approved German directive (TRBA 466), *S. plymuthica* is nowadays classified into risk group 1 by the DSMZ (German Collection of Microorganisms and Cell Cultures), indicating that the species does not inadvertently pose a threat to human health. In contrast to *Serratia marcescens*, which belongs to risk group 2, there is no compelling evidence that *S. plymuthica* is

capable of causing human infections. Furthermore, no pathogenicity factors have been identified so far and, in contrast to other nosocomial pathogens like *Burkholderia* and *Stenotrophomonas*, *S. plymuthica* does not inflict disease in alternative animal model systems such as the *Caenorhabditis elegans* assay (G. Berg, personal communication).

Serratia plymuthica, however, is most frequently associated with plants. This organism has been isolated from the rhizosphere of grass (Alstrom and Gerhardson, 1987), wheat (Astrom and Gerhardson, 1988), maize (Lucon and Melo, 2000), oilseed rape (Kalbe et al., 1996), grape (Chernin et al., 1995), melon (Kamensky et al., 2003), onion (Park and Shen, 2002), *Brassica* sp. (Carlot et al., 2002), *Cichorium intybus* (Stock et al., 2003), sugarbeet (Tenning et al., 1987), tomato (Frommel et al., 1991) and as an endophyte from the endorhiza of potato (Berg et al., 2005). It has been found on the edible parts of green onion, carrot and lettuce (Grimont et al., 1981), on the phyllosphere of spring wheat (Legard et al., 1994), on *Brassica* spp. (Leifert et al., 1993) and as a contaminant in a raw vegetable processing line (van Houdt et al., 2005).

A.2. *Serratia plymuthica* as a biocontrol agent of fungal plant pathogens

Over the last two decades, *S. plymuthica* has received steadily increasing attention as a biological control agent of mainly fungal pathogens. As such, *S. plymuthica* isolates have been used to control fungal soil-borne and leaf pathogens. Furthermore, some papers report the use of *S. plymuthica* to suppress post-harvest diseases. An overview of the *S. plymuthica* strains which have been reported to provide biocontrol of fungal plant pathogens *ad planta* is listed in Table A.1.

A.2.1. Using *Serratia plymuthica* to control soil-borne diseases

Since *Serratia plymuthica* strains are frequently associated with plant roots, they have most extensively been studied for their ability to control soil-borne fungal diseases. *Serratia plymuthica* strain IC1270 from the rhizosphere of grapes, previously described as *Enterobacter agglomerans* (Chernin et al., 1995) and later on attributed to *S. plymuthica* (Ovadis et al., 2004b), effectively controlled *Rhizoctonia solani* damping-off of cotton (Chernin et al., 1995), *R. solani* root rot of bean and *Pythium aphanidermatum* pre- and postemergence damping-off on cucumber (Ovadis et al., 2004) under greenhouse conditions. *Pythium* disease severity was reduced to about two-third in the IC1270-treated plants compared with control non-bacterized plants.

Table A.1. Overview of *S. plymuthica strains* providing biocontrol of plant pathogens *ad planta*

Strain	Plant	Pathogen	Reference
IC1270	*Gossypium barbardense* (cotton)	*Rhizoctonia solani*	Chernin et al., 1995
	Phaseolus vulgaris (bean)	*Rhizoctonia solani*	Ovadis et al., 2004
	Cucumis sativus (cucumber)	*Pythium aphanidermatum*	Ovadis et al., 2004
	Prunus persica (peaches)	*Monilinia fructicola*	Ritte et al., 2002
		Rhizopus stolonifer	Ritte et al., 2002
	Pirus malus (apples)	*Penicillium expansum*	Ritte et al., 2002
	Citrus sinensis (oranges)	*Penicillium digitatum*	Meziane et al., 2006b
		Penicillium italicum	Meziane et al., 2006b
	Phaseolus vulgaris (bean)	*Colletotrichum lindemuthianum*	Meziane et al., 2006a
	Phaseolus vulgaris (bean)	*Botrytis cinerea*	Meziane et al., 2006a
	Lycopersicon esculentum (tomato)	*Botrytis cinerea*	Meziane et al., 2006a
	Oryza sativa (rice)	*Magnaporthe grisea*	De Vleesschauwer and Höfte, unpublished results
		Xanthomonas oryzae pv. *oryzae*	
IC14	*Citrus sinensis* (oranges)	*Penicillium digitatum*	Meziane et al., 2006a
		Penicillium italicum	Meziane et al., 2006a
	Cucumis sativus (cucumber)	*Botrytis cinerea*	Kamensky et al., 2003
		Sclerotinia sclerotiorum	Kamensky et al., 2003
CL43	Dutch white cabbage	*Botrytis cinerea*	Leifert et al., 1993
		Alternaria brassicicola	Leifert et al., 1993
R1GC4	*Cucumis sativus* (cucumber)	*Pythium aphanidermatum*	McCullagh et al., 1996
	Cucumis sativus (cucumber)	*Pythium ultimum*	Benhamou et al., 2000
3Re4-18[1]	*Solanum tuberosum* (potato)	*Rhizoctonia solani*	Grosch et al., 2005a
	Lactuca sativa (lettuce)	*Rhizoctonia solani*	Faltin et al., 2004
	Beta vulgaris (sugarbeet)	*Rhizoctonia solani*	Faltin et al., 2004
R12	*Fragaria virginiana* (strawberry)	*Verticillium dahliae*	Berg et al., 2001
HRO-C48[2]	*Fragaria virginiana* (strawberry)	*Verticillium dahliae*	Kurze et al., 2001a
	Fragaria virginiana (strawberry)	*Phytophtora cactorum*	Kurze et al., 2001a
2-67	*Cucumis sativus* (cucumber)	*Colletotrichum orbiculare*	Gang et al., 1991
-	*grape*	*Eutypa lata*	Schmidt et al., 2001
B-781	*Cucumis sativus (cucumber)*	*Pythium perplexum*	Galland and Paul, 2001
A21-4	*Capsicum annuum* (pepper)	*Phytophtora capsici*	Park and Shen, 2002

[1] strain 3Re4-18 was also designated B4

[2] strain HRO-C48 has been deposited as DZMZ12502

Over 5000 bacterial isolates from the roots of oilseed rape were screened for antifungal properties against *Verticillium dahliae*. 146 of the active isolates were determined, 18 isolates belong to the genus *Serratia* (Kalbe et al., 1996). Of the 18 *Serratia* strains, 16 strains were identified as *Serratia plymuthica*. All the investigated isolates showed an antifungal acivity against *Verticillium dahliae*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* in bioassay (Kalbe et al., 1996). One of the isolates from this study is the well-characterized *S. plymuthica* strain HRO-C48 (indicated as isolate C48 in Kalbe et al. (1996) and as *S. plymuthica* strain DSMZ12502 in Berg (2000) and Kurze et al. (2001a). Dipping strawberry roots in a suspension of *S. plymuthica* HRO-C48 reduced the percentage of *Verticillium* wilt with 18.5% and the percentage of *Phytophtora cactorum* root rot with 33.4%. In three different field trials, *Verticillium* wilt was reduced compared with the nontreated control with an average of 24.2%, whereas the average yield increase was 296%.

Phytophthora root rot was reduced with an average of 9.6%, while the strawberry yield was increased by 60% compared with the nontreated control (Kurze et al., 2001a). A commercial product on the basis of HRO-C48 has been developed (European patent 98124694.5) and called RhizoStar® (e-nema GmbH, Raisdorf, Germany).

