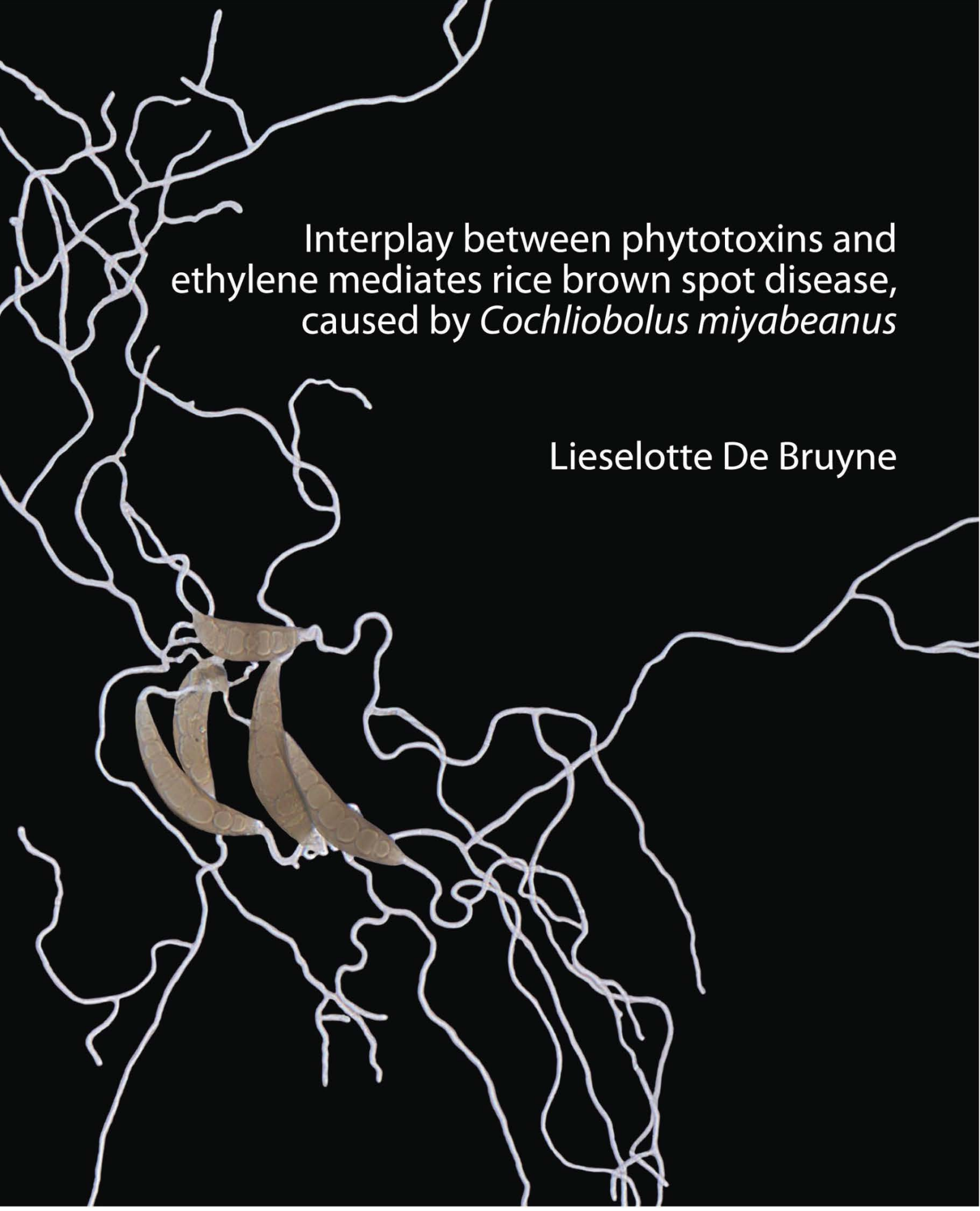
Interplay between phytotoxins and ethylene mediates rice brown spot disease, caused by *Cochliobolus miyabeanus*

Lieselotte De Bruyne



UNIVERSITEIT GENT

FACULTEIT BIO-INGENIEURSWETENSCHAPPEN

The important thing is not to stop questioning.
Curiosity has its own reason for existing.

Albert Einstein

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Interplay between phytotoxins and ethylene mediates rice brown spot disease, caused by *Cochliobolus miyabeanus*

Thesis submitted in fulfilment of the requirements for
the degree of Doctor (PhD) in Applied Biological Sciences

Dutch translation of the title:

Wisselwerking tussen fytotoxines en ethyleen stuurt rijst brown spot ziekte, veroorzaakt door *Cochliobolus miyabeanus*

Cover illustration:

Germinating conidia of *Cochliobolus miyabeanus*.

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Dankwoord

Zie hier: mijn doctoraat! Na een korte valse start heb ik de richting gevonden die me naar dit eindpunt heeft geleid. Hoewel de cover van dit boekje mijn naam draagt, is dit werk niet enkel mijn persoonlijke verdienste. Een woordje van dank is dus op zijn plaats.

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Contents

List of abbreviations

AACT	Acetoacetyl-CoA transferase
AAL-toxin	*Alternaria alternata* f. sp. *lycopersici* toxin
ABA	Abscisic acid
ABC	ATP-binding cassette
ACC	1-aminocyclopropane-1-carboxylic acid
ACP-domain	Acyl carrier protein domain
A-domain	Adenylation domain
AOA	Aminooxyacetic acid
AT-domain	Acyltransferase domain
AVG	Aminoethoxyvinylglycine
BI	Bax inhibitor
bZIP	Basic Leucine Zipper
CaM	Calmodulin
CDC	Conditionally dispensable chromosome
C-domain	Condensation domain
CDPK	Calcium-dependent protein kinase
CerS	Ceramide synthase
CFP	Cercosporin facilitator protein
CFU	Colony forming units
CMX	Complete medium with xylose
CTB	Cercosporin toxin biosynthesis
CWDE	Cell wall degrading enzymes
CYC-domain	Cyclase domain
CYP	Cytochrome P450
DAMP	Damage-associated molecular pattern
DMAPP	Dimethylallyl diphosphate
DH-domain	Dehydratase domain
DON	Deoxynivalenol
DNA	Deoxyribonucleic acid
Dpi/t	Days post inoculation/treatment
DW	Dry weight
DXP	Deoxyxylulose 5-phosphate
EFE	Ethylene forming enzyme
EGTA	Ethylene glycol tetra-acetic acid
ER(-domain)	Enoyl reductase (domain)
ESI	Electrospray ionization
ET	Ethylene
ETF	Electron transfer flavoprotein
FPPS	Farnesyl-pyrophosphate synthase
	flavoprotein
FS	Fusicoccadiene synthase
FW	Fresh weight
GC	Gas chromatography
GC-content	Guanine-cytosine content
GDC	Glycine decarboxylase
GFPP	Geranylfarnesyl pyrophosphate
GPCR	G-protein coupled receptor
HC-toxin	*Cochliobolus carbonum* toxin
HCTR	HC-toxin reductase
HDAC	Histone deacetylase complex
HGT	Horizontal gene transfer
HMGR/S	3-hydroxy-3-methylglutaryl-CoA reductase/synthase
Hpi/t	Hours post inoculation/treatment
HR	Hypersensitive response
HST	Host-selective toxin
HS-toxin	*Cochliobolus sacchari* toxin
HTS1	HC-toxin synthetase
HygB	Hygromycin B
IPP	Isopentenyl diphosphate
IPPI	IPP isomerase
JA	Jasmonic acid
KMBA	α-keto-γ-methylthiobutyric acid
KR-domain	Ketoreductase domain
KS-domain	Ketoacyl CoA synthase domain
LC	Liquid chromatography

LPAD	Laser photoacoustic detection
LysM	Lysin motif
m/z	Mass/charge
MDC	Mevalonate-5-pyrophosphate decarboxylase
MEP	Methylerythritol phosphate
MET	Methionine
MFS	Major facilitator superfamily
MK	Mevalonate kinase
MRM	Multiple reaction monitoring
MTA	5-methylthioadenosyl
MT-domain	Methyltransferase domain
MTR	5-methylthioribose
MTR-1P	5-methylthioribose-1-phosphate
MVA	Mevalonate
MW	Molecular weight
NIP	Necrosis inducing proteins
NADP	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NRP(S)	Non-ribosomal peptide (synthetase)
NST	Non-selective toxin
Ops1	Ophiobolin synthase
ORF	Open reading frame
OXO	2-oxoglutarate
PAL	Phenylalanine ammonia-lyase
PAMP	Pathogen-associated molecular pattern
PC(A)	Principal component (analysis)
PCD	Programmed cell death
PCR	Polymerase chain reaction
PDA/B	Potato dextrose agar/broth
P-domain	Peptidyl carrier protein domain (= T-domain)
PDR	Pleiotropic drugs resistance
PK(S)	Polyketide (synthase)
PM	Plasma membrane
PMK	Phosphomevalonate kinase
PPO	Polyphenol oxidase
PPTase	4'-phosphopantetheinyl transferase
PtrToxA	*Pyrenophora tritici-repentis* ToxA
QTL	Quantitative trait locus
R-domain	
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RuBisCO	Ribulose 1,5-bisphosphate carboxylase/oxygenase
SA	Salicylic acid
SAM	S-adenosyl-methionine
SE	Standard error
SNP	Single nucleotide polymorphism
SSP	Small secreted proteins
STS	Silver thiosulphate
SVM	Support vector machine
TC	Terpene cyclase
TCA	Tricarboxylic acid cycle (Citric acid cycle)
T-domain	Thiolation domain (=P-domain)
TE-domain	Thioesterase domain
TF	Transcription factor
TIC	Total ion chromatogram
Tmsc	Texas male sterile cytoplasm
UHPLC-MS/MS	Ultrahigh performance liquid chromatography – Tandem mass spectrometry
W5	N-(6-aminohexyl)-1-naphthalene sulfonamide hydrochloride
W7	N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide hydrochloride
WT	Wild type

Problem statement and research outline

Rice (*Oryza sativa*) is a major staple crop and is the main calorie source for more than half of the world population (Khush, 2005). Thanks to the Green Revolution, rice yields have steadily increased during the past decades, ensuring food security for a fast growing world population. The main focus during the Green Revolution was an increase in production capacity, which was achieved by wide scale adoption of high-yielding varieties, improvement of irrigation facilities and the application of fertilizers and other complementary inputs (Mew *et al.*, 2004; Khush, 2005). Today, production levels are reaching their limits, while the world population keeps growing. Knowing that up to 40% of the theoretical yield is lost due to pests, diseases and weeds, pivoting the focus from increasing yield to preventing losses can create new opportunities (Savary *et al.*, 2012a). It is estimated that optimal pest control could account for yield gains of 20% of the current actual yields (Savary *et al.*, 2012b).

Rice brown spot disease, caused by *Cochliobolus miyabeanus* (anamorph *Bipolaris oryzae*) is currently considered one of the most important yield reducers of rice, causing average losses of 10% wherever it appears (Savary *et al.*, 2012a). Brown spot is a necrotrophic fungal disease affecting leaves and seeds of rice plants (*Oryza sativa*). Each growing season the disease affects millions of hectares of rice on fields that suffer from drought and nutrient deficiencies (Barnwal *et al.*, 2013). These fields mainly belong to resource-poor farmers, therefore the disease is also called 'poor farmers disease'. Recently, the disease is gaining in importance due to climate change. As drought is becoming more frequent, conditions become more favourable to brown spot disease, especially in combination with periods of heavy rainfall (Savary *et al.*, 2005). Despite three reported severe outbreaks during the 20th century (brown spot contributed to the great Bengal famine in 1942) and the fact that brown spot has now become a chronic disease in many regions, brown spot has not received as much attention as it deserves (Padmanabhan, 1973; Barnwal *et al.*, 2013).

Past breeding efforts have not resulted in resistant varieties, although recently a few studies have identified promising quantitative trait loci, providing broad resistance to brown spot disease (Hossain *et al.*, 2004; Sato *et al.*, 2008; Katara *et al.*, 2010; Yaqoob *et al.*, 2011; Kumar *et al.*, 2013). Hence, chemical crop protection is still the most widely used control measure. In order to improve brown spot disease control, a thorough knowledge of the disease process is of paramount importance. Past research efforts have revealed few molecular characteristics of brown spot disease. Many necrotrophic pathogens produce phytotoxins that contribute strongly to pathogenicity and virulence by inducing plant cell death. *C. miyabeanus* produces phytotoxic ophiobolins that can reproduce brown spot symptoms on rice leaves, but the involvement in disease development is still unclear. Furthermore, ethylene seems to play a major role in plant susceptibility. The application of external ethylene strongly enhanced susceptibility, whereas ethylene-insensitive plants were highly resistant (De Vleesschauwer *et al.*, 2010). Surprisingly, ethylene biosynthesis is induced upon infection (Van Bockhaven *et al.*, 2015b). Therefore it seems plausible that the fungus deploys strategies to hijack the plant ethylene metabolism. The primary

aim of this study was to broaden our knowledge on the molecular interaction between rice and *C. miyabeanus* during brown spot disease. We started our research from the following questions:

1. Which virulence mechanisms are deployed by *C. miyabeanus* during rice brown spot disease?
2. What are the temporal dynamics of the interaction between *C. miyabeanus* and rice?
3. How can the fungus influence the plant ethylene metabolism?

In **Chapter 1** we review the most important literature on rice brown spot disease. We start with an introduction to the disease epidemiology and the current state of knowledge on the molecular interaction between *C. miyabeanus* and rice. The second part of this chapter discusses virulence mechanisms employed by Dothideomycetes plant pathogens and gives an introduction to fungal secondary metabolism.

For many years *C. miyabeanus* has been known to produce ophiobolin A, a sesterterpenoid non-host specific phytotoxin, and yet its role in the infection process has never been shown unambiguously. In **Chapter 2** we discuss the role of ophiobolin A during brown spot disease**.** We identified a key terpene cyclase in the ophiobolin biosynthesis pathway, CmOps1, and deleted the corresponding gene from a virulent *C. miyabeanus* strain. Infection experiments with ophiobolin-deficient mutants show that ophiobolin A is an important virulence factor during the first phase of infection. Furthermore we provide insight into the plant defense response and cell death triggered by ophiobolin A.

Like most fungal secondary metabolite genes, *CmOPS1* is part of a gene cluster. In **Chapter 3** we describe the broad genomic region surrounding *CmOPS1* and identify a cluster of seven genes, featuring two specific transcription factors, a P450 monooxygenase, two oxidoreductases and an ABC-type transporter, in addition to the key terpene cyclase CmOps1. The cluster is conserved in a diverse group of fungal species belonging to the Pezizomycotina. Although most of these species most likely acquired the cluster through vertical gene transfer, one horizontal gene transfer event might have happened.

In **Chapter 4** we used a metabolomics approach to identify new virulence factors produced by *C. miyabeanus*. We describe for the first time the production of tentoxin by *C. miyabeanus* and identified the corresponding non-ribosomal peptide synthetase CmNps3, an ortholog of the *C. heterostrophus* ChNps3. The corresponding gene was deleted and the resulting mutants were used to assess the contribution of tentoxin to disease development. We show that tentoxin is produced during the second phase of infection and is associated with the development of chlorosis.

Ethylene enhances susceptibility to brown spot disease, yet the plant increases biosynthesis upon infection. In **Chapter 5** we investigate how the fungus might hijack the plant ethylene metabolism. First, we show that ethylene emissions describe a biphasic pattern during infection.

Next we show that *C. miyabeanus* is able to produce fungal ethylene via two distinct pathways. Finally, we analyse the correlation between fungal phytotoxin and ethylene production and overall ethylene emissions throughout disease.

We conclude this work with an overall discussion of the main findings and future perspectives in **Chapter 6.** We propose a disease process characterized by two distinct phases, an early phase in which the fungus induces localized cell death via ophiobolin A, and a second phase in which the fungus triggers spreading senescence and chlorosis, assisted by fungal ethylene and tentoxin production. These two phases coincide with the biphasic ethylene emission pattern.

Our research project initially started as an investigation into the role of brassinosteroids in rice defense towards *C. miyabeanus*. Unfortunately, preliminary results were not promising and did not suggest an important role for brassinosteroids in this specific interaction. Although the research outline was redirected, we ended up reviewing the recent literature on brassinosteroids in plant defense responses. The **Addendum** at the end of this manuscript features a review on the role of brassinosteroids and gibberellins in switching between plant defense and plant growth.

Chapter 1

General introduction

Cochliobolus miyabeanus, the rice brown spot pathogen

Taxonomy of *Cochliobolus miyabeanus*

Cochliobolus miyabeanus is a filamentous ascomycete belonging to the Pleosporales order of the class Dothideomycetes. Although the sexual form has not been encountered in nature, the fungus is best known by its teleomorph name, *Cochliobolus miyabeanus* (Ito et. Kurib) Drechsler ex Dastur (Ito & Kuribayashi, 1927; Dastur, 1942). Currently, the anamorph is officially called *Bipolaris oryzae,* but has changed name many times since its first discovery. The asexual state was first described in 1900 as *Helminthosporium oryzae* Breda de Haan, but was renamed *Bipolaris oryzae* (Breda de Haan) in 1959, because of its typical bipolar germination pattern (Breda de Haan, 1900; Shoemaker, 1959) Later, *Drechslera oryzae* (Breda de Haan) was used temporarily after the observation of spore germination from intermediate segments (Subramanian & Jain, 1966).

Many *Cochliobolus* species are destructive plant pathogens on monocotyledons and are notorious for the production of phytotoxins (Panaccione, 1993; Condon *et al.*, 2013). Most of the pathogenic species are categorized as necrotrophs, but *C. sativus* is a hemibiotroph (Kumar *et al.*, 2002). Many *Cochliobolus* spp. can produce host-selective phytotoxins (HST), which are only active against a particular host species or cultivar. *C. carbonum* and *C. heterostrophus* are known pathogens of maize. *C. carbonum* race 1 is extremely virulent due to the production of the HST HC-toxin, a cyclic non-ribosomal tetrapeptide that inhibits plant defense gene expression. Without HC-toxin, *C. carbonum* is still weakly virulent (Pitkin *et al.*, 2000). *C. heterostrophus* race O is known as a minor pathogen on maize, but race T produces the HST T-toxin, a polyketide that renders the pathogen highly virulent towards Texas male sterile cytoplasm (Tmsc) maize. An outbreak of *C. heterostrophus* race T on Tmsc maize resulted in a major epidemic of Southern corn leaf blight in the USA in 1969 (Horwitz *et al.*, 2013; Condon *et al.*, 2013). *C. sativus* attacks different plant parts of cereals and grasses and different pathotypes have been described that vary significantly in virulence level (Condon *et al.*, 2013). *C. victoriae* can cause Victoria blight disease on oats and produces the HST victorin, a cyclic non-ribosomal pentapeptide that targets photorespiration and induces senescence (Navarre & Wolpert, 1999; Tada *et al.*, 2005).