Serratia plymuthica strain 3Re4-18 [indicated as *S. plymuthica* B4 in Grosch et al. (2005a)] is an endophyte, isolated from the endorhiza of potato (Berg et al., 2005). This isolate caused an average reduction of 25% in *Rhizoctonia solani* disease severity in two experiments on potato sprouts. Under field conditions, a disease suppression effect of 31% was achieved on potato, whereas the marketable tuber yield increased up to 17% compared with the pathogen control. The strain was thus more effective in the field than in the pot experiments. Strain B4 was also used to control bottom rot on lettuce, caused by *R. solani* AG1-1B on leaf disks (Faltin et al., 2004) and in two experimental fields. In both field experiments, soil application with the isolate increased the dry mass of lettuce as much as 31% and reduced disease severity 19% (Grosch et al., 2005a). However, strain B4 was not effective to control damping-off disease caused by *Rhizoctonia solani* AG4 on sugar beet seedlings (Faltin et al., 2004).

Alström and Gerhardson (1987) describe an isolate of *Serratia plymuthica* (G15), frequently isolated from roots of various plant species that showed strong antagonism against *Botrytis cinerea* and *Gerlachia nivalis* and moderate antagonism against *Rhizoctonia solani*, *Fusarium culmorum* and *Pythium* sp. In addition, this isolate significantly increased growth of lettuce plants when applied to the roots under non-sterile conditions.

Serratia plymuthica strain A153 was isolated from the rhizosphere of wheat (Astrom and Gerhardson, 1988). This strain was later on shown to suppress apothecia formation in *Sclerotinia sclerotiorum* (Thaning et al., 2001). Inhibition of apothecial formation appears to be due to the production of chlorinated macrolides (Levenfors et al., 2004; Thaning, 2000; Weissmann, 2002). Strain A153 has also been used for the biological control of weeds, both in the greenhouse (Weissmann, 2002) and the field (Weissmann et al., 2003).

The isolate *S. plymuthica* R1GC4 (origin could not be retraced) has been tested on rockwool-grown cucumbers for its ability to reduce *Pythium* root rot caused by *Pythium aphanidermatum*. Strain R1GC4 slightly increased the cumulative cucumber yields (McCullagh et al., 1996). Benhamou et al. (2000) also used this isolate in a later study in which the defense reactions of cucumber seedlings against *Pythium ultimum* with and without bacterial treatment were studied at the cellular level.

S. plymuthica strain A21-4 was isolated from the roots of onion and significantly inhibited mycelium growth, zoosporangia formation and cystospore germination of *Phytopthora capsici* in vitro. When pepper seedlings were dipped in a cell suspension of A21-4 and transplanted in the greenhouse, the bacteria successfully suppressed *Phytophthora* blight. Disease incidence 60 days after transplanting was 72.4% in the untreated plot, compared to 12.6% in the treated plants. A21-4 readily colonized the pepper roots and the bacterial density on the root was maintained above 10^6 CFU/g root until 3 weeks after transplanting (Park and Shen, 2002).

S. plymuthica strain B-781, which was isolated from a soil sample taken in the Burgundy region in France, effectively controlled damping-off disease of cucumber caused by *P. perplexum* (Galland and Paul, 2001).

A.2.2. Using *Serratia plymuthica* to control fungal post-harvest diseases

Only a few reports deal with the use of *Serratia plymuthica* to control post-harvest diseases. *Serratia plymuthica* CL43 (= *Serratia plymuthica* NCIMB40492), among other bacterial antagonists, has been used to control *Botrytis cinerea* and *Alternaria brassicicola* on Dutch white cabbage at cold store temperature (Leifert et al., 1992; Leifert et al., 1993; Stanley et al., 1994). The *Serratia plymuthica* strains used showed in vitro and in vivo antagonism at 4°C. The use of *Serratia plymuthica* CL43 and other bacteria to control post-harvest diseases on cabbages is the subject of three different patents (US patent no. 5780080, US patent no. 5869038 and US patent 5597565).

Serratia plymuthica strain IC1270 is an effective antagonist of *Penicillium expansum* (blue mould) on apple, and *Monilia fructicola* on peach (Ritte et al., 2002). In addition, this strain and *Serratia plymuthica* strain IC14, isolated from soil around melon roots (Kamensky et al., 2003) effectively suppressed *Penicillium digitatum* (green mould) and *Penicillium italicum* (blue mould) on orange (Meziane et al., 2006b). Both strains reduced disease incidence by about 30% compared with control treatments.

A.2.3. Using *Serratia plymuthica* to control fungal leaf pathogens

Only *Serratia plymuthica* strains IC14 and IC1270 have been used for foliar application. Strain IC14 protected cucumber seedlings against *Botrytis cinerea* grey mould and *Sclerotinia sclerotiorum* white mould diseases of leaves under greenhouse conditions. Disease incidence was reduced by 76 and 84%, respectively (Kamensky et al., 2003). The survival ability of strain IC14 on cucumber leaves is limited, however. The titer of bacteria decreased from 1 x 10^6 cells per 0.5 cm^2 of leaf tissue to 2.7 x 10^3 cells per 0.5 cm^2 of leaf tissue after 72 h. Leaf application with strain IC1270

decreased the number of *B. cinerea* spreading lesions from 92% in the control to 64%, and from 78% to 48% in bean and tomato, respectively (Meziane et al., 2006a).

Some *S. plymuthica* strains, however, can induce systemic resistance in plants and control leaf pathogens when inoculated on plant roots. Seed treatment with *S. plymuthica* strain 2-67 significantly reduced the number and diameter of lesions caused by *Colletotrichum orbiculare* on cucumber leaves in two of three trials under greenhouse conditions (Gang et al., 1991). Soil and seed treatment with *S. plymuthica* strain IC1270 induced systemic resistance to *B. cinerea* on tomato and bean leaves and to *Colletrichum lindemuthianum* on bean (Meziane et al., 2006a). Effective root colonization resulted in a 35% disease severity reduction. Strain 1270 was also able to induce systemic resistance to *Magnaporthe grisea* and *Xanthomonas oryzae* pv. *oryzae* on rice, causing reductions in disease severity of as much as 50% (De Vleesschauwer and Höfte, unpublished results).

A.3. Biocontrol mechanisms

A thorough understanding of the antimicrobial mechanisms employed by *S. plymuthica* is key to an efficient and long-lasting biocontrol. Rhizosphere competence and biocontrol activity of *S. plymuthica* are enabled by antibiosis, parasitism involving production of lytic enzymes, competition for nutrients and iron by secretion of siderophores, and induction of plant defense mechanisms. None of these mechanisms are mutually exclusive and frequently several modes of action are exhibited by a single *S. plymuthica* strain. For instance, the mode of action of strain HRO-C48 comprises a diverse set of biocontrol mechanisms facing both pathogen and host plant (Alstrom and Gerhardson, 1987; Berg, 2000; Chernin et al., 1996; Chernin et al., 1995; Kalbe et al., 1996; Kamensky et al., 2003; Levenfors et al., 2004; Shoji et al., 1989; Thaning et al., 2001). Table A.2. presents an overview of the spectrum of biocontrol-associated secondary metabolites produced by model strains of *S. plymuthica*.

A.3.1. Antibiosis

The production of organic antimicrobial secondary metabolites as a biocontrol mechanism of *S. plymuthica* has become increasingly better understood over the past decade. A variety of antibiotics have been identified, including compounds such as pyrrolnitrin, prodigiosin, the dipeptide antibiotic CB-25-I, 1-acetyl-7-chloro-1-H-indole and haterumalides (Alstrom and Gerhardson, 1987; Berg, 2000; Chernin et al., 1995; Kalbe et al., 1996; Kamensky et al., 2003).

Few *S. plymuthica* strains produce the non-diffusible red pigment and antifungal antibiotic prodigiosin (Alstrom and Gerhardson, 1987; Berg, 2000; Chernin et al., 1995; Kalbe et al., 1996; Kamensky et al., 2003). Pigmented *S. plymuthica* biotypes, which were rarely isolated from plants, seem to be toxic to protozoa (Grimont, 1992). Hence, production of prodigiosin might offer an ecological advantage in widely diverse ecological niches. However, a correlation between the production of prodigiosin and the level of resistance to several antibiotics as has been demonstrated for *S. marcescens* (Grimont, 1992) could not be confirmed for *S. plymuthica* (Berg, 2000).