C. miyabeanus mainly affects rice and grasses and is somehow atypical in that regard that it does not produce a known HST. However, *C. miyabeanus* can be highly virulent under favourable conditions and contributed partially to the great Bengal famine in 1942, resulting in the deaths of two million people (Padmanabhan, 1973). Like *C. heterostrophus*, *C. miyabeanus* is known to produce ophiobolins, non-selective phytotoxic sesterterpenes that can cause symptoms on many plant species (Nakamura & Ishibashi, 1958; Sugawara *et al.*, 1987; Au *et al.*, 2000).

Disease cycle

C. miyabeanus has a broad host-range, and has been isolated from several Poaceae plant species, in addition to a few dicotyledonous hosts (Manamgoda *et al.*, 2011). However, *C. miyabeanus* has the highest economic impact when it infects rice fields. The primary inoculum originates from infected rice seeds or from infected plant parts left in the field from a previous crop, in which it can survive for multiple years if conditions are favourable (Ou, 1985). Infected seeds can cause seedling blight, but subsequent leaves may stay lesion-free under rapid growth (Ou, 1985). A secondary inoculum originates from asexual spores or conidia produced at the end of a primary infection (Figure 1.1a).

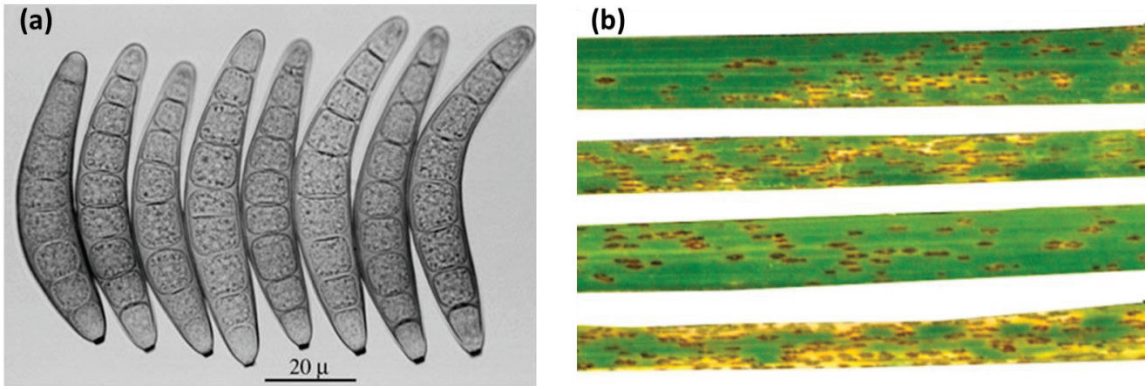


Figure 1.1. (a) *Cochliobolus miyabeanus* conidia. Picture adapted from Matsushima (1975). **(b)** Typical brown spot symptoms three days after inoculation of rice (*Oryza sativa)* plants with *C. miyabeanus* strain Cm988.

Conidia are spread through the air by wind and rain and land on the leaves of nearby rice plants. The spores germinate under favourable circumstances and germination is optimal at 25 - 30 °C and >89% humidity (Sunder *et al.*, 2014). Scanning electron microscopy pictures of the germination and infection process have been published by Locci (1969) and Hau & Rush (1982) (Figure 1.2). Germ tubes typically emerge from both ends, but can originate from the middle cells as well (Figure 1.2a). The infection biology of *C. miyabeanus* is very similar to the closely related species *C. heterostrophus* (Horwitz *et al.*, 2013). Around four hours after spore germination, appressoria are formed at the end of the germ tubes (Figure 1.2b). Most appressoria are formed over bulliform cells, but they are also commonly formed at cell junctions. Alternatively, penetration can happen through stomata without the formation of an appressorium (Hau & Rush, 1982). Similar to *C. heterostrophus*, germinating spores secrete an extracellular sheath with adhesive characteristics, allowing them to stick tightly to the rice leaf (Hau & Rush, 1982; Horwitz *et al.*, 2013). Beneath the appressoria the fungus develops peg-like structures, possibly representing the actual points of penetration. Direct penetration of the leaf tissue seems very smooth and no trace of laceration can be observed on the leaf tissue (Locci, 1969) (Figure 1.2c). No reports are available on the location of hyphae inside the leaf tissue, but our own observations indicate that *C. miyabeanus* behaves very similarly to *C. heterostrophus*. Hyphae grow mainly

between mesophyll cells and the first signs of cell death occur soon after penetration. Eventually, new conidiophores emerge from the infected areas and provide a new inoculum for a subsequent infection round (Figure 1.2d).

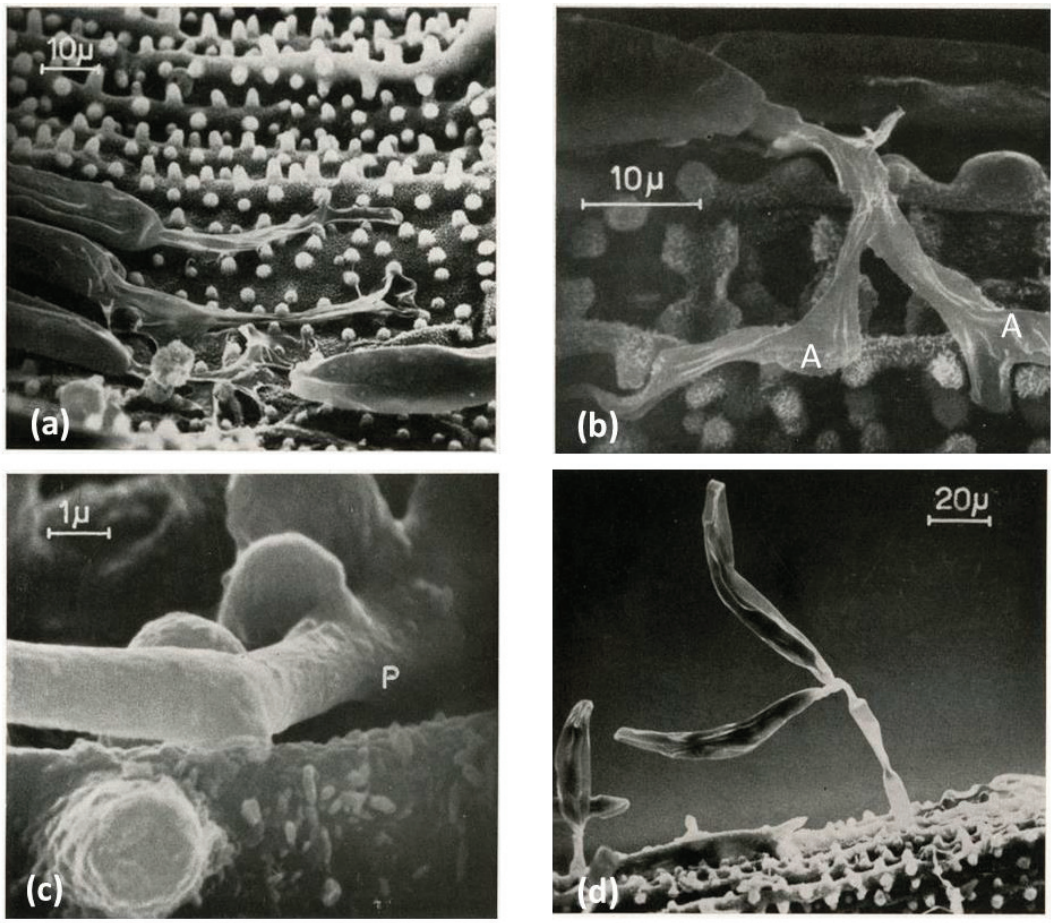


Figure 1.2. Scanning electron microscopy pictures of *C. miyabeanus* during infection of a rice leaf. (a) Spore germination after four hours on leaf surface. **(b)** Appresorium (A) formation by the germ tube. **(c)** Penetration (P) of the hyphae through the leaf cuticle. **(d)** Conidiophores emerging from the leaf surface (60 hours after inoculation). Pictures adapted from Locci (1969) and Hau & Rush (1982).

During the growing season *C. miyabeanus* mainly affects the leaves of rice plants were it causes brown necrotic oval lesions during the first 24 hours of infection, followed by spreading chlorosis around the lesions (Figure 1.1b). The disease results in a strong reduction of photosynthetic capacity and withering of the leaves. During the ear development, seeds can get infected too. Severely infected seeds will decolorize, show spots and shrivel and become unsuitable for consumption (Chakrabarti, 2001). If infected seeds are used for a following crop, seedlings can suffer heavily from seedling blight (Ou, 1985).

Impact of brown spot disease

Rice (*Oryza sativa*) is the main calorie source for more than half of the world population and is mainly produced in Asia. Recent assessments show that about 20% of the attainable rice yield is lost due to pests and diseases (Savary *et al.*, 2012a). Brown spot, caused by *Cochliobolus*

miyabeanus, causes average losses of 10% wherever it appears, making it one of the most important yield reducers of rice today. When inedible infected grains are taken into account, yield losses can be even higher, up to 52% (Chakrabarti, 2001).

Two major epidemics of brown spot occurred in India during the growing seasons of 1918 and 1942. The latter outbreak contributed partially to the great Bengal famine, which resulted in the deaths of two million people during the second world war (Stuthman, 2002). In 1961, a severe brown spot epidemic in Minnesota caused a complete crop of wild rice (*Zizania aquatic*) to fail (Bean & Schwartz, 1961). Although such acute and extreme outbreaks are generally rare, brown spot has now become a chronic disease that affects millions of hectares of rice every growing season. The disease is widespread and prevalent in all rice growing regions in the world, stretching from Asia to Australia over Africa and Southern Europe all the way to North and South America (Manamgoda *et al.*, 2011).

The highest incidence of the disease is reported in rainfed fields suffering from multiple nutrient deficiencies and drought, conditions that predispose plants to brown spot disease (Barnwal *et al.*, 2013). Several mineral abnormalities have been associated with brown spot susceptibility: phosphorus, nitrogen, potassium, silicon, manganese, magnesium, iron and calcium (Ou, 1985; Carvalho *et al.*, 2010). For this reason, the occurrence of brown spot can be seen as an indication of poor soil health. For most nutrients, optimal response patterns can be achieved, as different concentrations may correspond to opposite effects on disease severity. Silicon is a nutrient of special interest. It can induce resistance to many abiotic and biotic stresses and it has been shown convincingly that silicon application is particularly successful in making plants more resistant to brown spot (Epstein, 1994; Dallagnol *et al.*, 2009; Prabhu *et al.*, 2012; Van Bockhaven *et al.*, 2015a,b). Furthermore, silicon also has growth enhancing properties and does not become detrimental when applied in excess (Ma & Yamaji, 2008). Similar poor soil conditions also cause Akiochi disease, which is basically a nutritional disorder, associated with the presence of H_2S in reductive soils. Unsurprisingly, plants suffering from Akiochi disease are more susceptible to bown spot disease and the other way around. This creates opportunities to find resistant cultivars by screening germplasma for H_2S resistance (Ou, 1985).

Besides poor soil conditions, specific weather conditions can also precede disease. In years of normal rainfall, incidence is low. However when periods of drought are alternated by periods of heavy rainfall, disease incidence can be severe (Pannu *et al.*, 2005; Patel *et al.*, 2010; Barnwal *et al.*, 2013). This also explains the higher susceptibility of rain-fed crops compared to irrigated fields. Furthermore, drought is directly correlated to nutrient deficiencies as nutrients are taken up by the roots along with water. As drought is becoming more frequent due to climate change, brown spot is gaining importance, especially during short periods of heavy rainfall (Savary, 2005). It is clear that nutrient deficiency and drought, together with soil characteristics are interacting factors that determine disease predisposition. However, most resource-poor farmers lack the

means to improve field conditions; therefore they are often the first economic and social victims. Brown spot disease definitely deserves more attention than it has received so far.

Disease management

Because nutrient deficiencies underlie the predisposition to brown spot susceptibility, the main crop protection practice implies restoring soil constitution and repletion of mineral shortages. This is reflected by the fact that the disease is almost completely absent in healthy plants (Ou, 1985; Barnwal *et al.*, 2013). As farmers do not always have the means to improve field conditions, the second priority should be breeding for resistant varieties. So far, breeding programs have mainly focussed on high-yielding varieties and resistance to diseases prevalent under intensive farming conditions, such as rice blast. Thanks to those efforts, the problem of rice blast is well under control. However, good sources of resistance to brown spot are still lacking. Given that brown spot is a necrotrophic disease, qualitative resistance genes are not likely to be found. The few qualitative resistance genes found for true necrotrophs, encode phytotoxin detoxifying enzymes or R-gene like susceptibility genes, rather than real R-genes (Walton, 2006; Poland *et al.*, 2009). Therefore, breeding efforts should focus on quantitative disease resistance. Quantitative resistance is overall less strong but more durable in the long term; broad base resistance is less easily overcome by the pathogen. Fortunately, efforts are increasing and several available rice genotypes are being tested under different field conditions and new promising quantitative trait loci have been reported (Hossain *et al.*, 2004; Sato *et al.*, 2008; Katara *et al.*, 2010; Yaqoob *et al.*, 2011; Kumar *et al.*, 2013). Biological control measures may also proof useful in fighting brown spot disease. The use of *Trichoderma* species is a very promising technique in rice pest management. Additionally, several plant extracts have shown their usefulness in controlling brown spot disease (Harish *et al.*, 2008; Khalili *et al.*, 2012). Despite recent efforts to develop alternative crop protection measures, chemical crop protection is still the most widely used and most effective method to control brown spot disease (Lore *et al.*, 2007).

It is clear that all current management techniques require a certain amount of resources. Smallholder farmers mostly do not have the means to invest extensively in fertilizer or fungicides. Therefore new and more affordable methods to fight the disease are urgently needed, as well as efforts to allow farmers the use of resistant varieties without a high investment cost. In order to breed towards more resistant varieties, better knowledge is needed regarding the molecular interactions between plant and pathogen and the correlation with plant physiology and soil constitution.

Molecular interaction with rice

Necrotroph infection strategies

Plants are constantly threatened by attempted pathogen attack and have evolved ingenious ways to withstand infection in most cases (Glazebrook, 2005). Detection of pathogen- or damage-associated patterns (P/DAMPs) and other danger signals typically evokes a complex and energy-consuming immune response involving different molecular and biochemical events. The immune response is typically characterized by the induction of pathogenesis related-genes, antimicrobial compounds and a specific blend of plant hormones, which have been uncovered as essential mediators of disease signaling and resistance responses (Boller & Felix, 2009; Pieterse *et al.*, 2012; Denancé *et al.*, 2013; De Vleesschauwer *et al.*, 2014; De Bruyne *et al.*, 2014). Depending on the plant-pathogen interaction, specific hormone cross-talk results in either resistance or susceptibility. This also implies the existence of trade-offs; induction of a hormone signaling pathway in response to one pathogen changes the resistance towards other pathogens (Verhage *et al.*, 2010; De Vleesschauwer *et al.*, 2013). Whereas for Arabidopsis a rather straight forward binary concept has been suggested in which ethylene and jasmonic acid induce resistance to necrotrophs and salicylic acid enhances resistance to biotrophs, cross-talk in rice seems much more complex (De Vleesschauwer *et al.*, 2013, 2014). Furthermore, effective defense against biotrophic and many hemibiotrophic pathogens encompasses the induction of localized cell death, mediated by a gene-for-gene interaction. On the other hand, a similar defense response enhances susceptibility towards necrotrophs (Mengiste, 2012).

While plants invest energy to safeguard their integrity, pathogens strive to release nutrients from the plant for their proliferation. Thereto they need to evade plant defenses and at the same time induce a steady nutrient supply. Necrotrophs count on the induction of cell death to access plant nutrients and generally use phytotoxic compounds to induce localized programmed cell death (PCD) and/or senescence (van Kan, 2006; Laluk & Mengiste, 2010; Horbach *et al.*, 2011). These plant suicide processes are characterized by a shut-down of photosynthetic energy production and enhanced recycling of available cell content to preserve nutrients for healthy tissue (Belknap & Garbarino, 1996; Hörtensteiner & Feller, 2002). The cessation of carbohydrate production results in a down regulation of respiration through glycolysis. However, this is partially compensated for by the induction of lipid and protein respiration to preserve energy production through the TCA cycle. Proteins are broken down by activated proteasomes into separate amino acids (Hörtensteiner & Feller, 2002). Free amino acids are converted to pyruvate, acetyl-CoA or 2-oxoglutarate to be used for energy production via the TCA cycle or can provide electrons for energy production via the electron transfer flavoprotein (ETF) chain (Araújo *et al.*, 2011). At the same time, plants remobilize nitrogen by enhanced conversion of glutamate to glutamine by glutamine synthetases. The free amino acids released by protein breakdown during programmed cell death are not only reused by the plant, but also become available to the

pathogen. Interestingly, amino acids typically increase during infection, despite being drained by the fungus, indicating that the fungus manipulates the metabolism to maintain a supply of nitrogen compounds (Solomon *et al.*, 2003; Dulermo *et al.*, 2009). Furthermore, glutamine and ammonia are usually the preferred nitrogen source and as long as these nutrients are available the fungus will delay its own catabolic processes (Divon & Fluhr, 2007).

Rice – C. miyabeanus *molecular interaction*

Brown spot disease is similarly associated with significant changes in plant physiology, including a strong down-regulation of photosynthetic processes and a switch to nitrogen remobilization (Kim *et al.*, 2014; Van Bockhaven *et al.*, 2015b). These metabolic changes thus provide a constant flux of nutrients for *C. miyabeanus*. It is expected that *C. miyabeanus* also undergoes significant metabolic changes in order to evade defenses and cause disease. Although the pre-penetration dynamics of *C. miyabeanus* metabolism have been fairly well investigated, the dynamics upon penetration are still an unexplored field.