Table A.2. Overview of the production of biocontrol-related secondary metabolites by model *S. plymuthica* strains

	S. plymuthica strain					
Metabolite	HRO-C48	IC1270	IC14	A153	R12	3Re4-18
Prodigiosin	-	-	-	ND	ND	ND
Haterumalides	ND	ND	ND	+	ND	ND
Pyrrolnitrin	+	+	+	+	ND	ND
Glucanases	-	ND	-	ND	+	+
Chitinases	+	+	+	ND	+	+
Proteases	+	+	+	ND	+	+
Siderophores	+	+	+	ND	ND	+
IAA	+	-	+	ND	ND	-

ND = not determined

The chlorinated macrolides, haterumalide NA, B, NE and X, were among the first polyketide substances found to be produced by isolates belonging to the genus *Serratia* (Levenfors et al., 2004; Thaning et al., 2001). Isolated haterumalides, purified from the supernatant of *S. plymuthica* strain A 153, strongly suppressed apothecial formation, ascospore germination and mycelial growth of several filamentous fungi and oomycetes in vitro (Levenfors et al., 2004; Strobel et al., 1999). Haterumalides NA, B and NE were also isolated from an Okinawan *Ircinia* sponge as inhibitors of the cell division of fertilized sea urchin eggs (Takada et al., 1999). Structural similarities to other compounds suggest that the biosynthetic pathway of the haterumalides involves a type I polyketide synthase cluster, similar to the haterumalide biosynthesis in bacteria from the genus *Pseudomonas* (Nowak-Thompson et al., 1999; Rangaswamy et al., 1998). Nevertheless, further research regarding the genetic origin of haterumalides and the underlying biosynthetic pathway is needed to confirm the involvement of a type I polyketide synthase cluster in the biosynthesis of haterumalide antibiotics by *S. plymuthica*.

Pyrrolnitrin [3-chloro-4-(2´-nitro-3´-chlorophenyl) pyrrole] is a tryptophan-derived secondary metabolite which has been reported to suppress a wide range of fungal and bacterial pathogens (for review see Haas and Keel, 2003). Although a vast amount of isolated *S. plymuthica* strains has been demonstrated to produce pyrrolnitrin (Prn) *in vitro* (Chernin et al., 1996; Kalbe et al., 1996; Kamensky et al., 2003; Levenfors et al., 2004), several studies showed discrepancies regarding the role of Prn in the antagonistic activity of *S. plymuthica* strains. While Prn production was assumed to be a key factor of *S. plymuthica* IC1270-mediated biocontrol of several fungal post-harvest pathogens of peaches and apples (Ritte et al., 2002), no evidence was found for the involvement of Prn in post-harvest control of blue and green mould by the same strain (Meziane et al., 2006b). Likewise, IC1270-triggered resistance against *Botrytis cinerea* was shown to be independent of Prn, whereas Prn was demonstrated to play a prevalent role in direct antagonism towards distinct pathogens by the latter strain (Gavriel et al., 2004; Meziane et al., 2006a). However, these conflicting observations can be reconciled when considering that many biocontrol strains produce a pallet of secondary antimicrobial metabolites and that conditions favoring one compound may not favor another (Duffy and Defago, 1999). This varied arsenal of biocontrol traits may enable antagonists to efficiently fine-tune their biocontrol activity and perform their ultimate objective of pathogen suppression under a wide range of environmental conditions. As such, different mechanisms or combinations of mechanisms may be involved in the suppression of different plant diseases by a particular biocontrol agent (Haas and Defago, 2005).

Because Prn production is an important biocontrol mechanism against several plant pathogens, extensive work has been carried out to elucidate its gene expression and regulation in the model strain *Pseudomonas fluorescens* Pf-5. In addition to the identification of the Prn biosynthetic gene operon, which comprises four genes (Hammer et al., 1997; Kirner et al., 1998), Prn has been reported to be under global genetic control by a two-component regulatory system composed of the sensor protein ApdA (also called LemA) (Corbell and Loper, 1995) and the response regulator GacA (Gaffney et al., 1994; Laville et al., 1992). Moreover, a gene necessary for pyrrolnitrin production has been identified as *rpoS*, which encodes the stationary-phase sigma factor sigma(s) (Nowak-Thompson et al., 1999; Sarniguet et al., 1995). The interactions between the RpoS and the GacS/GacA regulons are, however, poorly understood. Elegant research by Ovadis and associates (2004a) demonstrated the involvement of *rpoS* and *gacA*/*lemA* homologues (tentatively designated *grrA*/*grrS* for global response regulation activator/sensor) in pyrrolnitrin regulation in *S. plymuthica* strain IC1270. Pyrrolnitrin-deficient *grrA*, *grrS* and *rpoS* gene replacement mutants were markedly less capable of suppressing *Rhizoctonia solani* and *Phytium aphanidermatum* under greenhouse conditions, indicating that IC1270-mediated biocontrol is

tightly modulated by the GrrA/GrrS global regulatory cascade and the sigma factor RpoS. In addition, pyrrolnitrin biosynthesis was very recently demonstrated to be subject to positive control by a LuxI/LuxR-type quorum-sensing system consisting of an N-acyl-homoserine lactone (AHL) synthase (SplI) and an AHL-responsive cognate transcriptional repressor, designated as SplR (Liu et al., 2007). Using an AHL and pyrrolnitrin double negative mutant of strain HRO-C48, which was deficient in suppressing the growth of several fungal plant pathogens in vitro, the authors first provided evidence for the involvement of quorum-sensing signalling in biocontrol exerted by *S. plymuthica*.

A.3.2. Parasitism

Parasitism relies on the excretion of extracellular cell wall-degrading enzymes, such as chitinases, proteases and β-1,3-glucanases that can lyse pathogen cell walls (Whipps, 2001).

Chitin, an insoluble β-(1,4)-linked polymer of *N*-acetyl D-glucosamine (GlcNAc), is a ubiquitous component of most fungal cell walls. Chitinases, which catalyze the hydrolysis of chitin, can be classified into two major categories. Endochitinases (EC 3.2.1.14) cleave chitin randomly at internal sites, generating low molecular mass multimers of GlcNAc, such as chitotetraose, chitotriose, and diacetylchitobiose. Exochitinases can be divided into two subcategories: chitobiosidases (EC 3.2.1.29) catalyze the progressive release of diacetylchitobiose units starting at the nonreducing end of chitin microfibrils, and *N*-acetyl- β-(1,4)-D-glucosaminidases (EC 3.2.1.30), which cleave the oligomeric products of endochitinases and chitobiosidases, generating monomers of GlcNAc (Patil et al., 2000). Based on this system of nomenclature, several types of chitinases have been identified in *S. plymuthica* strains. Strain IC1270 produces two *N*-acetyl- β-D-glucosaminidases of 89 and 67 kDa, an endochitinase with a molecular mass of 59 kDa and a 50 kDa chitobiosidase. Strains IC14 and HRO-C48, on the other hand, have been reported to secrete an endochitinase and a 100 kDa *N*-acetyl-β-1,4-D-hexosaminidase or chitobiase (Frankowski et al., 2001; Kamensky et al., 2003).