After landing on a suitable host leaf, the conidia germinate and eventually develop an infection structure or appressorium at the end of the germ tube. During the germination phase, the fungus has no access to the host metabolism and relies on its own reserves. During this phase, internal carbon sources such as glycogen, trehalose, sugar alcohols and lipids are broken down to provide energy (Divon & Fluhr, 2007). Especially lipid catabolism seems to be important throughout the whole pre-penetration phase, whereas the activity of glycolysis diminishes as the appressorium matures (Solomon *et al.*, 2003). Although fungi mainly rely on plant nitrogen sources, studies show that some turnover of amino acid metabolism occurs during the pre-penetration phase (Solomon *et al.*, 2003). High proteolytic activity was observed in non-germinated *C. miyabeanus* conidia, and this activity increased even more during infection. Proteins are broken down into amino acids and eventually converted to glutamine, which reached very high levels in germinating spores. At the same time trehalose is metabolised by the hexose monophosphate pathway and fed into the TCA cycle to produce energy (Matsubara & Kuroda, 1980). Interestingly, despite the limited internal reserves, germinating spores can already produce small amounts of ophiobolins (Xiao *et al.*, 1991). By the end of appressorium formation, most internal reserves are consumed. The pathogen penetrates the rice leaf by means of infection pegs from the appressorium or through the stomata, announcing the first phase of disease. Further investigations are needed to characterize what happens once the fungus enters the rice tissue.

Penetration of *C. miyabeanus* is likely assisted by cell wall degrading enzymes (CWDEs), as is the case during *C. heterostrophus* penetration, a pathogen with very similar disease biology (Horwitz *et al.*, 2013). This is supported by the detection of several fungal CWDEs in the apoplast at 12 hours post inoculation (hpi) (Kim *et al.*, 2014). The same study shows that additional CWDEs are secreted by the plant, besides chitinases and a putative LysM-containing protein. The

consequently produced PAMPs and DAMPs trigger an non-specific defense response in rice plants, characterized by the induction of the phenylpropanoid pathway, chitinases, H_2O_2 production and enhanced ethylene metabolism (Kim *et al.*, 2014; Van Bockhaven *et al.*, 2015b). Induction of the phenylpropanoid pathways is reflected in higher activity of phenylalanine ammonia lyase (PAL) and polyphenol oxidase (PPO) and a steep increase in phenolic compounds (Vidhyasekaran *et al.*, 1992; Van Bockhaven, 2014). Phenolics have been shown to actively counteract brown spot infection and are an effective plant defense strategy (Shabana *et al.*, 2008). Reactive oxygen species (ROS) regulate defense signalling and have antimicrobial activity, but can also induce host cell death (Mengiste, 2012). Besides, antimicrobial effects of ROS are not always effective against necrotrophs and they may even produce additional ROS themselves (Choquer *et al.*, 2007; Shetty *et al.*, 2008). To counteract this negative role of ROS in necrotroph resistance, the plant strongly induces production of the antioxidant enzymes ascorbate peroxidase and superoxide dismutase in an attempt to restore cell redox homeostasis (Kim *et al.*, 2014).

The induction of ethylene biosynthesis does not protect the plant but to the contrary, enhances disease. This ethylene-enhanced susceptibility can be counteracted by abscisic acid via inhibition of *OsMPK5*. Furthermore ethylene-insensitive *ein2* antisense plants were also much more resistant, underlining the importance of ethylene signaling in susceptibility (De Vleesschauwer *et al.*, 2010). The strong induction of ethylene during successful infection raises the question whether the pathogen can somehow steer the defense response and hijack ethylene biosynthesis and/or signaling. Other hormone signaling pathways seem of minor importance during rice - *C. miyabeanus* interaction (Ahn *et al.*, 2005).

It is expected that *C. miyabeanus* uses an array of phytotoxic compounds in addition to CWDEs to attack the plant and induce cell death. The production of phytotoxic ophiobolins by *C. miyabeanus* has been well documented *in vitro*, but their contribution to disease has not been confirmed. Ophiobolin A (Table 1.1) can reproduce some of the physiological changes observed during disease, such as permeability gain of the cell membranes, which results in the loss of electrolytes, carbohydrates and amino acids, as well as a decrease in CO_2 fixation and protein and RNA synthesis (Chattopadhyay & Samaddar, 1976). The effect of ophiobolin A on phenol production is obscure and positive as well as neutral effects have been reported (Oku, 1962; Chattopadhyay & Samaddar, 1980b). Ophiobolins may play a role during disease, but additional virulence factors likely determine the overall severity of brown spot disease. Interesting in this regard is the isolation of a so far uncharacterized toxic fraction that could reduce the phenolic contents in rice leaves (Vidhyasekaran *et al.*, 1992). Moreover, the increased activity of PPO may be attributed to fungal PPO production (Chattopadhyay & Samaddar, 1980a). The recent sequencing of the *C. miyabeanus* genome provides an invaluable tool to characterize new factors involved in disease (Condon *et al.*, 2013). Furthermore, it is not known how *C. miyabeanus* could trigger the ethylene response and how it may be interconnected with the action of *C. miyabeanus* phytotoxins.

Chemical weapons of fungal necrotrophs

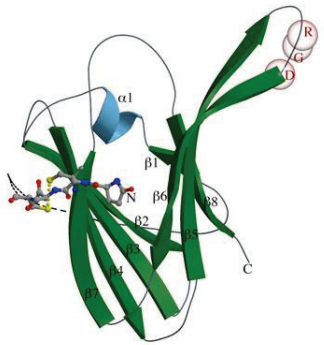
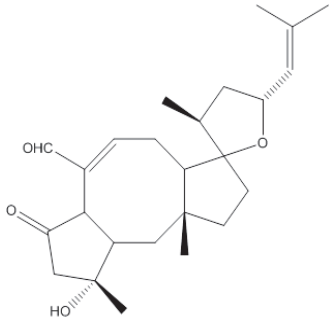
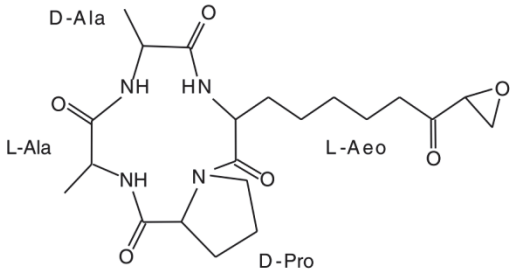
Introduction

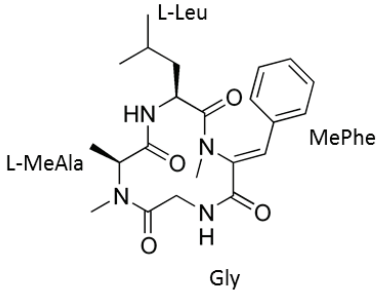
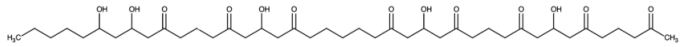
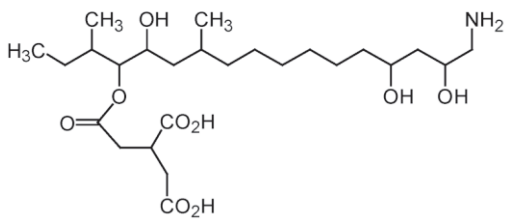
Pathogen attack involves an intimate interaction between the plant and the invader, characterized by production of pathogen-derived compounds and induction of plant defense responses. Pathogen-derived compounds typically interact with a host target or receptor and trigger a response that may lead to either suppression or disease development (Wolpert *et al.*, 2002; Muria-Gonzalez *et al.*, 2014). Depending on the life style of the fungus, secreted substances can include small secreted proteins, phytotoxins, mycotoxins, CWDEs, plant hormones or mimics thereof, small RNAs and reactive oxygen species (Stergiopoulos & de Wit, 2009; Horbach *et al.*, 2011; Stergiopoulos *et al.*, 2013; Muria-Gonzalez *et al.*, 2014; Weiberg *et al.*, 2014). Furthermore, CWDEs and lytic enzymes, produced by both plant and pathogen, release PAMPs and DAMPs that trigger a basal defense response in the plant (Boller & Felix, 2009; Thomma *et al.*, 2011).

In the case of biotrophs, the pathogen depends on nutrient uptake from living plant cells. They form complex feeding structures within living cells and produce small effector proteins that elegantly suppress effective plant defense responses, which typically involve induction of cell death (Horbach *et al.*, 2011; Thomma *et al.*, 2011; Stergiopoulos *et al.*, 2013). Necrotrophs on the other hand thrive on dead plant tissue and have long been considered the barbaric counterparts of the sophisticated biotrophs, blasting their way through the plant cells by means of CWDE to feed on the nutrients from the macerated tissue. However, it has become clear that the methods of necrotrophs are more sophisticated than that. Necrotrophs can subtly manipulate their host and induce programmed cell death, potentially after redirecting the host primary metabolism to their advantage (Howlett, 2006; Oliver & Solomon, 2010; Horbach *et al.*, 2011; Stergiopoulos *et al.*, 2013). A third, intermediary class of fungi is formed by the hemibiotrophs that first go through a distinct biotrophic phase before switching to a necrotrophic life style. Some hemibiotrophic fungi develop separate biotrophic and necrotrophic fungal structures, but hemibiotrophy can also be determined by an extended symptomless phase, followed by increasing tissue damage (Oliver & Ipcho, 2004). Many hemibiotrophic fungi, such as *Fusarium* spp., can produce phytotoxic compounds during the necrotrophic phase (Kazan *et al.*, 2012). The classification of fungi according to this system is often open to debate, as several necrotrophic fungi have been shown to go through a very short biotrophic phase upon penetration (Asselbergh *et al.*, 2007; Shlezinger *et al.*, 2011).

Necrotrophic fungi hence describe a group defined by their life style and are present in many fungal lineages, but are overrepresented in the class of Dothideomycetes, especially in the Pleosporales order comprising *Cochliobolus*, *Alternaria*, *Stagonospora* and *Pyrenophora* species (Friesen *et al.*, 2008; Oliver & Solomon, 2008; Ohm *et al.*, 2012). Some of the most devastating epidemics of the past century have been caused by necrotrophs.

Table 1.1. Different types of phytotoxins produced by Dothideomycetes plant-pathogens.

Type	Toxin	Structure	Example of producing organism	Host plant	Ref.
Proteinacous phytotoxin	PtrToxA		*Pyrenophora tritici-repentis*	Wheat	Sarma *et al.* (2005) Manning *et al.* (2009)
Terpene	Ophiobolin A		*Cochliobolus miyabeanus*	Rice	Au *et al.* (2000)
Non-ribosomal peptide	HC-toxin		*Cochliobolus carbonum*	Maize	Walton (2006)

Type	Toxin	Structure	Example of producing organism	Host plant	Ref.
Non-ribosomal peptide	Tentoxin		*Alternaria alternata*	Tomato	Yamagishi *et al.* (2006)
Polyketide	T-toxin		*Cochliobolus heterostrophus*	Maize	Inderbitzin *et al.* (2010)
Polyketide	AAL-toxin		*Alternaria alternata*	Tomato	Ramm *et al.* (1994)

Besides sporadic epidemics, pathogenic fungi are chronically prevalent on many fields, causing persistent and significant yield losses (Möbius & Hertweck, 2009). Hence, necrotrophic fungi can have enormous economic and social impact on agricultural systems. Virulence factors employed by necrotrophs typically encompass phytotoxins, plant hormones and CWDEs.

Phytotoxins

Phytotoxins comprise a diverse group of chemical structures that are capable of deranging vital activity of plant cells in order to trigger cell death (Berestetskiy, 2008; Möbius & Hertweck, 2009). They are historically classified based on their spectrum of bioactivity, rather than on their chemical properties, creating two groups: the host-selective toxins (HST) and the non-selective toxins (NST). Whereas HSTs interfere with the metabolism of only a particular host species or cultivar, NST are typically active against a broad spectrum of plant species (Berestetskiy, 2008; Stergiopoulos *et al.*, 2013). In general it is believed that HSTs are pathogenicity or virulence determinants and deficiency results in inability to infect the host, whereas NSTs are not necessary for pathogenicity but increase the overall virulence of the fungal strain and contribute to symptom development (Markham & Hille, 2001; Berestetskiy, 2008). However, this dichotomy is artificial, as both HSTs and NSTs ultimately interact with specific cell components, which differ in distribution among hosts (Ballio, 1991). A selective list of phytotoxins produced by Dothideomycetes plant-pathogens is presented in Table 1.1.

Secondary metabolite phytotoxins

Secondary metabolites are varied group of bioactive low molecular weight compounds, produced via unique and unusual biochemical pathways. Secondary metabolites are typically dispensable for normal fungal growth and development and are not constitutively produced. They provide the producing organism with an evolutionary advantage under specific conditions. These specific conditions together with the physiological and morphological state of the fungus regulate production (Keller *et al.*, 2005; Howlett, 2006; Stergiopoulos *et al.*, 2013). Several fungal secondary metabolites have pharmaceutically interesting activities (e.g. penicillin and cyclosporine) or can be detrimental to human health (e.g. aflatoxins and ergot alkaloids), and many have been identified as phytotoxins that act as important virulence factors during disease. (Keller *et al.*, 2005; Yu & Keller, 2005; Jegorov *et al.*, 2006; Georgianna & Payne, 2009; Lorenz *et al.*, 2010; Condon *et al.*, 2013). Fungal secondary metabolites can be subdivided in three main classes: non-ribosomal peptides (NRP), polyketides (PK) and terpenes. Furthermore, some secondary metabolites are hybrid NRP-PK or PK-NRP structures or indole alkaloids. Whereas NRPs have only be found in fungi and bacteria, PKs can be produced by fungi, bacteria as well as by plants, although their biosynthetic mechanisms differ (Staunton & Weissman, 2001; Bushley & Turgeon, 2010; Yu *et al.*, 2012).

Secondary metabolite biosynthesis

NRPs are small bioactive peptides biosynthesized via a thiotemplate mechanism independent of the ribosomes, catalysed by multimodular megasynthases: the non-ribosomal peptide synthetases (NRPS) (Figure 1.3a). NRPs are derived from both proteinogenic and non-proteinogenic components, such as 2-amino butyric acid, 2,3-dihydroxi-phenyl-glycine or even salicylic acid (Finking & Marahiel, 2004; Keller *et al.*, 2005; Rausch *et al.*, 2005). Several hundred substrates of NRPSs are known today, reflecting the structural diversity of NRPs. Each NRPS module is responsible for the incorporation of one building block and contains several catalytic domains (Figure 1.3a).

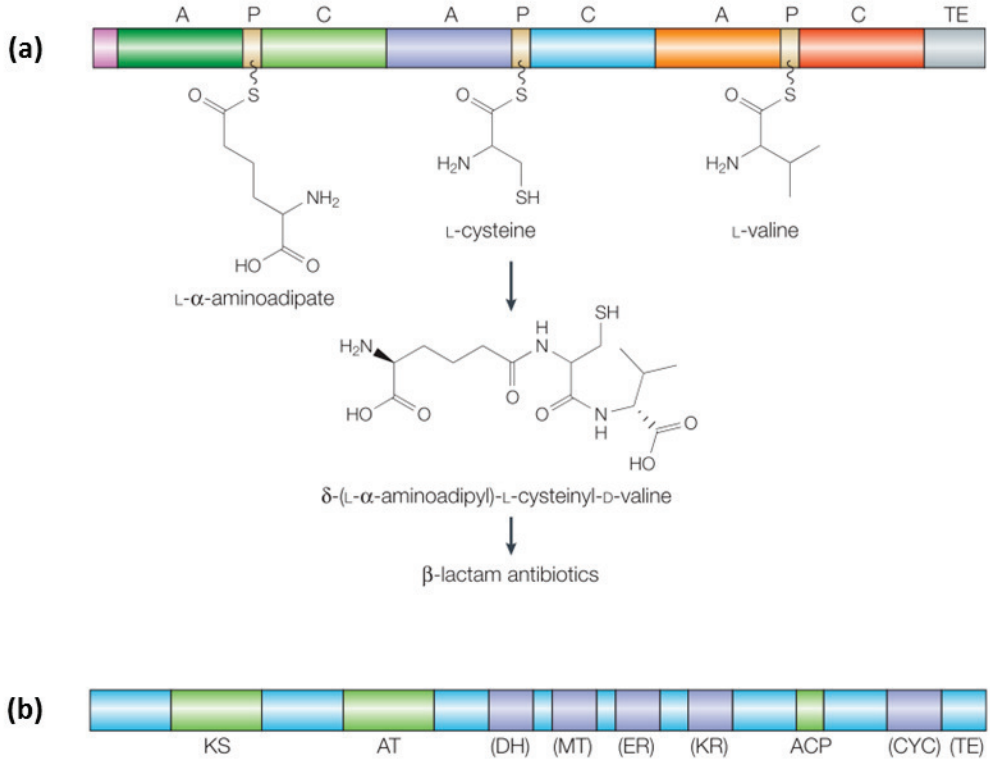


Figure 1.3. Fungal non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) structure. Figure adapted from Keller *et al.* (2005) **(a)** Structure of ACV synthetase, an example of a trimodular NRPS involved in penicillin biosynthesis. Each amino acid is recognized and activated by the cognate adenylation domain (A), and attached as a thioester to 4′-phosphopantetheine at the peptidyl carrier domain (P). Peptide bonds are formed with the involvement of the condensation domain (C). The final tripeptide, attached to the peptidyl carrier domain of the C-terminal module, is released by the integrated thioesterase domain (TE), with the L-valine isomerized to D-valine. The tripeptide is subsequently cyclized to isopenicillin N. **(b)** Fungal polyketide synthase (PKS) domain structure. The minimal structure is KS–AT–ACP. Optional domains are in brackets. Polyketide synthesis is initiated when acetyl and malonyl coenzyme A (CoA) are loaded as thioesters on to the 4′-phosphopantotheine of an acyl carrier (ACP) domain by means of the acyltransferase (AT) domain. Condensation then occurs with another thioester intermediate bound to the ketoacyl CoA synthase (KS) domain, and decarboxylation of the ACP-bound intermediate occurs. Optional domains: KR: ketoreductase, DH: dehydratase, ER: enoyl reductase, MT: methyltransferase, CYC: cyclase, TE: thioesterase.

The adenylation (A) domain is responsible for substrate recognition and specificity and the activation of the residue as its acyl adenylate. The activated amino acid is subsequently accepted

by the peptidyl carrier protein (P) domain (also known as thiolation (T) domain) via a thioester bond to the 4´-phosphopantetheine cofactor that is attached to the P-domain through a conserved serine residue. Finally, the condensation (C) domain catalyses the peptide bond formation between amino acyl substrates on P-domains of adjacent modules. The resulting NRP can be released from the template via a thioesterase (TE) domain located at the C-terminal end of the last module, either in its linear form or after cyclization (Finking & Marahiel, 2004; Keller *et al.*, 2005; Bushley & Turgeon, 2010). However, most fungal NRPs are released via a terminal C-domain or a thioesterase NADP(H) dependent reductase (R) domain (Bushley & Turgeon, 2010). Besides the classic domains, additional tailoring domains can be involved, such as methylation domains that are responsible for C or N methylation of amino acids via S-adenosyl methionine as methyl donor or glycosylation domains (Finking & Marahiel, 2004). Examples of phytotoxic NRPs are the HST HC-toxin produced by *C. carbonum* during infection of maize, and the NST tentoxin, isolated from *Alternaria alternata* (Ramm *et al.*, 1994; Horbach *et al.*, 2011) (Table 1.1).

PKs are the most abundant fungal secondary metabolites. The biosynthesis of fungal PKs is catalysed by type I polyketide synthases (PKS), unimodular multidomain proteins that are related to eukaryotic fatty acid synthases (Figure 1.3b). PKSs require at least three domains. The acyltransferase (AT) domain selects the substrate, acetyl or malonyl coenzyme A, and transfers it to the 4´-phosphopantetheine of an acyl carrier (ACP) domain. Eventually the residue is relocated to a cysteine moiety of the ketoacyl CoA synthase (KS) domain where it is condensed to another ACP-bound intermediate through a decarboxylation reaction. Despite being restricted to one module, fungal PKSs can carry out repeated biosynthetic reactions, called iterative PKSs (Finking & Marahiel, 2004; Keller *et al.*, 2005). The PCD-inducing AAL-toxin from *Alternaria alternata* is a well-studied PK phytotoxin (Table 1.1). Another notorious highly toxic PK is T-toxin, which renders the toxin-producing *C. heterostrophus* race T extremely virulent towards maize lines with texas male sterile germplasm (Horwitz *et al.*, 2013) (Table 1.1).

The importance of NRP and PK metabolism in fungal phytopathogenicity is clear from experiments with Sfp-type 4´-phosphopantetheinyl transferase (PPTase) knockout mutants. PPTases are responsible for the activation of P-domains in NRPSs and ACP-domains in PKSs via phosphopantetheinylation. Deletion of PPTase genes resulted in significant reduction of fungal pathogenicity in *Colletotrichum graminicola*, *Magnaporthe oryzae*, *Fusarium fujikuroi* and *C. sativus* (Horbach *et al.*, 2009; Leng & Zhong, 2012; Wiemann *et al.*, 2012).

A third group of secondary metabolites are the terpenes. Terpenes or isoprenoids are produced by all living organisms and are derived from the universal precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) (Figure 1.4). Plants possess two parallel pathways to produce these precursors, the mevalonate (MVA) pathway in the cytoplasm starting from acetyl-CoA and the methylerythritol phosphate (MEP) pathway starting from glyceraldehyde-3-phosphate and pyruvate in the plastids (Vranová *et al.*, 2013). Fungi on the other hand, only

retained the MVA pathway (Disch & Rohmer, 1998; Lange *et al.*, 2000). IPP and DMAPP are combined to form geranyl-pyrophosphate (C10) and farnesyl-pyrophosphate (C15), which eventually act as substrates for specific terpene cyclases (TC) (Keller *et al.*, 2005; Christianson, 2006; Vranová *et al.*, 2013). Most TCs generate one unique cyclic hydrocarbon product. TCs feature two independent domains. The first domain is a prenyltransferase that catalyses the synthesis of the full length linear isoprenoid backbone chain. This carbon chain is then accepted by the terpenoid cyclase domain that catalyses the cyclization reaction to yield the unique terpenoid ring structure. This backbone can subsequently be modified to yield a range of analogues terpenoids (Christianson, 2006). The phytotoxic ophiobolins, produced by *C. miyabeanus* and *C. heterostrophus* are sesterterpenoid phytotoxins, biosynthesized from geranylfarnesyl pyrophosphate (Figure 1.4).

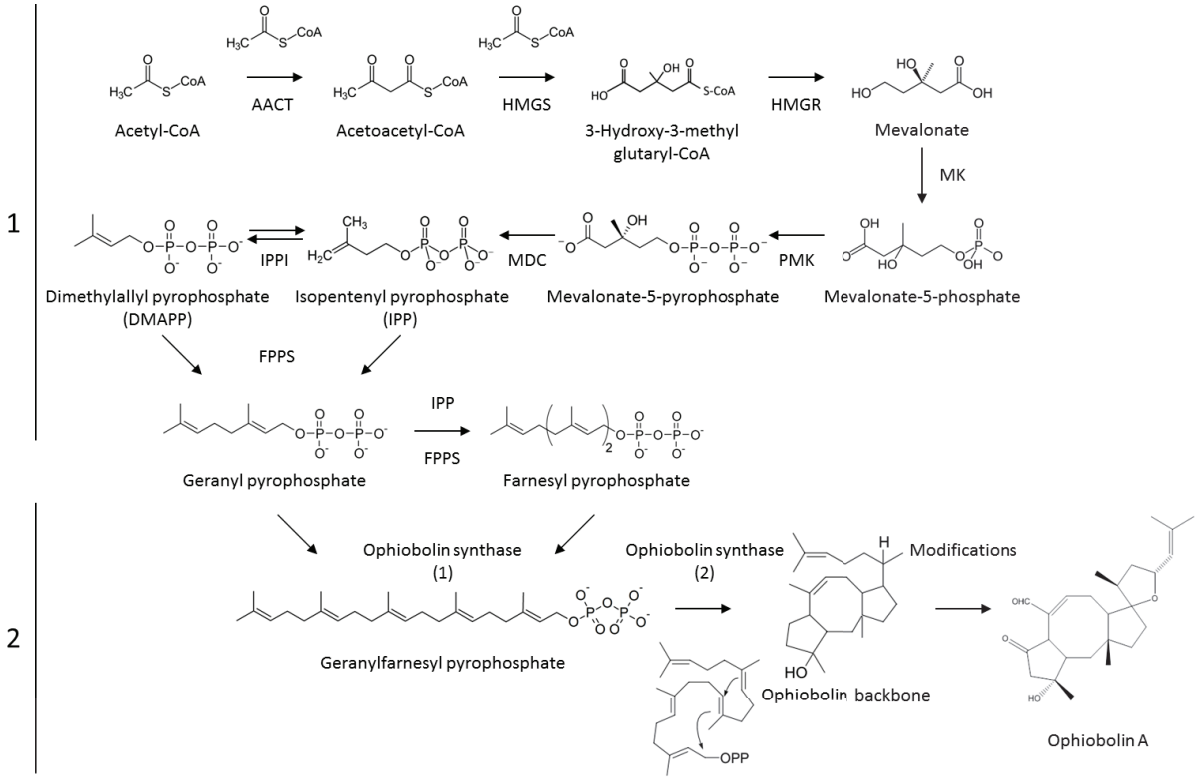


Figure 1.4. Terpene biosynthetic pathway leading to the sesterterpene ophiobolin A. 1: Production of geranyl-pyrophosphate and farnesyl pyrophosphate via the mevalonate pathway, 2: ophiobolin biosynthesis steps. The ophiobolin synthase is a terpene cyclase featuring a prenyltransferase domain, catalyzing geranylfarnesyl pyrophosphate formation and a cyclase domain that catalyzes the cyclization reaction. The bare ophiobolin backbone is subsequently modified by unidentified tailoring enzymes to yield different ophiobolin analogues, such as ophiobolin A. AACT: Acetoacetyl-CoA transferase, HMGS: 3-hydroxy-3-methylglutaryl-CoA synthase, HMGR: 3-hydroxy-3-methylglutaryl-CoA reductase, MK: Mevalonate kinase, PMK: phosphomevalonate kinase, MDC: Mevalonate-5-pyrophosphate decarboxylase, IPPI: IPP isomerase, FPPS: Farnesyl-pyrophosphate synthase.

Secondary metabolite biosynthesis gene clusters: regulation and evolution

Secondary metabolite biosynthesis genes are typically organized in gene clusters of pathways specific genes. The recent boom in fungal genome sequencing has revealed the existence of far

more secondary metabolite gene clusters than had ever been predicted based on the number of metabolites characterized so far (Kroken *et al.,* 2003; Bushley & Turgeon, 2010; Osbourn, 2010; Ohm *et al.*, 2012; Condon *et al.*, 2013). The analysis of these gene clusters reveals a common arrangement in which a NRPS, PKS or TC has a central position and is surrounded by tailoring enzymes, specific regulators and/or transporter proteins (Keller *et al.*, 2005; Brakhage, 2013). Following biosynthesis of the bare skeleton structure by the backbone genes, the tailoring enzymes such as oxidoreductases, methyltransferases, acyltransferases and glycosyltransferases can modify the structure, typically resulting in a range of analogues compounds (Osbourn, 2010; Brakhage, 2013). Most secondary metabolite gene clusters are conserved in only a limited number of species (Ohm et al., 2012; Condon et al., 2013). Often the gene order in the orthologues clusters has changed through evolution, but this does not appear to affect function (Carbone *et al.,* 2007; Osbourn 2010)

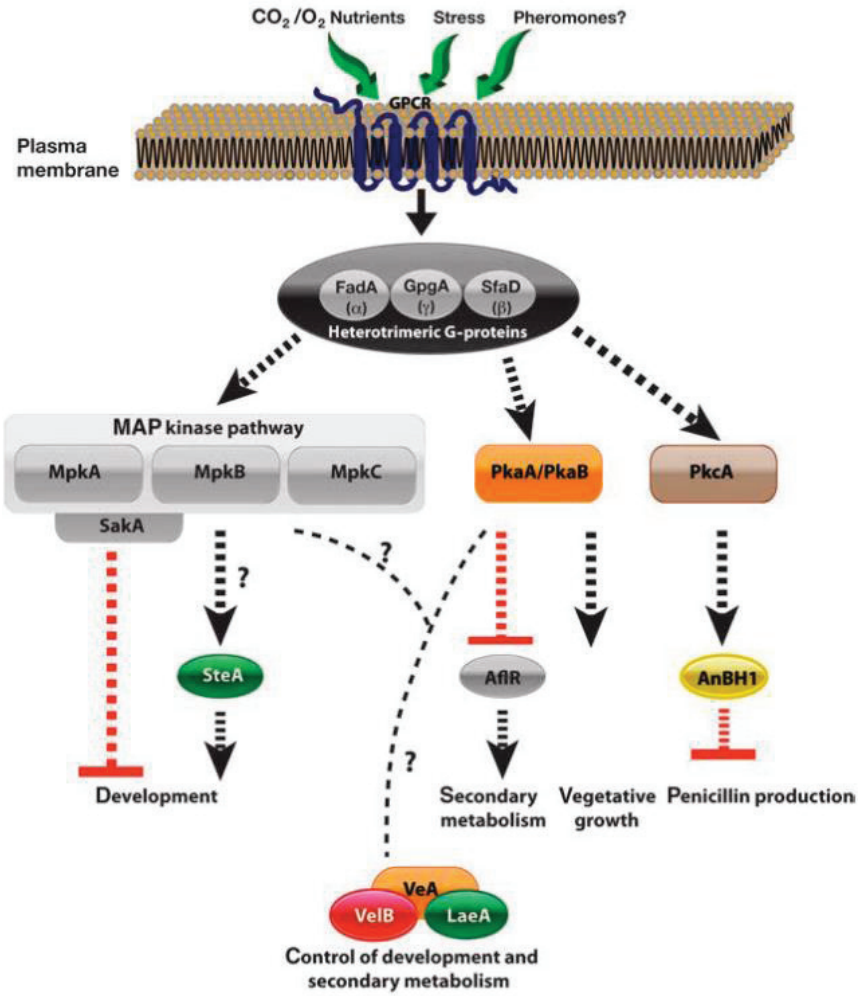


Figure 1.5. Example of complex regulatory networks that integrate environmental cues to steer fungal development and secondary metabolism in *Aspergillus nidulans*. Figure adapted from Bayram & Braus (2012). External signals are perceived, for example by G-protein coupled receptors (GPCRs), transmitted to heterotrimeric G-proteins such as FadA/GpgA/SfaD and transferred within the fungal cells by various signal transduction pathways that activate or inhibit fungal regulators such as SteA, AflR, AnBH1 or the trimeric velvet complex VelB–VeA–LaeA. '?' means that the functional connection is unknown.'

As mentioned earlier, secondary metabolites are not constitutively produced. Biosynthesis is activated by physiological or environmental triggers. This is reflected in the failure to produce most secondary metabolites under laboratory conditions. The signalling cascades leading to activation or repression of these clusters are mediated by global and specific transcription factors (Keller *et al.*, 2005; Yu & Keller, 2005; Osbourn, 2010). Global transcription factors are essential in integrating cellular responses to environmental cues, including pH, light, temperature, but also the carbon and nitrogen source (Keller *et al.*, 2005). These global regulators are conserved and can regulate several secondary metabolite gene clusters at the same time as well as steer fungal development. Specific regulators may or may not be integrated in the gene cluster. They fine-tune the phytotoxin production and can be controlled by global regulators. Complex interaction networks emerge that allow the fungus to sense the environment and to easily switch between physiological states (Figure 1.5) (Bayram & Braus, 2012).

Surprisingly few secondary metabolism gene clusters have been thoroughly characterized. The gene cluster of the carcinogenic mycotoxin aflatoxin has been particularly well studied in *Aspergillus flavus*. The total biosynthetic gene cluster contains no fewer than 25 genes, including two transcriptional regulators AflR and AflS. The global regulators LaeA, VeA and VelB work together on top of these transcription factors and regulate the aflatoxin production, as well as 12 additional gene clusters (Tilburn *et al.*, 1995; Tudzynski *et al.*, 1999; Bayram & Braus, 2012; Brakhage, 2013). LaeA has been shown to regulate the transcription of about 10% of the *A. flavus* genes, making it a master regulator. Interestingly, putative LaeA and VeA homologs are present in several additional filamentous fungi, including *Cochliobolus* spp. In *C. heterostrophus*, LaeA and VeA orthologs globally regulate the production of T-toxin, as well as pathogenicity, super virulence, oxidative stress responses and sexual development. However, no specific regulators have been identified among the nine genes belonging to the T-toxin gene cluster (Inderbitzin *et al.*, 2010; Wu *et al.*, 2012).

Clustering of genes has the advantage that regulation can be accomplished at the level of chromatin. Interestingly, the LaeA global regulator has a direct role in chromatin remodelling. In this regard, it is interesting to note that different gene clusters are often juxtaposed and can consequently be activated at the same time through changes in the chromatin configuration (Batada *et al.*, 2007; Osbourn, 2010). In addition, G-proteins and protein kinases also act in the signalling pathways that regulate fungal development and pathogenicity (Turrà *et al.*, 2014). The G-protein subunit FadA and the protein kinase A PkaA work together in *A. nidulans* to stimulate colony growth and negatively regulate aflatoxin production via inhibition of LaeA (Keller *et al.*, 2005).

Several gene clusters feature transporter encoding genes, which are likely involved in the efflux of toxic compounds to protect the producer on one hand, and to deliver the phytotoxin in plant cells on the other hand (Keller *et al.*, 2005; Brakhage, 2013). Typically, transporters belong

to the ATP-binding cassette (ABC) or major facilitator superfamily (MFS) (Stergiopoulos *et al.*, 2002). The HC-toxin gene cluster in *C. carbonum* is organized around the key gene for biosynthesis of the NRPS, HTS1 (HC-toxin synthetase), and contains a MFS transporter-encoding gene (ToxA), besides the specific regulatory gene ToxE (Stergiopoulos *et al.*, 2013). The *Tri5* gene cluster in *Fusarium* spp. similarly features a specific MFS transporter-encoding gene, *Tri12* (Kimura *et al.*, 2014). Tri12p efflux pumps not only export trichothecene toxins (e.g. DON) outside the cell, but are also involved in the transport of trichothecenes from toxisomes to the vacuole within the fungal cell (Menke *et al.*, 2013). Alternatively, specific transporter-encoding genes can be located outside the biosynthesis gene cluster as is the case for cercosporin efflux. Cercosporin, produced by *Cercospora* spp. is transported out of the cell via the MFS-transporter CFP (cercosporin facilitator protein), but the encoding gene is not linked with the CTB (cercosporin toxin biosynthesis) gene cluster (Thines *et al.*, 2006; Chen *et al.*, 2007).

Clustering of genes is advantageous because of the ease of coactivation and regulation of genes involved in biosynthesis. However, an additional advantage is the likely coinheritance of genes within a cluster, assuring preservation of the functionality (Osbourn, 2010). An additional pressure driving the conservation of gene clusters is the possible production of a highly toxic product if certain genes on a cluster are lost (Campbell *et al.*, 2012). The evolutionary mechanisms behind the origin and preservation of gene clusters in fungal genomes are still unclear. Phytotoxic gene clusters typically show low sequence conservation and are present in local plastic regions surrounded by overall well-conserved sequences (Raffaele & Kamoun, 2012). Secondary metabolite genes and clusters can arise and disappear through complex processes of gene duplications, relocations or deletions (Friesen *et al.*, 2008; Raffaele & Kamoun, 2012; Condon *et al.*, 2013). Furthermore, repetitive DNA and transposable elements in the flanking regions could be involved in cluster formation and regulation (Osbourn, 2010; Bushley & Turgeon, 2010). The clusters can be transferred through vertical gene transfer, but also horizontally transmitted within or between species and even between kingdoms (Rosewich & Kistler, 2000; Friesen *et al.*, 2006; Khaldi *et al.*, 2008; Akagi *et al.*, 2009; Mehrabi *et al.*, 2011; Campbell *et al.*, 2012; Gardiner *et al.*, 2013). The incidence of horizontal gene transfer (HGT) events suggests that not only selective pressure within the genome preserves the cluster, but that propagation of the cluster in itself drives the evolution ('selfish cluster hypothesis') (Walton, 2000). Furthermore, acquisition of foreign metabolic gene clusters may not only contribute to novel metabolism, but can also provide new metabolic regulators (Campbell *et al.*, 2012). Apart from gene clusters, horizontal transfer of entire chromosomes has been reported (Raffaele & Kamoun, 2012). Many *Alternaria alternata* pathotypes carry a conditionally dispensable chromosome (CDC) bearing host-specific toxin-encoding gene clusters. The origin and evolution of these CDCs is as yet unknown, but it has been suggested that the CDCs are horizontally transferred across *A. alternata* pathotypes and can explain their host range and host-range adaptation (Tanaka *et al.*, 1999; Hatta *et al.*, 2002; Thomma, 2003; Mehrabi *et al.*, 2011; Raffaele & Kamoun, 2012).