To date, only one chitinase-encoding gene from *S. plymuthica* has been cloned (Chernin et al., 1997). Sequencing of the cloned gene *chiA*, which encodes the endochitinase from strain IC1270, yielded an open reading frame coding for 562 amino acids of a 61-kDa precursor protein with a putative leader peptide at the N terminus. Homology modelling of the deduced enzyme's three-dimensional structure revealed high structural similarities with the corresponding enzyme from *S. marcescens*. Both structures consisted of an all-β-strand amino-terminal fibronectin III (FnIII)-type domain, an α + β fold domain, and an α/β-barrel domain. While the first domain has been suggested to facilitate the binding of chitinase to chitin, the last domain is catalytic retaining the

conserved residues Glu315 and Asp391 which are located in the active site (Chernin et al., 1997). The antifungal activity of the secreted endochitinase was demonstrated in vitro using recombinant DNA techniques. The recombinant strain *E. coli* JM109/pCHITEa1, expressing the *S. plymuthica chiA* gene, acquired the ability to suppress *Rhizoctonia solani* and spore germination of *Fusarium oxysporum* f. sp. *meloni* in vitro. Furthermore, the transformed strain also abrogated root rot disease caused by *R. solani* in cotton seedlings under greenhouse conditions (Chernin et al., 1997).

The 58 kDa endochitinase of *S. plymuthica* IC14 (ChiA) differs from this of strain IC1270 in that it not only hydrolyzes chitin but also EGC, a chitin derivative usually used as a test substrate for lysozyme activity, suggesting that IC14 ChiA belongs to the class of bifunctional chitinase/lysozyme enzymes (Kamensky et al., 2003). Such bifunctional enzymes have been suggested to enable bacteria to compete efficiently with fungi and other bacteria in a limited-nutrient environment. Alternatively, broader substrate specificity of chitinases has been related to other aspects of their function such as modulating the intricate relationships between biocontrol bacteria and their host organism (Kamensky et al., 2003).

So far, research aimed at elucidating the regulatory mechanisms underlying chitinase production in *S. plymuthica* has been confined to a limited number of strains. The GacS/GacA two-component system has previously been shown to positively regulate the expression of genes coding for secreted enzymes such as chitinases in a group of root-colonizing, plant-beneficial bacteria including *Pseudomonas chlororaphis* PCL1391 and *Pseudomonas fluorescens* BL915 (for review see Heeb and Haas, 2001). *GrrA* and *grrS* gene replacement mutants of IC1270, however, were deficient in production of the 58 kDa ChiA endochitinase but not in that of the 89-kDa and 67-kDa exochitinases. As the *rpoS* mutant of IC1270 still secretes ChiA, the mutation in *grrA* or *grrS* is unlikely to exert its effect via repression of the stationary sigma factor RpoS, whose expression is positively regulated by the GacS/GacA system in *P. fluorescens* strain Pf-5 (Whistler et al., 1998) and *Escherichia coli* (Mukhopadhyay et al., 2000). In addition, regulation of chitinase production seems to act independently of the quorum sensing machinery of IC1270 because synthesis of *N*-acyl-homoserine lacton signal molecules was blocked in both *rpoS* and *grrA*/*grrS* gene replacement mutants (Ovadis et al., 2004). However, Müller and associates (2006) very recently reported that expression of chitinase is regulated positively by quorum sensing in strain HRO-C48. Likewise, an extracellular chitinase in *S. plymuthica* strain RVH1 is synthesized under the positive control of the SplIR quorum-sensing system (Van Houdt et al., 2007), suggesting that the regulatory cascades that modulate chitinase production are strain-specific. Recently, it has been demonstrated that the GacS/GacA system partly steers its effects via posttranscriptional control

exerted by small regulatory RNAs such as RsmB, RsmZ and RsmY (Cui et al., 2001; Heeb et al., 2002). In *Erwinia carotovora* subsp. *carotovora* and *P. fluorescens*, these regulatory RNAs sequester the RNA-binding protein RsmA and thereby relieve translational repression of target mRNAs. Based on the taxonomic resemblance between *S. plymuthica* and *E. carotovora*, Ovadis and associates (2004) hypothesize that translation of ChiA mRNAs involves similar posttranscriptional regulators.

Several studies have investigated the role of chitinases in biocontrol activity of *S. plymuthica* strains. Chitinases produced by *S. plymuthica* HRO-C48 played an important role in the antifungal activity of the latter strain both in dual culture assay and *ad planta* (Frankowski et al., 2001). However, chitinolytic activity appears less essential for *S. plymuthica* IC14; when used to suppress *S. sclerotiorum* and *B. cinerea*, synthesis of proteases and other biocontrol traits were involved (Kamensky et al., 2003). Likewise, *S. plymuthica* IC1270-mediated biocontrol against *R. solani* and *P. aphanidermatum* was demonstrated to be independent of chitinase production (Gavriel et al., 2004). Similar results were obtained in IC1270-modulated biocontrol assays with different post-harvest pathogens (Meziane et al., 2006b; Ritte et al., 2002). Hence, the contribution of chitinolytic activity in *S. plymuthica*-mediated biocontrol is clearly strain-specific and further illustrates the heterogeneous multifaceted character of biocontrol mechanisms employed by distinct bacterial strains against a diverse set of pathogens.

Glucanases and proteases are cell-wall degrading enzymes that are produced by a wide range of *S. plymuthica* strains (Berg et al., 2001; Berg et al., 2005; Faltin et al., 2004; Kalbe et al., 1996; Kamensky et al., 2003). However, to date, no studies regarding the regulation or precise role of these antifungal compounds in biocontrol by *S. plymuthica* have been conducted.

A.3.3. Competition for iron and the role of siderophores

Iron is an essential growth element for all living organisms. The scarcity of bioavailable iron in soil habitats and on plant surfaces foments a furious competition (Höfte et al., 1993). Under iron-limiting conditions, bacteria produce a range of low-molecular-weight compounds or siderophores to competitively acquire ferric iron. These bacterial iron chelators are thought to sequester the limited supply of iron available in the rhizosphere, thereby depriving pathogenic fungi of this essential element and consequently restricting their growth (Loper and Henkels, 1999; Osullivan and Ogara, 1992). Several *S. plymuthica* strains including IC1270, IC14, 3Re4-18 and HRO-C48, have been shown to secrete potent siderophores *in vitro* when grown on iron-poor media (Berg et al., 2005; Faltin et al., 2004; Frankowski et al., 1998; Kalbe et al., 1996; Kamensky et al., 2003; Ovadis et al., 2004). Additionally, the residual biocontrol activity of

distinct pyrrolnitrin- and/or endochitinase-negative mutants of IC1270 have been partially attributed to the unaltered ability of the latter strains to compete for nutrients such as iron (Gavriel et al., 2004; Ovadis et al., 2004). Nevertheless, more detailed studies using siderophore-deficient mutants and application of purified compounds will be required to unequivocally delineate the involvement of bacterial iron chelators in *S. plymuthica*-mediated biocontrol.

A.3.4. Induction of plant resistance mechanisms

An additional mechanism by which *S. plymuthica* can reduce plant diseases is by activating the host plant's defensive repertoire. Although the concept of rhizobacteria-mediated systemic resistance (ISR) has received increasing attention over the last decade, reports about *S. plymuthica* strains mounting systemic resistance are scarce. Gang and associates (1991) first reported evidence that the *S. plymuthica* strain 2-67 induces ISR in cucumber to *Colletotrichum orbiculare*. Further evidence showing the ISR-triggering capacity of *S. plymuthica* was provided by Benhamou and associates (2000). Using electron microscopy, the authors demonstrated that *Pythium*-challenged induced cucumber root cells undergo significant ultrastructural and biochemical modifications that correlate with the formation of structural barriers that likely prevent pathogen ingress towards the vascular stele accompanied by the deposition of a phenolic-enriched occluding material. Such responses associated with the onset of induced resistance would include the oxidation and polymerization of pre-existing phenols and the synthesis of new phenolic compounds via an activation of the phenylpropanoid pathway. Hence, *S. plymuthica* R1CG4 reduces *Pythium* root rot by priming susceptible cucumbers plants to elaborate a wide range of defense mechanisms. Recently, *S. plymuthica* strain IC1270 was shown to mount ISR against *B. cinerea* and *C. lindemuthianum* in bean and tomato (Meziane et al., 2006a). In rice, however, IC1270 plays an ambivalent role in mounting induced resistance responses. While IC1270 conferred enhanced resistance to *M. grisea* and the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae*, bacterial colonization significantly promoted subsequent infection with the necrotrophic pathogens *R. solani* and *Bipolaris oryzae*. The differential effectiveness of IC1270 with respect to ISR-mediated disease resistance in rice is most likely due to its capacity to modulate the plant's oxidative machinery. Biochemical and histochemical studies demonstrated that IC1270 primes rice seedlings for a potentiated generation of reactive oxygen species in response to pathogen infection and wounding (Chapter 4).