Proteinacous phytotoxins and small secreted proteins

Beside secondary metabolites, some phytotoxins are normal ribosome-derived peptides. Some are small secreted proteins (SSP) that play major roles during infection, for example as phytoalexin inactivators or inducers of chlorosis and necrosis (Horbach *et al.*, 2011). However, fungal genomes feature many more genes encoding for putative small secreted proteins that are yet to be functionally identified (Horbach *et al.*, 2011). For example, the genome of *C. miyabeanus* carries about 143 SSPs of which 51 are strain unique (Condon *et al.*, 2013). Some proteins have non-specific functions, like the necrosis inducing proteins (NIP) in *Rhynchosporium secalis*, whereas other proteinacous phytotoxins are notorious HSTs. Proteinacous HSTs have been shown to work in a similar gene-for-gene mechanism as their biotrophic effector counterparts. In the case of necrotrophic HSTs, the function of the R-protein is subverted in order to promote disease. The loss of a susceptibility gene can render plants resistance to certain pathogens (Oliver & Solomon, 2010; Stergiopoulos *et al.*, 2013).

There are strong indications that fungi can acquire proteinacous phytotoxins through (cross-kingdom) HGT events, similar to secondary metabolite gene clusters. The Ave1 effector from *Verticillium dahliae* for example, might have been acquired from plant species, where its homolog plays a role in water and ion homeostasis. Furthermore, the NIP proteins have likely been exchanged between oomycetes, bacteria and fungi (Gardiner *et al.*, 2013). Similarly, a peptide HST from *S. nodorum* ToxA has recently been horizontally transferred to *P. tritici-repentis* (Friesen *et al.*, 2006) (Table 1.1).

Modes of action of fungal phytotoxins

Typically, phytotoxins can interact with a range of cellular targets in order to derange the plant metabolism and eventually induce cell death and release nutrients. Some toxins hijack plant defense responses and trigger the cells own suicide machinery (Markham & Hille, 2001). A recurring theme is a disruption of photosynthetic processes or energy production, resulting in chlorosis, PCD and/or senescence (Möbius & Hertweck, 2009) (Figure 1.6).

A first cellular target is the membrane integrity of plant cells. Phytotoxins like fumonisin and AAL-toxin from *A. alternata* inhibit ceramide biosynthesis. This results in hampered lipid biosynthesis and increased membrane permeability, but also deranges defense signalling and eventually leads to PCD (Markham & Hille, 2001; Möbius & Hertweck, 2009). Similarly, the sesquiterpene glucoside HS-toxin from *Cochliobolus sacchari* is a membrane depolarizer, resulting in electrolyte leakage and lesion formation (Stergiopoulos *et al.*, 2013). The *C. miyabeanus* phytotoxin ophiobolin A too has been shown to interfere with membrane integrity and cause ion leakage (Chattopadhyay & Samaddar, 1976). Changes in membrane permeability can disrupt the electrochemical gradient and ultimately cause an energy breakdown.

Some phytotoxins are able to tamper with plant energy production directly. Victorin from *C. victoriae* specifically inhibits the glycine decarboxylase complex, involved in photorespiration. The inhibition of photorespiration induces senescence in infected leaves (Navarre & Wolpert, 1999). The rapid plant response to victorin includes RuBisCO cleavage, but also lipid peroxidation, respiratory burst, extracellular alkalinization, phytoalexin synthesis and ethylene production,

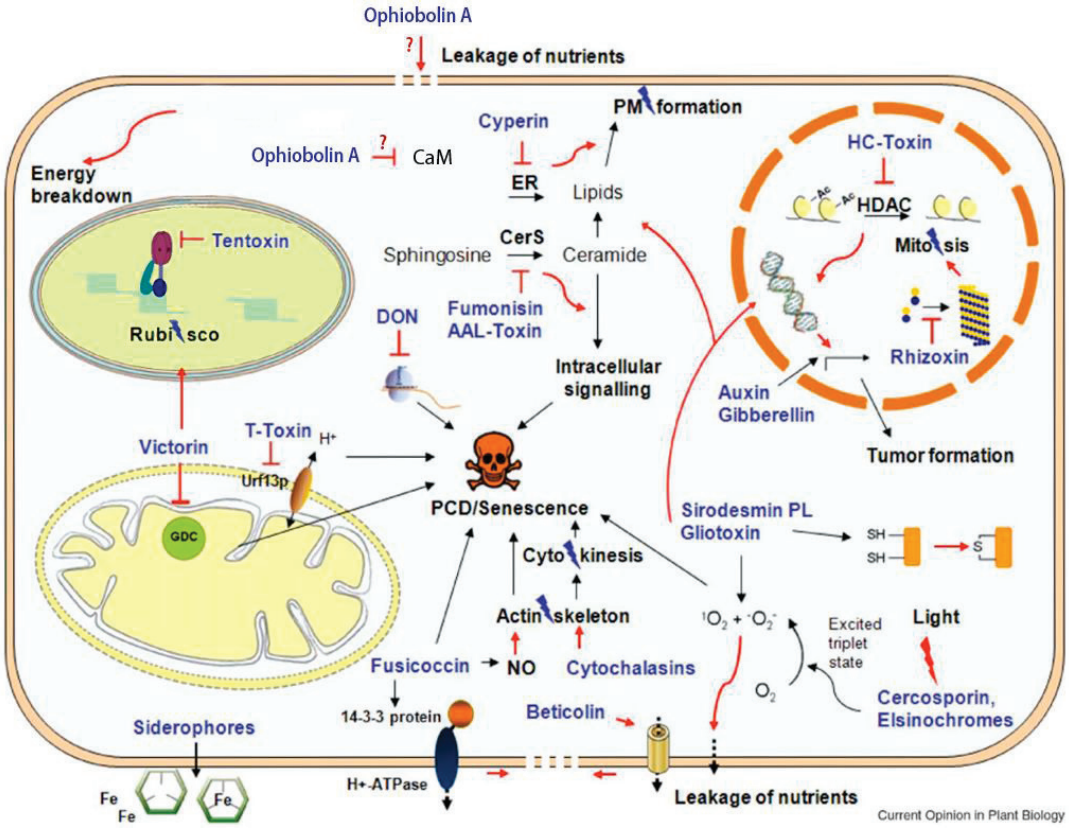


Figure 1.6. Overview of cellular targets and the mode of action of several fungal phytotoxins. Figure adopted and modified from Möbius & Hertweck (2009). GDC: glycine decarboxylase, CerS: ceramide synthase, ER: enoyl reductase, HDAC: histone deacetylase complex, NO: nitric oxide, PCD: programmed cell death, PM: plasma membrane

reminiscent of a classic plant defense response (Markham & Hille, 2001; Wolpert *et al.*, 2002; Lorang *et al.*, 2012).T-toxin from *C. heterostrophus* race T targets the mitochondrial membrane of male sterile T-cms maize, causing pore formation and uncoupling of the oxidative phosphorylation (Stergiopoulos *et al.*, 2013). Tentoxin, produced by *A. alternata* has been shown to inhibit photophosphorylation and cause chlorosis by hampering the F1-subunit of chloroplast ATP-synthase and preventing incorporation of PPO into the chloroplasts in seedlings of sensitive species (Klotz, 1988). Furthermore, PtrToxA targets the chloroplasts where it alters photosystems I and II, leading to light-dependent accumulation of ROS, disruption of photosynthesis and ultimately PCD (Manning *et al.*, 2009; Ciuffetti *et al.*, 2010).

Other phytotoxins are activated by light energy and generate ROS, damaging membrane lipids and inducing PCD. These so-called photosensitizers include the perylenequinone cercosporin

produced by *Cercospora* spp., whose biosynthesis is regulated by light (Möbius & Hertweck, 2009). Another mode of action is through the repression of transcription of defense related genes. HC-toxin, produced by *C. carbonum*, inhibits secretion of inhibitory compounds by the plant by repressing histone deacetylases, but cannot kill cells by itself (Markham & Hille, 2001; Möbius & Hertweck, 2009).

Besides targeting plant metabolism, some phytotoxins are detected by plant receptors in a way that is reminiscent of the effector-R-gene interaction between biotrophs and their hosts. As mentioned earlier, in this way some phytotoxins actually serve as necrotroph effectors (Oliver & Solomon, 2010). For example, the proteinacous PtrToxA targets the chloroplasts as primary site, but may also trigger a defense response mediated by Tsn1 (Manning *et al.*, 2009; Ciuffetti *et al.*, 2010). The NRP victorin primarly targets a protein in the glycine decarboxylase complex in the mitochondria of oat, but can also induce disease symptoms in Arabidopsis by interaction with Lov1 (Sweat *et al.*, 2008). Furthermore it has been shown that the terpenoid toxin deoxynivalenol (DON) interacts with both primary and secondary metabolism resulting in PCD. Although its specific cellular target is so far not known, DON triggers H_2O_2 production and elicits plant defense responses characterized by the production of antimicrobial products (Desmond *et al.*, 2008; Kazan & Lyons, 2014).

Metabolic perturbation as well as interaction with plant receptors induces plant responses that result in PCD, often regulated by hormone and MAPK signaling pathways. Interestingly, several toxins rely on ethylene, a plant hormone that has been shown to be involved in processes related to senescence and PCD (Hoeberichts & Woltering, 2003; Lim *et al.*, 2007). Fumonisin B1 and AAL-toxin are known PCD-inducers that rely on an active ethylene signaling pathway to exert their effect and can induce ethylene signaling by themselves (Moore et al., 1999; Asai et al., 2000a; Hoeberichts & Woltering, 2003; Gechev et al., 2004). Similarly, the senescence inducing phytotoxin victorin seems to trigger ethylene biosynthesis (Wolpert et al., 2002; Gilbert & Wolpert, 2013). In this regard it is interesting to mention that some phytotoxin producers can also biosynthesize microbial ethylene, which may act as a toxin synergist (Chagué, 2010; Tzima et al., 2010).

Some plants have developed mechanisms to protect their cells from the detrimental effects of phytotoxins. These mechanisms can comprise detoxification by structural modifications, compartmentalization or elimination of the phytotoxic compound (Berthiller *et al.*, 2013; Muhovski *et al.*, 2014). Chemical modifications involve one or more enzyme-catalysed reactions that result in the conversion of the foreign molecule to derivatives that are chemically distinct from the original phytotoxin (Coleman *et al.*, 1997; Pedras *et al.*, 2001; Berestetskiy, 2008; Berthiller *et al.*, 2013). Besides hydrolysis or oxidation, structural modification can among others encompass the conjugation with glucosyl, malonyl or glutathione residues in a reaction catalysed by glucosyl-, malonyl- and glutathione-S-transferases, respectively (Berestetskiy, 2008; Berthiller

et al., 2013). The resulting conjugates are less toxic and can subsequently be eliminated to the vacuole or apoplast (Edwards *et al.*, 2000; Berthiller *et al.*, 2013). HCTR1 and 2 are HC-toxin reductases that can detoxify HC-toxin from *C. carbonum* via a NADPH-dependent reduction of an essential carbonyl group (Han *et al.*, 1997). Maize plants possessing the HCTR-encoding dominant Hm1 or Hm2 alleles are resistant to HC-toxin (Walton, 2006; Dehury *et al.*, 2014). Another example is the detoxification of DON in barley and wheat. DON can be transformed to a DON-3-O-glucoside by an UDP-glucosyltransferase and could also be conjugated to glutathione (Gardiner *et al.*, 2010; Kazan *et al.*, 2012). Furthermore, Muhovski *et al.* (2014) identified two ABCG transporters in wheat that are potentially involved in wheat resistance to DON. Alternatively, the absence or structural modification of the molecular plant target can prevent successful interaction with and subsequent detrimental effects of the corresponding phytotoxin (Berestetskiy, 2008). For example, maize cultivars that do not possess the mitochondrial URF13 protein are insensitive to T-toxin from *C. heterostrophus* (Panaccione, 1993).

Plant hormones produced by fungal pathogens

Besides steering plant defense responses, including hormone signaling pathways, through the secretion of phytotoxins, some fungal pathogens are able to produce plant hormones or mimics thereof themselves. Different plant hormones typically mediate defense responses to specific pathogens, characterized by trade-offs in which resistance to one pathogen is accompanied by enhanced susceptibility to another. Hence, feeding into this complex network can be a successful virulence strategy for biotrophs and necrotrophs alike (Robert-Seilaniantz *et al.*, 2007; Kazan & Lyons, 2014). Surprisingly, fungi evolved different hormonal biosynthetic pathways from plants. Deficiency in plant hormone production typically does not affect fungal development, suggesting that their main goal may be to modulate the hormonal balance of the host (Robert-Seilaniantz *et al.*, 2007).

Ethylene was previously considered to provide resistance towards necrotrophs, but it is now clear that in several cases the opposite is true (Adie *et al.*, 2007). As discussed earlier, ethylene is involved in several plant PCD and senescence signaling pathways; it is therefore not surprising that some necrotrophs are able to produce ethylene. Fungi can produce ethylene via three possible biosynthesis routes, although most species only possess one or two pathways if any at all (Chagué, 2010)(Figure 1.7). Ethylene can be synthesized from methionine via either 1-aminocyclopropane-1-carboxylic acid (ACC) or via α-keto-γ-methylthiobutyric acid (KMBA). Production of ethylene via KMBA requires a first deamination step via a specific methionine transaminase to convert methionine to KMBA, followed by aspecific oxidation of KMBA in the presence of hydroxyl radicals to release ethylene. This last step requires the action of a NADH:Fe(III)oxidoreductase (Primrose, 1977; Ogawa *et al.*, 1990). In a third pathway ethylene is generated from the TCA cycle intermediate 2-oxoglutarate by an ethylene forming enzyme (EFE), an enzymatic step that requires additional amino acids, arginine and Fe^{2+} as cofactors (Fukuda *et*

al., 1992; Chagué *et al.*, 2006; Eckert *et al.*, 2014). Although ethylene is involved in resistance to *B. cinerea* in tomato, ethylene can promote spore germination in *B. cinerea* and *Colletotrichum gloeosporioides,* and several plant pathogens specifically produce ethylene as a virulence factor to promote host colonization (Chagué, 2010).

Furthermore, *B. cinerea* and *Fusarium oxysporum* are able to produce abscisic acid, a hormone that can enhance susceptibility in tomato by repressing SA-defense responses (Audenaert *et al.*, 2002; Denancé *et al.*, 2013). *Gibberella fujikuroi* produces gibberellins to interfere with the physiology of the plant, resulting in yellow elongated seedlings with stunted roots. Fungal gibberellins likely trigger DELLA-degradation and negatively regulate necrotroph defense responses (Achard *et al.*, 2008; Laluk & Mengiste, 2010). The hemibiotroph *Colletotrichum gloesporioides* f. sp. *aeschynomene* uses external tryptophan to produce auxins and can do so *in planta*. The exact function is still elusive, although auxins seem to be mainly important during the biotrophic phase (Maor *et al.*, 2004; Robert-Seilaniantz *et al.*, 2007; Ludwig-müller, 2015). Whereas many biotrophic fungi can produce cytokinins, this has never been reported for necrotrophic fungi (Murphy *et al.*, 1997; Walters & McRoberts, 2006).

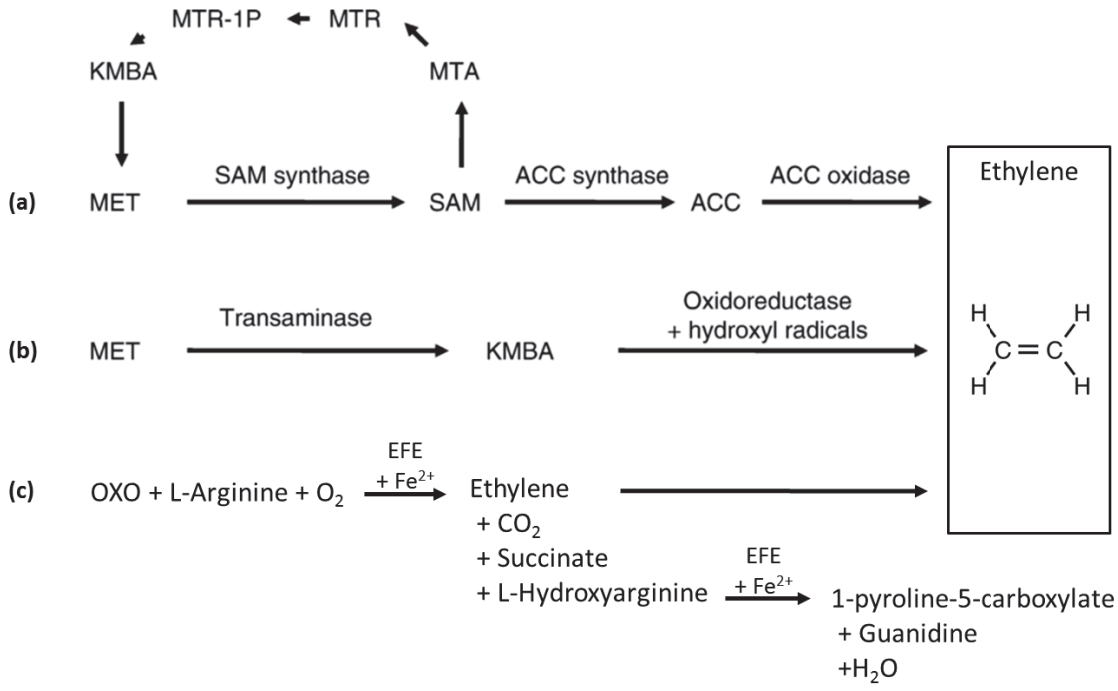


Figure 1.7. Different fungal ethylene biosynthesis pathways. Figure adopted and modified from Chagué (2010). **(a)** The ACC pathway, the same pathway is active in plants. **(b)** The KMBA pathway. **(c)** The EFE pathway. MET: L-methionine, SAM: S-adenosylmethionine, ACC: aminocycloprpane-1-carboxylic acid, MTA: 5-methylthioadenosyl, MTR: 5-methylthioribose, MTR-1P: 5-methylthioribose-1-phosphate, KMBA: α-keto-γ-methylthiobutyric acid. OXO: 2-oxoglutarate, EFE: ethylene forming enzyme.