A.3.5. Phytostimulation

Plant growth is affected by a plethora of abiotic and biotic factors. Most plant growth-promoting bacteria (PGPRs) increase plant growth indirectly either by the suppression of well-established diseases caused by major pathogens or by reducing the deleterious effects of minor pathogens. Alternatively, PGPRs may directly affect plant metabolism resulting in increased plant growth, seed emergence or improved crop yield (Whipps, 2001). Several *S. plymuthica* strains have been demonstrated to exert plant growth-promoting effects in phytochamber, greenhouse and field trials (Berg et al., 2001; Faltin et al., 2004; Kurze et al., 2001). The plant growth-stimulating ability of the latter strains has often been linked to their capacity to produce the auxin phytohormone indole-3-acetic acid (IAA) *in vitro*. IAA is the main auxin in plants, controlling many fundamental physiological processes including cell enlargement and division, tissue differentiation, and responses to light and gravity (Teale et al., 2006; Woodward and Bartel, 2005). However, in several independent studies it was shown that IAA biosynthesis alone cannot account for the overall plant growth-promoting effect of *Azospirillum* (Spaepen et al. 2007). Furthermore, Faltin and associates (2004) found no correlation between IAA production *in vitro* and the plant growth-promoting effect on lettuce seedlings of several antagonistic bacteria, including the *S. plymuthica* strain 3Re4-18. In view of these data, the growth and yield promotion observed might be explained by the 'additive hypothesis' (Bashan and Holguin 1997), postulating that growth promotion is the result of multiple coordinated mechanisms such as associative nitrogen fixation, modulation of phytohormonal balances, phytohormone biosynthesis, and solubilisation of phosphate. Thus, a balanced interplay of different factors including bacterial IAA biosynthesis rather than IAA production *per se* is most likely needed to stimulate plant growth. The use of mutant and transgenic strains and the analysis of inoculants' supernatant might shed more light on the diverse role played by different bacterial factors involved in *S. plymuthica*-mediated phytostimulation.

A.4. Conclusions and future considerations

Over the past two decades, several *S. plymuthica* strains have been demonstrated to be effective biocontrol agents against soil-borne and foliar diseases. Some *S. plymuthica* strains can also be used to control post-harvest diseases given their ability to antagonize pathogens at cold store temperatures (Leifert et al., 1992; Leifert et al., 1993; Stanley et al., 1994). In addition, *S. plymuthica* strains have been described as entomopathogens (Tan et al., 2006) and are employed for biological control of weeds (Weissmann, 2002; Weissmann et al., 2003). Many strains produce a variety of allelochemicals, including antibiotics, lytic enzymes and iron-chelating siderophores

(Chernin et al., 1995; Faltin et al., 2004; Kalbe et al., 1996; Levenfors et al., 2004; Ovadis et al., 2004; Shoji et al., 1989). Moreover, the diverse origin of *S. plymuthica* isolates demonstrates that these bacteria are able to colonize widely diverse ecological niches. Hence, *S. plymuthica* strains might be ideal candidates for use as broad-spectrum biocontrol agents in integrated crop management.

Despite their potential as low-input practical agents of plant protection, widespread application of *S. plymuthica* strains as commercial biocontrol products has been hampered by several reasons such as the limited number of field tests conducted so far, the difficult formulation of the bacteria, and their emergence as facultative pathogens. Chief among concerns is the often reported inconsistent performance of biocontrol agents in the field, which is usually attributed to their poor rhizosphere competence (Weller, 1988). Biocontrol strains can only be used optimally if the molecular basis of their beneficial effects, and the way these traits are influenced by a myriad of biotic and abiotic factors are unraveled. As many studies demonstrated discrepancies between the antagonistic potential of the biocontrol agent in vitro and its efficacy under field conditions (Faltin et al., 2004), successful reproducible biocontrol on the basis of plant-associated *S. plymuthica* also requires profound knowledge of the ecological and molecular interplay taking place in bacterial communities in order to predict the conditions under which biocontrol can be achieved. Revelations about the modes of action of *S. plymuthica* biocontrol strains will open new doors to design strategies for improving the efficacy of biocontrol products (Walsh et al., 2001). For instance, identifying different modes of action will facilitate the combination of biocontrol strains to hit pathogens with a broader spectrum of microbial weapons (de Boer et al., 1999; Olivain et al., 2004). Identification of key antimicrobials produced by *S. plymuthica***,** such as chitinases or pyrrolnitrin, and elucidation of their biosynthetic pathways can be exploited for streamlining biocontrol strain discovery by targeting selection of new isolates that carry relevant biosynthetic genes (Compant et al., 2005).

Despite the fact that genotypic and phenotypic diversity occuring in natural populations of biocontrol agents provides an enormous resource for improving biological control of plant diseases (Keel et al., 1996), exploitation of such diversity among bacterial biocontrol agents of fungal plant pathogens has received little attention. Yet, knowledge of the diversity within a group of strains sharing a common biocontrol trait can be exploited to select biocontrol strains that are superior with respect to rhizosphere competence and biocontrol activity. Recent studies by Berg (2000) demonstrated that populations of plant-associated and antifungal *S. plymuthica* strains can be highly diverse and thus have great potential for improving biological control. For instance, by matching bacterial genotypes with crops or varieties for which they have a

preference, genotypic differences among strains could be exploited to face the biotic and abiotic complexity of natural environments

A salient feature of *S. plymuthica* is that some strains are able to colonize the endorhiza (Berg et al., 2005). Given the intimate relationships with their hosts, endophytic bacteria hold great potential to further our understanding of the multiple facets of disease suppression. As indicated by Compant et al. (2005), continued work with endophytic bacteria might play a fundamental role in the development of biocontrol agents that are self-perpetuating by colonizing hosts and being transferred to progeny much as is the case with the nonsymbiotic endophyte bacterium *Burkholderia phytofirmans* PsJN (Sessitsch et al., 2005) or associative nitrogen-fixing bacteria on sugarcane (Boddey et al., 2003).

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Summary

To cope with the constant threat of a variety of pathogenic microorganisms, plants have evolved sophisticated strategies to perceive microbial attack and to translate this perception into an appropriate adaptive response. Apart from reacting locally, plants can also mount a systemic immune response, establishing an enhanced defensive capacity in plant parts distant from the site of initial invasion. A classic example of such a systemically induced resistance response is activated upon primary infection with a necrotizing pathogen. Once initiated, this so-called systemic acquired resistance (SAR) is generally durable and broad-spectrum, rendering plants more resistant towards a wide range of otherwise virulent pathogens. Colonization of plant roots by selected strains of nonpathogenic rhizobacteria leads to a phenotypically similar form of induced resistance, commonly referred to as induced systemic resistance (ISR). Although both SAR and ISR hold great potential as an environmentally sound and economically viable mean of disease control, research aimed at elucidating the underlying molecular mechanisms has been polarized towards the use of experimentally tractable dicot plants, such as *Arabidopsis thaliana* and tobacco. Conversely, in the class of the *Monocotyledoneae,* including the most important agronomic cereals, our understanding of the regulatory mechanisms controlling induced resistance is still in its infancy, this knowledge being key to effective utilization of SAR and ISR in an agricultural context.