Cell wall degrading enzymes (CWDEs)

On top of phytotoxic compounds, necrotrophs can produce a range of enzymes involved in cell wall degradation. Cell wall degradation can aid the pathogen during the penetration process, but can also provide the fungus with an important nutrient source (Annis & Goodwin, 1997; Glass *et al.*, 2013). Furthermore, the release of cell wall derived compounds can act as DAMPs and trigger plant defense responses (Boller & Felix, 2009). During brown spot infection, an array of *C. miyabeanus* produced CWDE has been detected in the apoplast, including α-amylase, α-N-arabinofurosidase, α-galactosidase, endo-1,4-ß-xylanase and exo-1,3-ß-glucanase (Kim *et al.*, 2014). The production of CWDEs increased during infection, suggesting that the enzymes are mainly used to provide a carbon source, rather than to induce cell death. Furthermore, galactosidases and arabinosidases typically release sugar moieties that are good nutritional sources. Although exo-1,3-ß-glucanase can cleave callose, a plant polysaccharide associated with defense responses, deletion of an exo-1,3-ß-glucanase encoding gene from *C. carbonum* did not alter virulence (Schaeffer *et al.*, 1994). Some pathogens use pectinases to directly kill plant cells, but similar enzymes were not detected during brown spot disease (Annis & Goodwin, 1997).

Chapter 2

Cochliobolus miyabeanus ophiobolin synthase mediates production of ophiobolin A, a major virulence factor during the early phase of rice brown spot disease

Authors

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Summary

C. miyabeanus is a plant-pathogenic fungus with a necrotrophic lifestyle and relies on the induction of cell death to successfully infect rice plants. Although *C. miyabeanus* is currently the biggest yield reducer of rice, little is known about the virulence strategies employed during disease development. *C. miyabeanus* produces ophiobolins, cyclic sesterterpenoid non-selective phytotoxins, but their importance during the infection process is not clear. Therefore we set out to clarify their role in brown spot disease development. In this chapter we identified an ophiobolin synthase (CmOps1*)* responsible for ophiobolin biosynthesis in *C. miyabeanus*. We confirmed its role in ophiobolin biosynthesis by creating *Cmops1* deletion mutants. Infection tests with wild type and *Cmops1* mutants combined with analysis of the temporal ophiobolin A production dynamics using UPLC-MS/MS show convincingly that ophiobolin A acts as a major virulence factor during the first phase of infection. Furthermore differential plant gene expression analysis, cell death bioassays and pH measurements provide insight in the plant responses triggered by ophiobolin A. These include medium alkalinisation and induction of *OsPAL1, OsMPK5, OsNAC4* and *OsBI-1* expression. Eventually, ophiobolin A causes plant cell death in a Ca^{2+} dependent and independent fashion.

Author contributions

Lieselotte De Bruyne: *Study conception and design, genome analysis, fungal transformations, data acquisition in all experiments, data analysis and interpretation, writing of the chapter.*

Christof Van Poucke**:** *Development of the ophiobolin A detection method, assistance with UPLC-MS/MS analysis, contribution to materials and methods section, critical revision of the chapter*

Dongliang Wu: *Assisted with the transformations*

Evelien De Waele: *Assisted with qPCR experiments*

Chien-Jui Huang, David De Vleesschauwer, Sarah De Saeger, B. Gillian Turgeon and Monica Höfte: *Critical revision of the chapter*

Introduction

Cochliobolus miyabeanus is a filamentous ascomycete belonging to the Pleosporales order of the class Dothideomycetes. Many species belonging to this order are notorious for the production of phytotoxins (Panaccione, 1993; Condon *et al.*, 2013). *C. miyabeanus* produces multiple ophiobolins, a family of sesterterpenoid non-host specific phytotoxins, also described in *C. heterostrophus* and some *Alternaria* spp. (Orsenigo, 1957; Nakamura & Ishibashi, 1958; Yun *et al.*, 1988; Shen *et al.*, 1999; Liu *et al.*, 2011; Chiba *et al.*, 2013). Ophiobolin A (Figure 2.1) is the ophiobolin analogue with the highest bio-activity. Application of pure ophiobolin A on rice leaves can reproduce necrotic lesions, similar to brown spot disease symptoms (Yun *et al.*, 1988; Xiao *et al.*, 1991). Furthermore, ophiobolin A induces programmed cell death (PCD) when applied to tobacco suspension cells. Unlike hypersensitive response (HR)-related cell death, the ophiobolin A-induced PCD is independent of an early H_2O_2-production (Bury *et al.*, 2013). Other fungal toxins can induce a similar H_2O_2-independent PCD, e.g. fumonisin B1 and AAL toxin (Lachaud *et al.*, 2011). Comparison of the physiological effects caused by *C. miyabeanus* infection and ophiobolin A leaf treatment, suggests a role for ophiobolin A during disease development. Upon either *C. miyabeanus* infection or ophiobolin A treatment, permeability changes in rice cell membranes have been observed, as well as changes in tissue respiration, photosynthesis and protein, RNA and DNA synthesis (Chattopadhyay & Samaddar, 1976, 1980a). Furthermore, contradictory reports are available whether or not plant phenolic content might be impacted (Oku, 1962; Chattopadhyay & Samaddar, 1980b). Nevertheless, until now it has not been proven with certainty that ophiobolins are responsible for the observed physiological changes and that ophiobolin A is an important virulence factor.

Apart from the phytotoxic effects, ophiobolins can provide the fungus with a competitive advantage over other microorganisms, as they display additional antibacterial, antifungal, nematocidal and even antiviral bioactivity (Singh *et al.*, 1991; Au *et al.*, 2000; Krizsán *et al.*, 2010). Moreover, ophiobolins can induce apoptosis in animal cells and a potential use in cancer treatment is currently being investigated (Fujiwara *et al.*, 2000; Bladt *et al.*, 2013; Wang *et al.*, 2013; Bury *et al.*, 2013; Bencsik *et al.*, 2014).

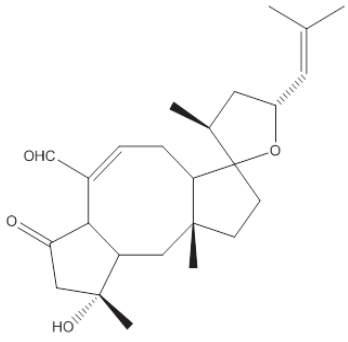


Figure 2.1. Chemical structure of ophiobolin A

Ophiobolins are biosynthesized from geranyl-pyrophosphate and farnesyl-pyrophospate, two end products of the isoprenoid biosynthesis pathway (Figure 1.4). In nature, two distinct isoprenoid biosynthesis pathways exist, the mevalonate pathway (MVA) and the deoxyxylulose 5-phosphate pathway (DXP) (Lange *et al.*, 2000). Whereas plants possess both pathways, fungi have only the MVA pathway available for the production of isoprenoid secondary metabolites (Disch & Rohmer, 1998; Lange *et al.*, 2000). The biosynthetic origins of ophiobolin A from the MVA pathway have been suggested a long time ago (Canonica *et al.*, 1967; Nozoe *et al.*, 1968). Combination of one geranyl-pyrophosphate unit with one farnesyl-pyrophosphate unit results in the C25 linear isoprenoid geranylfarnesyl pyrophosphate (GFPP), which serves as the backbone for the typical 5-8-5 ophiobolin ring structure. Recently, Chiba et al (2013) identified the first ophiobolin synthase gene in *Aspergillus clavatus*, *AcOS* (XP_001276070.1), responsible for ophiobolin F biosynthesis (Chiba *et al.*, 2013). Until now, no ophiobolin synthase has been characterized in other species, including the known ophiobolin producer *C. miyabeanus*.

In this study, the role of ophiobolin A in the interaction of *C. miyabeanus* with rice was investigated. We identified and confirmed the function of an ophiobolin synthase gene from *C. miyabeanus (CmOPS1)*, by deleting the gene from an ophiobolin A producing strain. We show that ophiobolin A is a major virulence factor during rice brown spot disease and that ophiobolin-deficient mutants cause less disease symptoms than the wild type (WT). Furthermore, we measured ophiobolin A production at different time points post inoculation and show a pattern in which ophiobolin A is induced upon penetration and is produced during the first 24 hours post inoculation (hpi). Finally we show that ophiobolin A triggers a stress response and induces cell death in both a Ca^{2+} independent and dependent way.

Results

Identification of a putative ophiobolin synthase encoding gene *CmOPS1*

We hypothesized that the enzyme responsible for ophiobolin production in *C. miyabeanus* might be a homolog of the recently identified AcOS from *A. clavatus* (Chiba *et al.*, 2013). A candidate AcOS ortholog was identified in the genome of *C. miyabeanus* strain WK1C based on a BLAST search and a subsequent *in silico* domain analysis of the corresponding predicted protein (CmOps1, *C. miyabeanus* ophiobolin synthase, JGI protein ID 4383). Alignment of the complete protein sequence of AcOS and CmOps1 showed 63% amino acid conservation. Another 19% of the amino acids represents conserved substitutions and 7% were semi-conserved substitutions, adding up to 89% overall similarity (Figure 2.2).

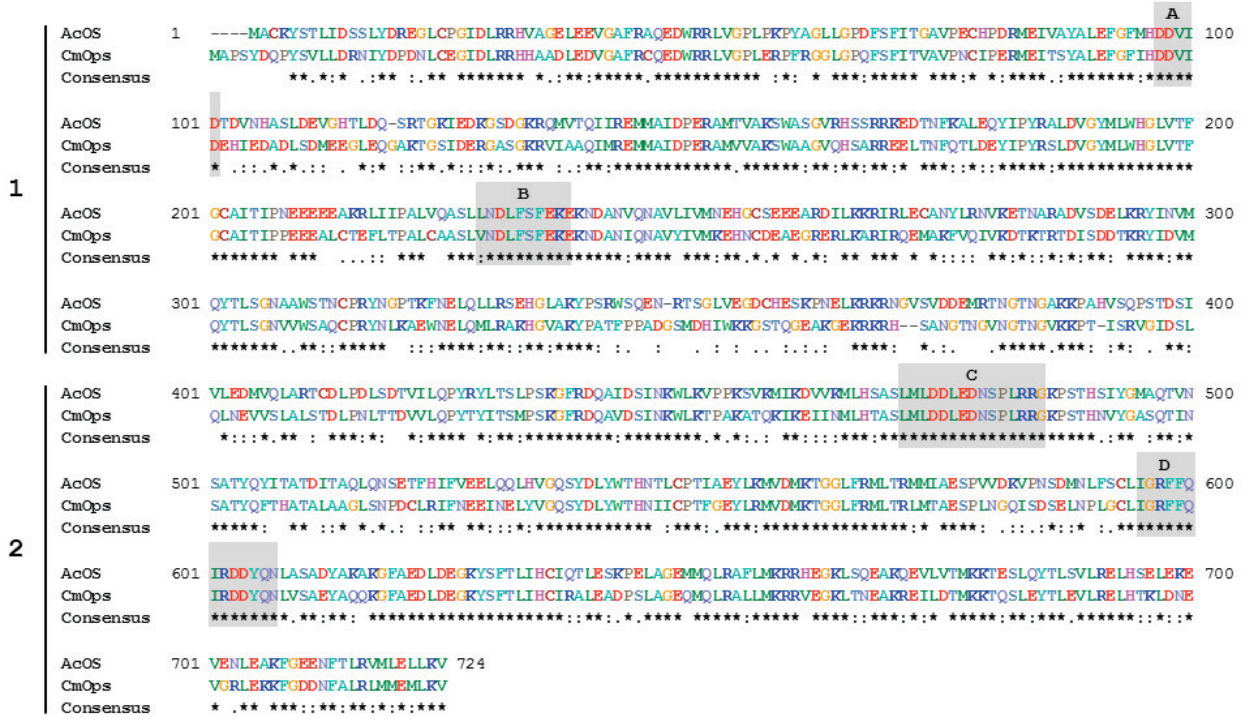


Figure 2.2. Pairwise alignment of AcOS from *Aspergillus clavatus* NRRL 1 and CmOps1 from *Cochliobolus miyabeanus* WK1C. The consensus sequence depicts conservation among amino acids: '*' identical, ':' conserved substitution, '.' semi-conserved substitution. Shaded positions mark motifs in the active domains. 1: terpene cyclase domain featuring motifs A and B; 2: prenyltransferase domain featuring motifs C and D.

The TC-domain of AcOS and CmOps1 belongs to class I terpenoid cyclases, exhibiting two typical motifs: an aspartate rich sequence DDxx(DE) on helix D and a consensus sequence of (LV)(VLA)(ND)D(LIV)x(ST)xxxE on helix H (also designated the NSE/DTE motif) (Christianson, 2006). In the case of AcOS and CmOps1, these motifs are DDxxD (motif A) and LVNDLxSxxxE (motif B) respectively (Figure 3). The PT-domain features two aspartate rich motifs, the ProSite Polyprenyl synthase signature 1 (LIVM)(LIVM)xDDxxDxxxxRR(GH) (PS00723) and 2 (LIVMFY)Gxx(FYL)Q(LIVM)xDD(LIVMFY)x(DNG) (PS00444) respectively. In the case of AcOS and CmOps1 these motifs are formed by LMxDDxxDxxxxRRG (motif C) and IGxxFQIxDDYxN

(motif D). Amino acids of all four motifs were conserved between AcOS and CmOps1, apart from the valine in motif B. Based on the strong conservation level of the active domains we decided that *CmOPS1* was a good candidate for an ophiobolin synthase gene in *C. miyabeanus*.

CmOps1 is involved in ophiobolin biosynthesis

To verify the function of CmOps1, *CmOPS1* was deleted from the highly virulent *C. miyabeanus* strain Cm988 by homologous recombination using protoplast transformation. The candidate transformants were purified by single spore selection and exchange of the *CmOPS1* gene for the Hygromycin B resistance gene was confirmed by fungal growth on $HygB^+$ medium and by PCR-verification (Figure S2.2). Initially, five confirmed knock-out mutants were selected for phenotyping.

Ophiobolin A production by the WT strain and five selected mutant strains was quantified using ultrahigh performance liquid chromatography tandem-quadrupole mass spectrometry (UPLC-MS/MS), based on ion transitions 401.0 > 365.2 and 401.0 > 109.0 (Figure S2.1). Whereas the WT strain produced high amounts of Ophiobolin A in liquid culture, production by all mutant strains was below the limit of detection (Figure 2.3a). Of the confirmed mutants, two were selected for this study, *Cmops9.1* and *Cmops13.1*. Application of pure ophiobolin A in a toxin bioassay resulted in necrotic lesions at the application site (Figure 2.3b). A sterile culture filtrate of the WT strain causes similar necrotic lesions, whereas a culture filtrate of the mutant strains could no longer induce these symptoms (Fig. 2.3b). This visualises the lack of ophiobolin A in mutant liquid culture.

The total ion chromatogram (TIC) generated by UPLC-MS analysis of the liquid culture extract shows that besides ophiobolin A (peak 1, m/z 401.0), three additional peaks are lacking from the mutant cultures (peak 2, m/z 401.0; peak 3, m/z 400.0; peak 4, m/z 401.0) (Figure 2.3c). The m/z values of the main ions in these peaks are in the range of ophiobolin analogues and the peaks eluted between 15 and 18 min, indicating similar chemical characteristics. Those peaks may represent ophiobolin analogues, but their identity has not been confirmed. Furthermore, new peaks are present in the mutant TICs between 9 and 15 min, which may represent uncharacterized precursors of ophiobolin biosynthesis.

Fungal growth rate and conidiation are not affected by *CmOPS1* deletion

Cmops1 mutants did not show major differences in growth rate or conidiation when grown on PDA medium, compared to the WT strain (Figure 2.3d and Figure 2.3e). After three days of growth, no significant differences could be observed ($p > 0.05$). After seven days, the diameter of *Cmops9.1* (80.0 ± 0.9 mm) was slightly, but significantly bigger than the WT (72.9 ± 1.6 mm). For *Cmops13.1*, no difference could be observed. Sporulation after 14 days of growth on PDA was not significantly different, with an average of 1.2 ± 0.1 x 10^6 spores per plate for the WT strain, 1.3 ± 0.05 x 10^6 spores for *Cmops9.1* and 1.0 ± 0.06 x 10^6 spores for *Cmops13.1*.