In the present work, we have explored the mechanistic basis and regulation of biologically and chemically induced pathogen resistance in rice, a central monocot plant model and the staple food for half the world's population. In the first part of this dissertation, several rhizobacteria known to elicit ISR in dicot plants were assessed for their capacity to convey protection against various rice pathogens exhibiting distinct parasitic habits. A potent activator of ISR in bean, tomato, and Arabidopsis, *P. aeruginosa* 7NSK2 proved able to significantly reduce infection caused by the hemibiotrophic rice blast pathogen *Magnaporthe oryzae*, thereby producing a resistance phenotype resembling that of quantitative trait loci-conditioned partial resistance. Although 7NSK2 is known to induce resistance in dicots plants through a synergistic interaction of the phenazine pigment pyocyanin and the iron-chelating compound pyochelin, only mutations interfering with pyocyanin production impaired ISR to *M. oryzae*, whereas *in trans* complementation of pyocyanin synthesis restored to ability to mount resistance. Intriguingly, pyocyanin-deficient mutants, unlike the wild-type, triggered ISR against the necrotrophic pathogen *R. solani*. These results pinpoint pyocyanin as a two-faced ISR elicitor, acting as a positive regulator of induced resistance to *M. oryzae* while facilitating infection by *R. solani*. Experiments using purified pyocyanin further revealed that transient enhancement of *in planta*

H_2O_2 levels priming naïve leaves for expression of hypersensitive response-like cell death upon pathogen attack accounts for the dual role of the latter compound in 7NSK2-mediated ISR. The differential effectiveness of such ROS-fueled systemic immune response was also reflected in experiments utilizing the *Serratia plymuthica* strain IC1270 as an inducing agent. Triggering high levels of resistance against *M. oryzae*, plant colonization by IC1270 rendered plants hypersusceptible to attack by *R. solani* and *C. miyabeanus*, both of which are considered necrotrophic fungi. Artificial enhancement of ROS levels in inoculated leaves faithfully mimicked the opposite effects of IC1270 bacteria on aforementioned pathogens, confirming a central role for oxidative events in the IC1270-induced resistance mechanism. Besides tagging reactive oxygen species and the associated hypersensitive response as a double-edged sword in the rice induced resistance program, these findings add weight to previous reports claiming that rice requires distinct mechanisms for defense against *M. oryzae* and the necrotrophs *R. solani* and *C. miyabeanus*.

Aiming to further dissect the induced systemic resistance response of rice, we next analyzed the bacterial traits and host defense mechanisms underpinning ISR elicited by the biocontrol bacterium *P. fluorescens* WCS374r. Similar to 7NSK2 and IC1270, root treatment with WCS374r induced an enhanced level of protection against *M. oryzae*. Nevertheless, the underlying regulatory mechanisms appeared to be substantially different. Using salicylic acid-nonaccumulating NahG rice, an ethylene-insensitive *OsEIN2* antisense line and the jasmonate-deficient mutant *hebiba*, we demonstrated that WCS374r-induced resistance, unlike 7NSK2-ISR, is regulated by an SA-independent but JA/ET-modulated signal transduction pathway, thereby mimicking the classic *P. fluorescens* WCS417r-inducible ISR pathway in Arabidopsis. Moreover, bacterial mutant analysis uncovered a pseudobactin-type siderophore as the crucial determinant responsible for ISR elicitation. Root application of WCS374r-derived pseudobactin (Psb374) sensitized naïve leaves for accelerated expression of a pronounced multifaceted defense response, comprising, amongst others, the rapid recruitment of phenolic defense compounds at sites of attempted pathogen entry, concerted expression of a diverse set of structural defenses, and a timely yet highly restricted hyperinduction of H_2O_2 in the epidermis. Strikingly, in sharp contrast to WCS374r-mediated ISR but similar to SA-dependent SAR in dicotyledons, chemical induction of blast resistance by the SA mimic benzothiadiazole was independent of JA/ET signaling and involved potentiation of SA-responsive gene expression. These findings not only strengthen the contention that monocots and dicots share evolutionary conserved plant-inducible defense pathways, but also suggest that rice is endowed with multiple blast-effective resistance

mechanisms, the simultaneous activation of which may provide an attractive tool for improvement of blast control.

In the second part of this work, attention was shifted to the complex and ambiguous role of the plant hormone abscisic acid (ABA) in rice disease resistance, thereby focusing on the rice- *C. miyabeanus* interaction. Historically most comprehensively studied as a key component in many aspects of plant development as well as in the regulation of stomatal aperture and in the initiation of adaptive responses to various environmental cues, emerging evidence also implicates ABA as an important factor integrating and fine-tuning responses to biotic challenges. Some exceptions notwithstanding, ABA predominantly behaves as a negative regulator of pathogen defense with basal or elevated plant ABA levels commonly being associated with disease susceptibility. Under our experimental conditions, however, topical application of ABA led to a significant restriction of *C. miyabeanus* progression in the mesophyll, culminating in less severe disease symptoms compared to noninduced controls. Acting independently of SA-, JA-, or callose-controlled defense mechanisms, this ABA-inducible brown spot resistance (ABA-IR) was found to be compromised in the Gα-deficient *d1* mutant, which points to a mechanistic connection between G protein signaling at the cell surface and ABA-inducible pathogen defense responses. Besides the involvement of Gα, exogenous ABA treatment was also found to steer its positive effects on *C. miyabeanus* resistance through negative cross-talk with the ET signaling pathway. Disease tests with ET-insensitive *OsEIN2*-knockout plants revealed enhanced resistance, whereas plant treatment with Ethephon, an ET-releasing chemical, favored subsequent infection. Moreover, transcriptional activation of the ET reporter gene *OsEBP89* was markedly lower in ABA-induced plants following pathogen challenge, suggesting that ABA specifically targets the ET pathway to promote *C. miyabeanus* resistance. Finally, RNAi-mediated knockdown of OsMPK5, a rice mitogen-activated protein kinase gene demonstrating a potentiated expression pattern in ABA-induced wild-type leaves, severely attenuated ABA-mediated repression of *EBP89* transcription and consequently compromised ABA-IR. Considering these findings, we propose that ABA recruits OsMPK5 to trigger resistance against *C. miyabeanus* by preventing the fungus from hijacking the ET signaling pathway. Whether or not *C. miyabeanus* disease resistance shares substantial overlap with the ABA-dependent signaling cascade(s) driving abiotic stress tolerance, and how upregulation of *OsMPK5* interferes with ET-responsive biotic stress signaling remains to be explored.

In conclusion, the results denoted in this thesis have provided several novel insights into the molecular machinery governing rhizobacteria- and chemical-induced disease resistance in rice and provide an excellent primer for future studies aimed at elucidating the tapestry of networks

underlying the innate, multicomponent defense response in rice and other cereals Such conceptual advances will not only advance our fundamental understanding of how plants cope with pathogen assault, but may also guide novel strategies to improve crop performance in suboptimal environments, and help identify appropriate contexts for the optimal deployment and commercial acceptance of induced resistance phenomena in certain agricultural contexts.