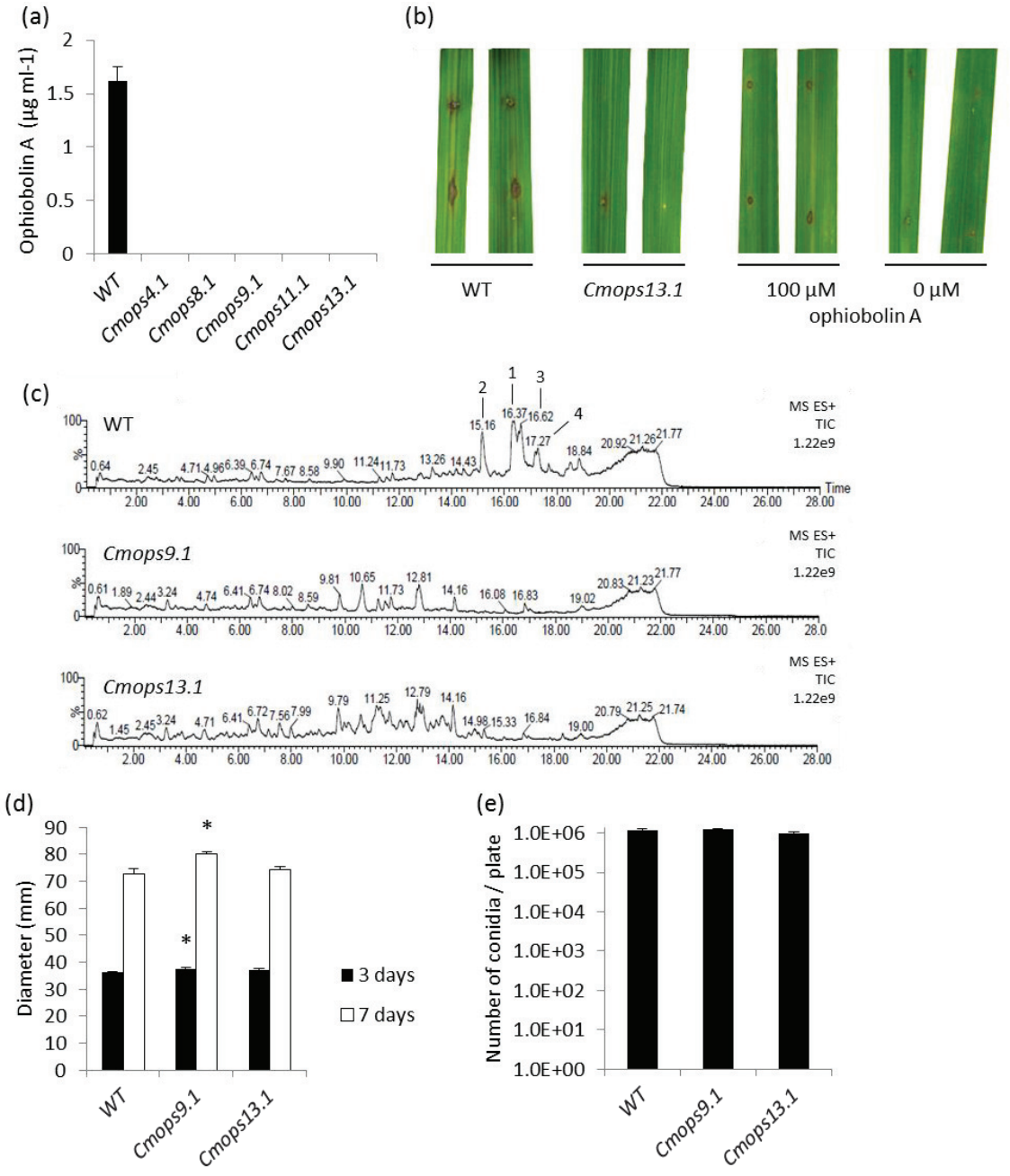


Figure 2.3. **Phenotypical and chemical characterization of *Cmops1* deletion mutants of wild type (WT) *Cochliobolus miyabeanus* strain Cm988 (a)** Fungi were grown in liquid Fries medium for 7 days, ophiobolin A was extracted from the filter sterilized supernatant and quantified using UPLC-MS/MS. *Cmops4.1-Cmops13.1* are independent genetically confirmed mutants. **(b)** Drops of a filter sterilized crude culture filtrate from the WT strain grown in liquid Fries culture for 7 days causes necrotic lesions on rice leaf pieces, whereas drops from the *Cmops13.1* mutant strain no longer induces any symptoms. In comparison, 100 µM pure ophiobolin A in 1 % DMSO causes small necrotic lesions. Pictures are taken at 48 hours post treatment. **(c)** Full scan UPLC-MS Total Ion Chromatograms (TIC) of compounds extracted from a liquid culture filtrate of WT and mutants *Cmops9.1* and *Cmops13.1* and detected by UPLC-MS in ESI^+ mode. Fungi were grown in liquid Fries medium for 7 days, compounds were extracted from the filter sterilized supernatant. Ophiobolin A elutes at 16.37 min (peak 1). Peak 2-4 correspond to putative ophiobolin analogues. **(d)** *Cmops1* mutants do not show major changes in growth rate when compared to the WT. Fungi were grown on PDA plates in dark at 28° C. The colony diameter was measured at three and seven days. **(e)** *Cmops1* mutants do not show changes in conidiation after 14 days of growth on PDA medium when compared to the WT. Results are means (± SE) of the pooled data of two independent experiments (n = 6). * indicate significant differences with WT by Wilcoxon Rank-Sum non-parametric test ($p < 0.05$).

CmOps1 plays an important role in brown spot disease development

Cmops1 mutants cause less severe disease symptoms than the wild type strain

To investigate the role of ophiobolin A during infection, we compared the ability to induce disease symptoms of ophiobolin-deficient mutants and the WT strain. In a first experiment, leaf pieces were spray-inoculated with WT or *Cmops9.1* spores respectively. Lesions typically appeared during the first 48 hpi. Significantly less lesions were counted at 48 hpi on leaves infected by *Cmops9.*1 (31.1 ± 5.4) compared with the WT (58.5 ± 6.6) ($p < 0.05$) (Figure 2.4a).

Additionally, the percentage affected leaf surface was measured from two until six days post inoculation (Figure 2.4b). The leaves infected by *Cmops9.1* were significantly less affected compared to the WT from day 2 onwards ($p < 0.05$), resulting in a final 50.8 ± 6.8 % of the leaf surface affected by WT infection, but only 22.1 ± 4.3 % after *Cmops9.1* inoculation. Subsequently, we spray-inoculated intact plants. Similar to the leaf piece bioassays, the affected area of the youngest fully developed leaf was significantly reduced after *Cmops9.1* (16.5 ± 2.1 %) and *Cmops13.*1 (19.8 ± 1.9 %) infection respectively, compared to the WT (30.9 ± 2.9 %) (Figure 2.5).

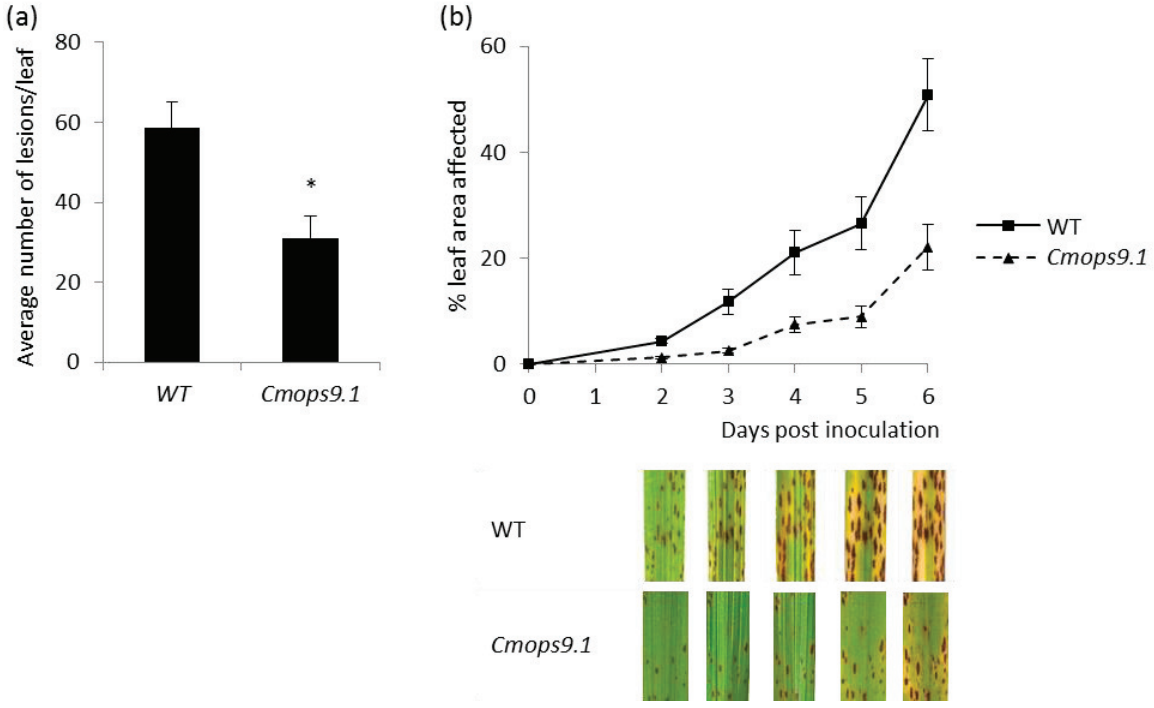


Figure 2.4. Ophiobolin-deficient *Cochliobolus miyabeanus* Cm988 mutants cause significantly less disease symptoms on rice (*Oryza sativa)* leaf pieces. Leaf pieces of 5 week old plants were spray inoculated with a spore solution ($1x10^4$ ml^{-1} spores) of *C. miyabeanus* Cm988 wild type or *Cmops9.1* respectively. **(a)** Inoculation with mutant spores resulted in significantly less lesions per leaf at 48 hpi. * indicate significant difference with WT by Wilcoxon Rank-Sum non-parametric test ($p < 0.05$). **(b)** Inoculation with mutant spores resulted in a significantly smaller affected leaf area. % affected leaf area was measured using APS Assess software at 2, 3, 4, 5 and 6 dpi. Differences were statistically significant at each time point by Wilcoxon Rank-Sum non-parametric test ($p < 0.05$). Pictures showing representative symptoms were taken at the corresponding time point. All results are means (± SE) of the pooled data of three independent experiments (n = 18).

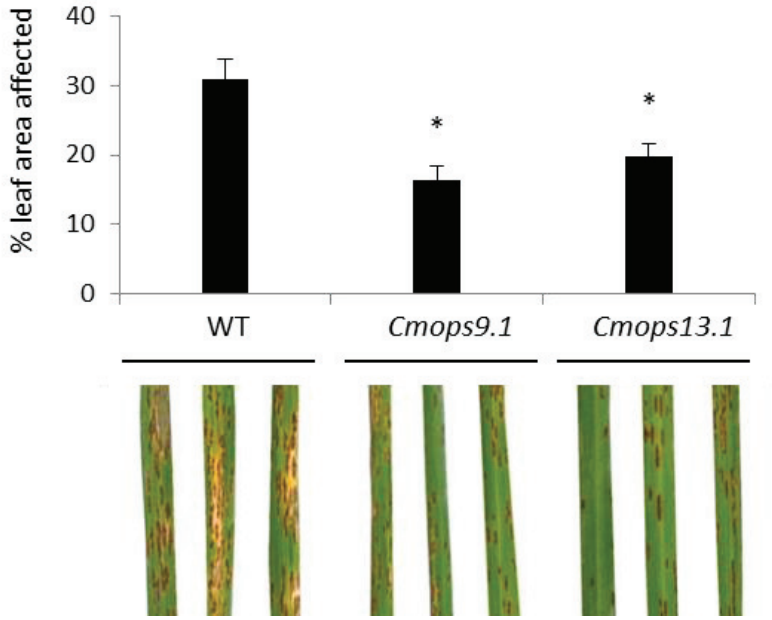


Figure 2.5. **Ophiobolin-deficient *Cochliobolus miyabeanus* Cm988 mutants cause significantly less disease symptoms on intact rice (*Oryza sativa*) plants.** Five week old plants were spray inoculated with a spore solution ($1x10^4$ ml^{-1} spores) of Cm988 wild type, *Cmops9.1* or *Cmops13.1* respectively. Percentage affected area on the youngest fully developed leaf was measured using APS Assess software at 3 dpi. Inoculation with *Cmops9.1* or *Cmops13.1* resulted in a significantly smaller affected leaf area compared to the wild type. * indicate significant differences compared to wild type by Wilcoxon Rank-Sum non-parametric test ($p < 0.05$). All results are means (± SE) of the pooled data of two independent experiments ($n = 24$). Pictures showing representative symptoms were taken at 3 dpi.

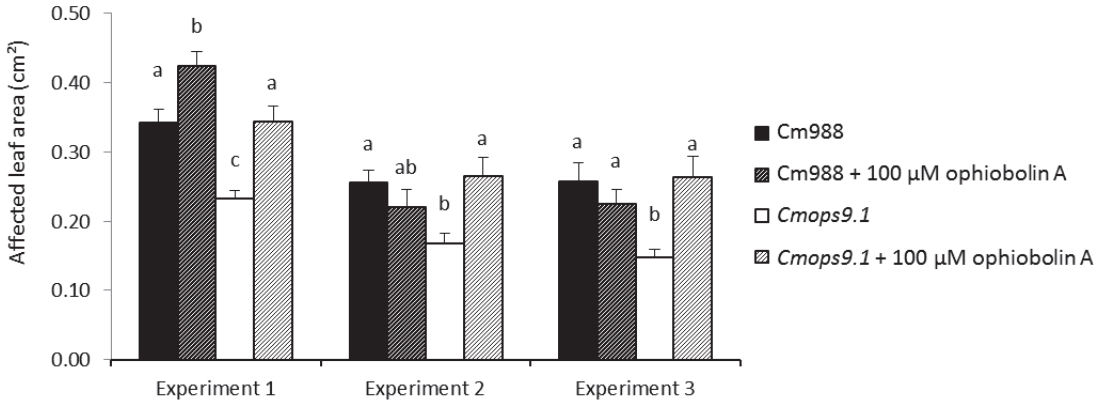


Figure 2.6. **Chemical complementation with 100 µM ophiobolin A restores the level of disease severity caused by of *Cochliobolus miyabeanus Cmops9.1* mutants to the wild type level.** Rice (*Oryza sativa*) leaf pieces were drop inoculated with a control inoculum or an ophiobolin A complemented inoculum with wild type and *Cmops9.1* spores ($1x10^4$ ml^{-1} spores) respectively. The affected leaf area around the infection sites was measured using APS Assess software at 4 dpi. Results are means (± SE) of three independent experiments. Symbols indicate significant differences between treatments within each experiment by Kruskal-Wallis non-parametric test and Mann-Withney U multiple comparison tests after Bonferroni correction ($\alpha = 0.05$).

To test whether lack of ophiobolin A was responsible for the change in disease severity upon infection with deficient mutants, we complemented the *Cmops9.1* inoculum with 100 µM pure

ophiobolin A before drop-inoculating leaf pieces (Figure 2.6). Similar to the spray-inoculation tests, the affected leaf area around *Cmops9.1* control droplets was significantly smaller compared to WT control droplets. However, the affected area was restored to WT level after chemical complementation with ophiobolin A. Because of biological variation between experiments, we could not pool the data. However, the result was statistically significant in each separate experiment ($p < 0.05$). Complementation with 10 µM ophiobolin A did not affect symptom development (Figure S2.3).

Ophiobolin A is produced during the first phase of infection and is correlated to the virulence level of different field strains

To find out if ophiobolin A is produced *in situ* during infection, we extracted the toxin from Cm988 spore-infected plant material. At 6 hpi, we detected 5.93 ± 0.64 ng ophiobolin A in 1 g leaf material, and at 16 hpi ophiobolin A had reached a concentration of 10.45 ± 1.72 ng /g $FW_{leaf.}$ By contrast, we could not detect ophiobolin A in the *Cmops9.1* or *Cmops13.1* infected leaf piece at 6 and 16 hpi (data not shown). Additionally, we monitored the *CmOPS1* gene expression during infection with WT Cm988. *CmOPS1* expression peaked at 6 hpi and was completely abolished at 48 hpi (Figure 2.7). These results suggest that ophiobolin A plays an important role during the first stage of infection.

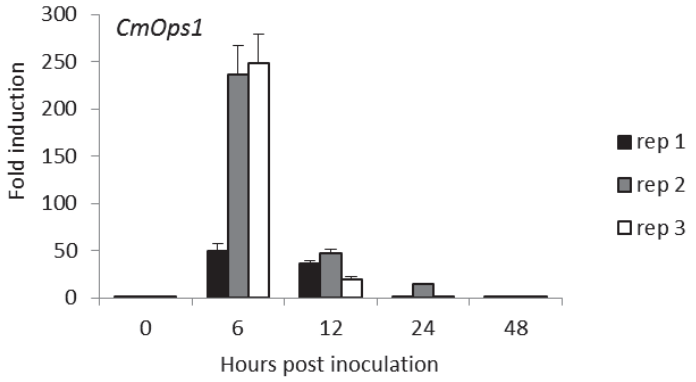


Figure 2.7. ***CmOPS1*** **gene expression is strongly induced at six hours after inoculation of rice (*Oryza sativa*) leaf pieces with wild type *Cochliobolus miyabeanus* Cm988 spore solution (1x10^4 ml^{-1} spores).** Gene expression levels were normalized with *CmActin* as internal reference and expressed relative to gene expression at 0 h. Three independent biological repeats are presented (error bars represent SE of two technical repeats).

To elucidate if *in planta* ophiobolin A production is linked to virulence ability, we measured *CmOPS1* gene expression and production for several less virulent WT strains (Fonteyne, 2011) (Figure 2.8). Because some of these strains do not conidiate easily in culture, all inoculations were done using a mycelial suspension. When mycelium is used, penetration of the plant tissue is delayed by 6 - 10 h compared to inoculation with a spore suspension (data not shown). This delay was also reflected in a difference of 6 h between maximal induction of *CmOPS1* after Cm988 spore inoculation (Figure 2.7) and mycelium inoculation (Figure 2.8a), respectively. Cm988 is a

highly virulent field strain, WK1C and G513 are intermediately virulent and S4 is almost avirulent (Fonteyne, 2011). Interestingly, *CmOPS1* fold induction is correlated with this order of virulence ability (Figure 2.8a). *CmOPS1* was weakly induced during S4 (3.9x) and WK1C (3.9x) infection, slightly more induced in G513 (6.6x) and highly induced in Cm988 (42.1x). *CmOPS1* expression peaked in all strains at 12 hpi, except for S4.