Samenvatting

Om aan de constante dreiging van een brede waaier aan pathogene micro-organismen te weerstaan, hebben planten een breed gamma aan gesofisticeerde afweermechanismen ontwikkeld. Sommige van deze mechanismen zijn constitutief, terwijl anderen slechts tot expressie komen als reactie op de belager. Behalve het activeren van afweermechanismen in lokaal geïnfecteerd weefsel, zijn planten tevens in staat een verhoogd niveau van resistentie te verwerven tegen toekomstige pathogeeninfecties. Een klassiek voorbeeld van dergelijke geïnduceerde resistentie is de zogenaamde 'systemisch verworven resistentie' (SVR). Geactiveerd door een primaire infectie met een necrose-inducerende pathogeen, is SVR niet alleen effectief tegen de initiële ziekteverwekker maar ook tegen een breed spectrum aan andere, doorgaans virulente, pathogenen. Bovendien verspreidt SVR zich systemisch doorheen de plant, wat leidt tot een bescherming van de volledige plant tegen toekomstige belagers. Een resistentie die fenotypisch vergelijkbaar is met SVR wordt geïnduceerd na kolonisatie van de wortels door bepaalde niet-pathogene rhizobacteriën. Deze vorm van resistentie wordt ook wel geïnduceerde systemische resistentie (ISR) genoemd. Hoewel SVR en ISR een uitgelezen target vormen ter ontwikkeling van een duurzame, economisch rendabele, en ecologisch verantwoorde biologische gewasbescherming, werd fundamenteel onderzoek inzake de onderliggende moleculaire mechanismen tot op heden voornamelijk toegespitst op dicotyle modelplanten zoals *Arabidopsis thaliana*, eerder dan op monocotyle voedselgewassen zoals gerst, tarwe en rogge. Het doel van voorliggend proefschrift bestond in het ontrafelen van de moleculaire mechanismen met betrekking tot biologisch en chemisch geïnduceerde pathogeenresistentie in rijst, een modelplant voor onderzoek naar graangewassen alsmede het basisvoedsel voor bijna de helft van de totale wereldbevolking.

In het eerste luik van dit werk werden verschillende rhizobacteriën die in staat zijn resistentie op te wekken in dicotyle planten getest op het vermogen om rijst te beschermen tegen diverse ziekteverwekkers. Behandeling van de wortels met *Pseudomonas aeruginosa* 7NSK2, een goed gekarakteriseerde induceerder van resistentie in boon, tomaat en Arabidopsis tegen tal van pathogenen, zorgde voor een significante reductie van ziekte veroorzaakt door de hemibiotrofe schimmel *Magnaporthe oryzae*. Hoewel uit voorgaand onderzoek in tomaat gekend is dat 7NSK2 resistentie induceert door een synergistische wisselwerking tussen respectievelijk het fenazine antibioticum pyocyanine en het siderofoor pyocheline, waren enkel pyocyanine-deficiënte mutanten niet langer in staat ISR op te wekken tegen *M. oryzae*. Echter, in tegenstelling tot wild type (WT) bacteriën, zorgden deze pyocyanine-negatieve mutanten voor een verhoogde afweer tegen de necrotrofe pathogeen *Rhizoctonia solani*. Deze resultaten tonen duidelijk aan dat

pyocyanine een duale rol vervult in het door 7NSK2 opgewekte resistentiemechanisme. Enerzijds fungeert dit pigment als een positieve regulator van resistentie tegen *M. oryzae*, anderzijds leidt de productie ervan tot een verhoogde weefselkolonisatie door *R. solani*. Uit experimenten met opgezuiverd pyocyanine bleek bovendien dat de ambivalente rol van pyocyanine in de door 7NSK2-geinduceerde ISR te wijten is aan een verhoogde accumulatie van waterstofperoxide (H_2O_2) *in planta*, een fenomeen dat gepaard gaat met *priming* van de plant voor een versnelde expressie van hypersensitieve celdood op de plaats van pathogeenaanval. Een gelijkaardig werkingsmechanisme bleek aan de grondslag te liggen van de differentiële effectiviteit van de *Serratia plymuthica* stam IC1270 met betrekking tot het induceren van ISR. Hoewel kolonizatie van de wortels door IC1270 resulteerde in de hyperinductie van H_2O_2-afhankelijke hypersensitieve celdood en een significant verhoogd resistentieniveau tegen *M. oryzae*, impliceerde dezelfde behandeling tevens een stijgende gevoeligheid voor zowel *R. solani* als de necrotrofe 'brown spot' pathogeen *Cochliobolus miyabeanus*. Deze resultaten tonen duidelijk aan dat, afhankelijk van het type ziekteverwekker, de accumulatie van reactieve zuurstofvormen en expressie van de geassocieerde hypersensitieve respons zowel een positieve als negatieve invloed kunnen uitoefenen op de geïnduceerde afweerrespons in rijst. Verder kan op basis van deze bevindingen worden geconcludeerd dat verschillende, onderling antagonistische, resistentiemechanismen vereist zijn voor een efficiënte plantafweer tegen de voornaamste rijstpathogenen: *M. oryzae*, *R. solani* en *C. miyabeanus*.

Om het mechanisme van door rhizobacteriën-geïnduceerde systemische resistentie in rijst verder te ontleden werden in een volgend stadium van het onderzoek de bacteriële determinanten en plant afweermechanismen geanalyseerd die betrokken zijn bij ISR opgewekt door de *P. fluorescens* stam WCS374r. Behandeling van de wortels met WCS374r leidde tot een verhoogde bescherming tegen *M. oryzae*, zoals ook waargenomen na behandeling met 7NSK2 en IC1270. Niettemin lijkt het onderliggende werkingsmechanisme aanzienlijk te verschillen. Door gebruik te maken van NahG rijst die geen salicylzuur accumuleert, een ethyleen ongevoelige *OsEIN2* antisense lijn en de jasmonaat deficiënte mutant *hebiba*, werd aangetoond dat, in tegenstelling tot 7NSK2-ISR, de resistentie geïnduceerd door WCS374r gereguleerd is door een SA-onafhankelijke maar JA/ET-afhankelijke signaaltransductieweg. Deze observaties bekomen in rijst, stemmen overeen met de klassieke ISR-pathway geïnduceerd door *P. fluorescens* WCS417r in Arabidopsis. Verder toonde analyse van verschillende bacteriële mutanten aan dat een siderofoor van het pseudobactine type de cruciale determinant is voor het opwekken van de ISR respons. Het toedienen aan de wortels van het opgezuiverde pseudobactine (Psb374) resulteerde in priming van de bladeren voor een versnelde afweerrespons gekenmerkt door onder meer een

snelle recrutering van fenolische componenten op plaatsen waar de pathogeen tracht binnen te dringen, evenals een gecombineerde expressie van verschillende structurele afweercomponenten en een hyperinductie van H_2O_2 in de epidermis. Verder bleek dat chemische inductie van resistentie tegen *M. oryzae* door benzothiadiazole, een synthetisch salicylzuur analoog, onafhankelijk is van jasmijnzuur en ethyleen maar gepaard gaat met een geprimede expressie van salicylzuur-gevoelige afweergenen. Dit type resistentie staat in scherp contrast met de ISR respons geïnduceerd door WCS374r maar is gelijkaardig aan de SA-afhankelijke SAR in dicotylen. Deze gegevens ondersteunen de idee dat monocotylen en dicotylen evolutionair geconserveerde signaaltransductieroutes delen en suggereren verder dat rijst voorzien is van verschillende effectieve resistentiemechanismen tegen *M. oryzae*. Gelijktijdige activatie van deze verschillende mechanismen is mogelijk een handig instrument in de ontwikkeling van nieuwe bestrijdingsstrategieën tegen deze destructieve pathogeen.

In het tweede luik van dit onderzoek werd het belang van het plantenhormoon abscisinezuur (ABA) bestudeerd in de afweer van rijst tegen *C. miyabeanus*. Tot op heden werd de rol van ABA voornamelijk onderzocht met betrekking tot abiotische stresstolerantie. Recent onderzoek bracht echter aan het licht dat ABA ook een belangrijke functie vervult in plantafweer tegen pathogenen. Hoewel er uitzonderingen gekend zijn, resulteert ABA deficiëntie vaak in verhoogde ziekteresistentie, terwijl basale of verhoogde ABA gehaltes doorgaans geassocieerd worden met een toegenomen gevoeligheid voor pathogenen. In voorliggend werk echter bleek exogene toediening van ABA een positief effect te hebben op het basale resistentieniveau van rijst tegen *C. miyabeanus*. Preventieve behandeling van de bladeren met ABA resulteerde in een significante reductie van schimmelgroei in de mesofyllaag, met verminderde symptoomontwikkeling tot gevolg. Hoewel deze ABA-induceerbare resistentie (ABA-IR) onafhankelijk is van door salicylzuur, jasmijnzuur, of callose-bemiddelde afweerreacties, waren rijstmutanten met een defect in de alfa subeenheid van het heterotrimerisch G proteïne niet langer in staat resistentie te genereren. Hoewel verder onderzoek noodzakelijk is, laat deze bevinding vermoeden dat er een mechanistisch verband bestaat tussen de door G proteïnen-bemiddelde signalisatie ter hoogte van de celperiferie en ABA-induceerbare ziekteresistentie. Verdere experimenten toonden tevens aan dat ABA-IR tegen *C. miyabeanus* gestoeld is op een negatieve interactie met de ethyleen pathway. Ethyleen ongevoelige *OsEIN2* knockout planten bleken minder ziektegevoelig dan wild type (WT) planten, terwijl behandeling van de bladeren met Ethephon, een ethyleen producerende groeiregulator, resulteerde in een verlaagd resistentieniveau. Verder bleek de transcriptionele activiteit van *OsEBP89*, een merker voor ethyleen-afhankelijke afweer in rijst, beduidend lager te zijn na pathogeeninfectie in door ABA-

geïnduceerde planten dan in onbehandelde controleplanten. Het door ABA-induceerbare MAP kinase gen *OsMPK5* speelt een cruciale rol in dit proces, aangezien transgene *OsMPK5* RNAi planten een analoog *OsEBP89* expressiepatroon vertoonden als onbehandelde controleplanten en ook niet in staat bleken om ABA-IR tot expressie te brengen. Deze bevindingen suggereren dat ABA resistentie induceert tegen *C. miyabeanus* door misbruik van de ethyleen signaaltranductieweg door de pathogeen te verhinderen. Of ziekteresistentie tegen *C. miyabeanus* al dan niet verloopt via een analoge ABA-bemiddelde signaaltransductieroute als deze vereist voor de activatie van plantreacties tegen abiotische stressfactoren, en hoe inductie van *OsMPK5* leidt tot een repressie van ethyleen-afhankelijke afweer is stof voor verder onderzoek.

Tot besluit kan gesteld worden dat deze thesis verscheidene nieuwe inzichten verleent in de fundamentele mechanismen die ten grondslag liggen aan door rhizobacteriën en abscisinezuur geïnduceerde pathogeenresistentie in rijst. De bekomen resultaten vormen een uitstekende basis voor verder fundamenteel onderzoek inzake de regulatie van de natuurlijke immuniteitsrespons in rijst en andere graangewassen, en kunnen aldus een belangrijke bijdrage leveren tot het ontwikkelen van nieuwe strategieën met het oog op een optimale exploitatie en implementatie van geïnduceerde resistentie in het kader van een geïntegreerde gewasbescherming.

Curriculum Vitae

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2000: Bachelor degree in Applied Biological Sciences, Faculty of Bioscience Engineering, Ghent University

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III. Professional Record

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IWT specialization fellowship entitled "Broad-spectrum control of rice pathogens by means of induced resistance"

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BOF project at the laboratory of Phytopathology, Ghent University, entitled "Unraveling the underlying mechanisms of biologically induced pathogen resistance in rice"

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IV. Publications

Peer reviewed

De Vleesschauwer D, Bakker PAHM, Djavaheri M, Höfte M. (2008). *Pseudomonas fluorescens* WCS374r-induced systemic resistance in rice against *Magnaporthe oryzae* is based on pseudobactin-mediated priming for a salicylic acid-repressible multifaceted defense response. *Plant Physiology*, accepted October 17th.

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

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Book chapters

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Conference proceedings

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V. Participation to Conferences and Symposia

De Vleesschauwer D, Bakker PAHM, Djavaheri M, Höfte M. (2008). Molecular insights into rhizobacteria-induced systemic resistance in rice. 9th International Congress of Plant Pathology, August 25-29, 2008, Turin, Italy. Poster presentation by D De Vleesschauwer:

De Vleesschauwer D, Höfte M. (2007). Bacterial determinants and defense mechanisms underpinning rhizobacteria-mediated systemic resistance in rice. 4th International Rice Blast Conference, October 9-14, 2007, Changsha, Hunan, China. Oral presentation by D De Vleesschauwer.

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De Vleesschauwer D, Höfte M. (2006). Redox-active pyocyanin: two-faced player in *Pseudomonas aeruginosa* 7NSK2-mediated ISR. 58th International symposium on crop protection, May 23, 2006, Ghent, Belgium. Oral presentation by D De Vleesschauwer.

De Vleesschauwer D, Höfte M. (2005). Induction of systemic resistance to *Magnaporthe grisea* and *Rhizoctonia solani* in rice by *Pseudomonas aeruginosa* 7NSK2. 12th International congress on Molecular Plant-Microbe Interactions, December 14-19, 2005, Merida, Yucatan, Mexico. Poster presentation by D De Vleesschauwer.

De Vleesschauwer D, Van Heirzeele B, and Höfte, M. (2005). Induction of systemic resistance to *Magnaporthe grisea* and *Rhizoctonia solani* in rice by *Pseudomonas aeruginosa* 7NSK2: the role of salicylic acid and pyocyanin. 57th International symposium on crop protection, May 10, 2005, Ghent, Belgium. Oral presentation by D De Vleesschauwer.

De Vleesschauwer D, Audenaert K, Höfte M. (2005). Suppression of bacterial blight on rice by induced systemic resistance and manipulation of endogenous abscisic acid levels. 1st International Symposium on Biological Control of bacterial diseases, 23rd – 26th October 2005, Seeheim-Jugenheim, Germany. Oral presentation by K Audenaert.

De Vleesschauwer D, Höfte M. (2004). Influence of major resistance genes on induced resistance to *Pyricularia grisea* on rice. International Joint workshop on PR proteins and induced resistance, May 5-9, 2004, Helsingor, Denmark. Oral presentation by D De Vleesschauwer.

VI. Supervision of undergraduate students

Jeroen Seaux (2003-2004). Moleculaire karakterisatie en pathotype analyse van een Indische *Pyricularia grisea* populatie. Promotor: Prof. M. Höfte. Thesis to obtain the degree of Bio-Engineer, master Cell and Gene biotechnology.

Bram Van Heirzeele (2004-2005). Inductie van resistentie in rijst door wortelkoloniserende bacteriën en avirulente pathogenen. Promotor: Prof. M. Höfte. Thesis to obtain the degree of Bio-Engineer, master Cell and Gene biotechnology.

Daphnis Dutoit (2004-2005). Inductie van systemische resistentie in rijst en boon met de wortelkoloniserende bacterie *Serratia plymuthica*. Promotors: Prof. M. Höfte and H. Meziane. Thesis to obtain the degree of Bio-Engineer, master Cell and Gene biotechnology.

Frederic Devos (2005-2006). Interactie van het *Mycosphaerella fijiensis* toxine juglone met resistente en gevoelige bananencultivars. Promotor: Prof. M. Höfte. Thesis to obtain the degree of Bio-Engineer, master Agriculture.

Hanne Decuypere (2006-2007). Basale en geïnduceerde resistentie in rijst (*Oryza sativa* L.) tegen necrotrofe en hemibiotrofe pathogenen. Promotors: Prof. M. Höfte and D. De Vleesschauwer. Thesis to obtain the degree of Bio-Engineer, master Cell and Gene biotechnology.

Bert Beck (2007-2008). De effecten van siliciumbehandeling op ziekteresistentie in rijst en banaan. Promotors: Prof. M. Höfte and D. De Vleesschauwer. Thesis to obtain the degree of Bio-Engineer, master Agriculture.