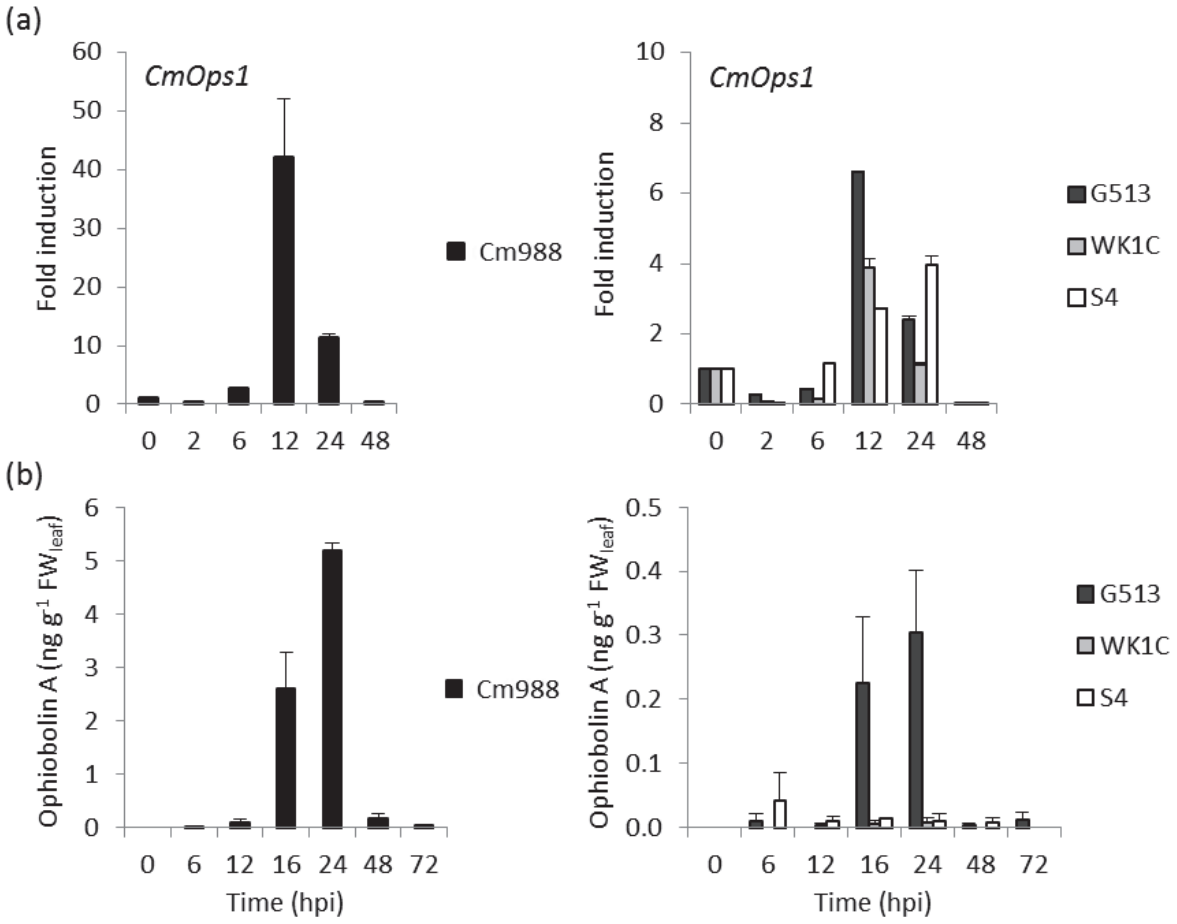


Figure 2.8. ***CmOPS1*** **gene expression and ophiobolin A production during infection of rice (*Oryza sativa*) leaves is correlated with the virulence level of different *Cochliobolus miyabeanus* wild type strains.** hpi: hours post inoculation. Cm988 is highly virulent, G513 and WK1C are intermediately virulent and S4 is almost avirulent. **(a)** Expression of *CmOPS1* by different field strains at several time points after mycelium inoculation ($5x10^4$ ml^{-1} CFU) of leaf pieces. Gene expression levels were normalized using actin as an internal reference and expressed relative to gene expression at time point 0 h. Results represent means (± SE) of two technical repeats of one experiment. Repetition of the experiment led to results very similar to those shown. **(b)** Ophiobolin A was extracted from infected leaf pieces at several time points after mycelium-inoculation, and quantified using UPLC-MS/MS. Results represent means (± SE) of 2 biological repeats (n = 6).

A similar picture emerged when we extracted ophiobolin A from infected tissue (Figure 2.8b). Although biological variation between experiments was substantial, the overall result was congruent. The highest levels of ophiobolin A were detected for Cm988, with a maximum at 24 hpi. Almost no ophiobolin A was detected in S4 or WK1C inoculated plant tissue, while intermediate levels were detected for G513. Together our data clearly show that ophiobolin A is a

major virulence factor during rice brown spot disease. Strains that produce more ophiobolin A during the early phases of infection cause more damage to the plant.

Ophiobolin A triggers a defense response, resulting in plant cell death

Ophiobolin A can induce PCD in plant suspension cells (Bury *et al.*, 2013). When we treated rice suspension cells with 100 µM ophiobolin A, 60 – 80% of the cells died within 24 hours post treatment (hpt) (Figure S2.4). In comparison, treatment with 1 or 10 µM ophiobolin A did not trigger cell death (Figure S2.5). In an attempt to characterize molecular pathways involved in ophiobolin A induced cell death, we treated rice suspension cells with 100 µM ophiobolin A and analysed the gene expression of three PCD marker genes, *OsNAC4*, *OsJAMyb* and *OsBI-1*, and two general defense markers genes, *OsMPK5* and *OsPAL1*. The expression pattern was then compared with differential expression in leaf pieces after inoculation with spores of Cm988 WT and *Cmops9.1* respectively.

OsNac4 is a plant-specific transcription factor that can induce HR in rice and OsJAmyb is activated by pathogen attack or JA and may act as a transcriptional regulator of signalling pathways leading to host cell death during HR (Lee *et al.*, 2001; Kaneda *et al.*, 2009). OsBI-1 is a negative regulator of PCD and is up regulated during pathogen-triggered cell death to protect the plant (Matsumura *et al.*, 2003). When rice suspension cells were treated with ophiobolin A, *OsNAC4* and *OsBI-1* mRNA levels started to rise from 2 hpt onward, and peaked at 6 hpt (Figure 2.9a and Figure 2.9b). This correlates with the time course of cell death induced by ophiobolin A, with 50% of total cell death reached by 3 hpt (Figure S2.4). Contrarily, *OsJAMyb* was down-regulated compared to control treated cells at 2 hpt and returned to untreated levels 4 hours later (Figure S2.6). Strikingly, *OsNAC4* was also strongly induced by Cm988 infection at 16 hpi, whereas infection by ophiobolin A-deficient strains only weakly induced *OsNAC4* (Figure 2.9c). The strong effect of ophiobolin A on *OsBI-1* expression was less clear during infection, and only a small difference in gene expression was observed between WT and mutant infection (Figure 2.9d). Although our data show that *OsJAMyb* expression is not strongly affected by ophiobolin A, post-translational activation of latent OsJaMyb cannot be excluded.

OsMPK5 is involved in MAPK signalling induced by several biotic and abiotic stresses, OsPAL1 catalyzes the first step in phenylpropanoid biosynthesis and is typically induced upon pathogen attack (Agrawal *et al.*, 2002; Jwa *et al.*, 2006). Both defense marker genes were activated in rice suspension cells after ophiobolin A treatment as early as 2 hpt, reaching maximal induction at 12 hpt (Figure 2.10a and Figure 2.10b). Similarly, these genes were induced by *C. miyabeanus* inoculation, with higher expression levels during WT infection (Figure 2.10c and Figure 2.10d). This confirms that ophiobolin A can trigger defense responses during *C. miyabeanus* infection.

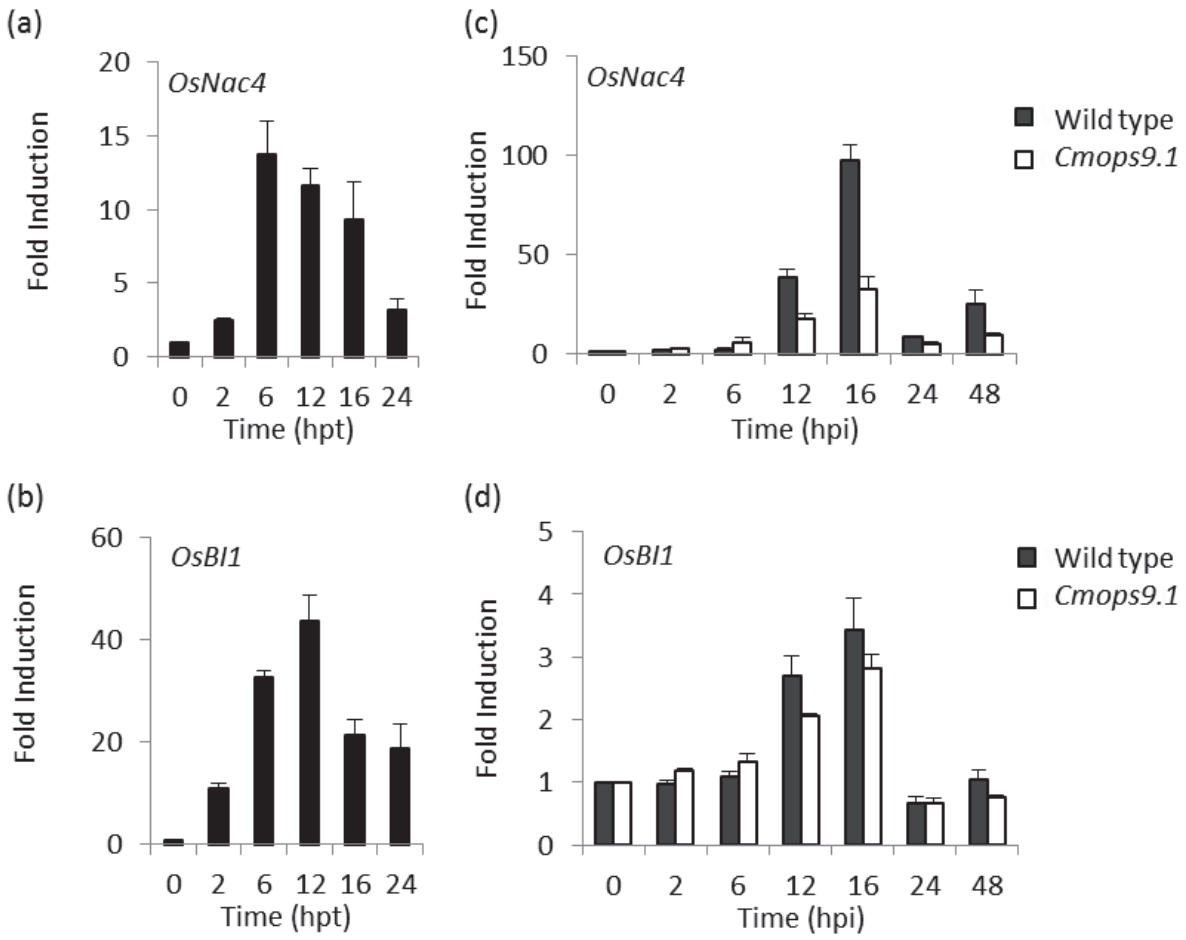


Figure 2.9. **Cell death-related genes are up-regulated in rice (*Oryza sativa*) upon treatment of suspension cells with 100 µM ophiobolin A and upon inoculation of rice leaf pieces by *Cochliobolus miyabeanus* spore suspension (1x10^4 ml^{-1} spores).** hpt/i: hours post treatment/inoculation. Gene expression levels were normalized using *OseIF1α* as an internal reference and expressed relative to control gene expression at each time point. Results represent means (± SE) of the pooled data of two independent experiments (2 biological x 2 technical repeats). The experiment was repeated one more time with results very similar to those presented. **(a)** Fold induction of the positive cell death regulator *OsNAC4* in rice suspension cells after treatment with 100 µM ophiobolin A. **(b)** Fold induction of the negative cell death regulator *OsBI-1* in rice suspension cells after treatment with 100 µM ophiobolin A. **(c)** Fold induction of the positive cell death regulator *OsNAC4* in rice leaf after inoculation with wild type or *Cmops9.1* mutant spores. **(d)** Fold induction of the negative cell death regulator *OsBI-1* in rice leaf pieces after inoculation with wild type or *Cmops9.1* mutant spores.

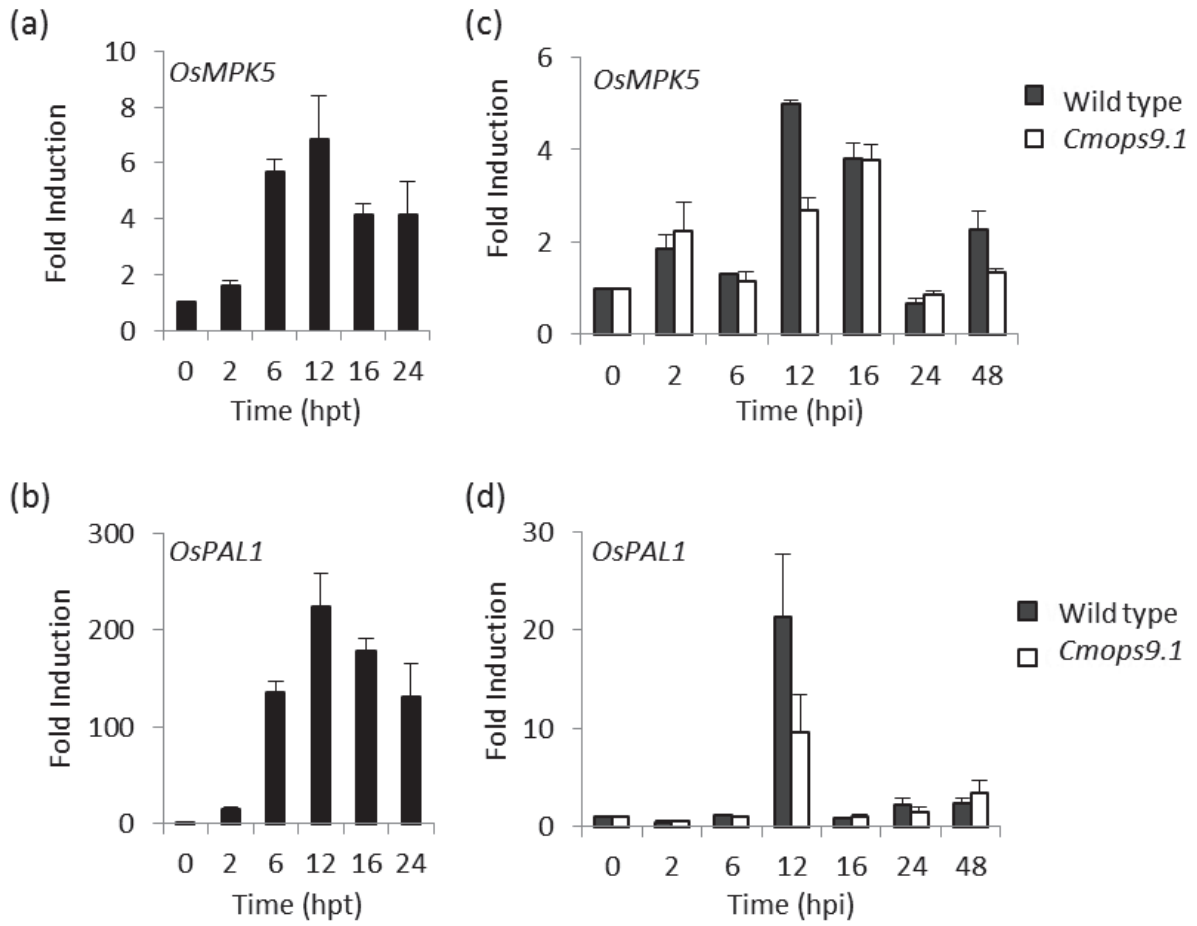


Figure 2.10. **General defense related genes are up-regulated in rice (*Oryza sativa*) upon treatment of suspension cells with 100 µM ophiobolin A and upon inoculation of rice leaf pieces by *Cochliobolus miyabeanus* spore suspension (1x10^4 ml^{-1} spores).** Gene expression levels were normalized using *OseIF1α* as an internal reference and expressed relative to control gene expression at each time point. Results represent means (± SE) of the pooled data of two independent experiments (2 biological x 2 technical repeats). The experiment was repeated one more time with results very similar to those presented. **(a)** Fold induction of *OsMPK5* in rice suspension cells after treatment with 100 µM ophiobolin A at different time points after treatment. **(b)** Fold induction of *OsPAL1* in rice suspension cells after treatment with 100 µM ophiobolin A at different time points after treatment. **(c)** Fold induction of *OsMPK5* in rice leaf pieces at different time points after inoculation with wild type or *Cmops9.1* mutant spores. **(d)** Fold induction of *OsPAL1* in rice leaf pieces at different time points after inoculation with wild type or *Cmops9.1* mutant spores.

Ophiobolin A induces both Ca^{2+} dependent and Ca^{2+} independent cell death

Plant responses to elicitors often feature Ca^{2+}-signatures and extracellular alkalinisation, leading to the activation of plant defense. Besides, ophiobolin can inhibit calmodulin (CaM) *in vitro* in a Ca^{2+} dependent manner (Leung *et al.*, 1985), a function that has never been confirmed *in vivo*. We were interested in clarifying the involvement of these mechanisms in the ophiobolin A triggered response. Ca^{2+} indeed played an important role during ophiobolin A-induced cell death (Figure 2.11a). Addition of the Ca^{2+}-channel blocker $LaCl_3$ significantly counteracted ophiobolin A triggered cell death compared to the control (Figure 2.11a). The extracellular Ca^{2+} chelator EGTA had a similar effect, but addition of $CaCl_2$ did not stimulate cell death (Figure S2.7). These data show that Ca^{2+} fluxes are needed for a strong induction of plant cell death by ophiobolin A, but ophiobolin A could also induce Ca^{2+} independent cell death.The Ca^{2+}- independent cell death is associated with a pH increase (Figure 2.11b). It was previously reported that ophiobolin A can change plasma membrane permeability of maize seedling roots to K^+. This resulted in a K^+ efflux and H^+ influx and alkalinisation of the medium (Tipton et al. 1977; Cocucci et al. 1983). A similar alkalinisation was observed when we treated rice suspension cells with ophiobolin A, resulting in a strong and lasting pH increase within 3 hpt (Figure 2.11b). Although addition of $LaCl_3$ resulted in a strong reduction of medium pH in the control cells, it did not affect the pH-inducing capacity of ophiobolin A. This suggests that pH-effects are independent of Ca^{2+} and that Ca^{2+}-independent cell death may be associated with ophiobolin A induced changes of plasma membrane permeability.

Next, we tested whether inhibition of CaM by ophiobolin A plays a role in cell death. To this end we compared the effect of ophiobolin on cell viability in the presence or absence of the CaM antagonist W7 (N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide hydrochloride) (Figure 2.11a). W5 (N-(6-aminohexyl)-1-naphthalene sulfonamide hydrochloride) is an inactive structural analogue of W7 and was used as a negative control. Interestingly, W7 treatment did not affect cell viability, suggesting that inhibition of CaM is not sufficient to trigger the cascade leading to cell death. When W7 was combined with ophiobolin A cell death was enhanced. This additive effect was neutralized by $LaCl_3$. Thus, ophiobolin A treatment seems to trigger a Ca^{2+} signature and makes cells vulnerable to CaM inhibition. Although the effect of CaM-inhibition was additive in terms of cell death, it was opposite in terms of pH induction (Figure 2.11c). When W7 and ophiobolin A were combined, pH decreased slightly compared to ophiobolin A alone. Although we cannot exclude that ophiobolin A interacts with CaM *in vivo*, from our results it is clear that induction of rice cell death by ophiobolin A is only weakly associated with CaM inhibition.

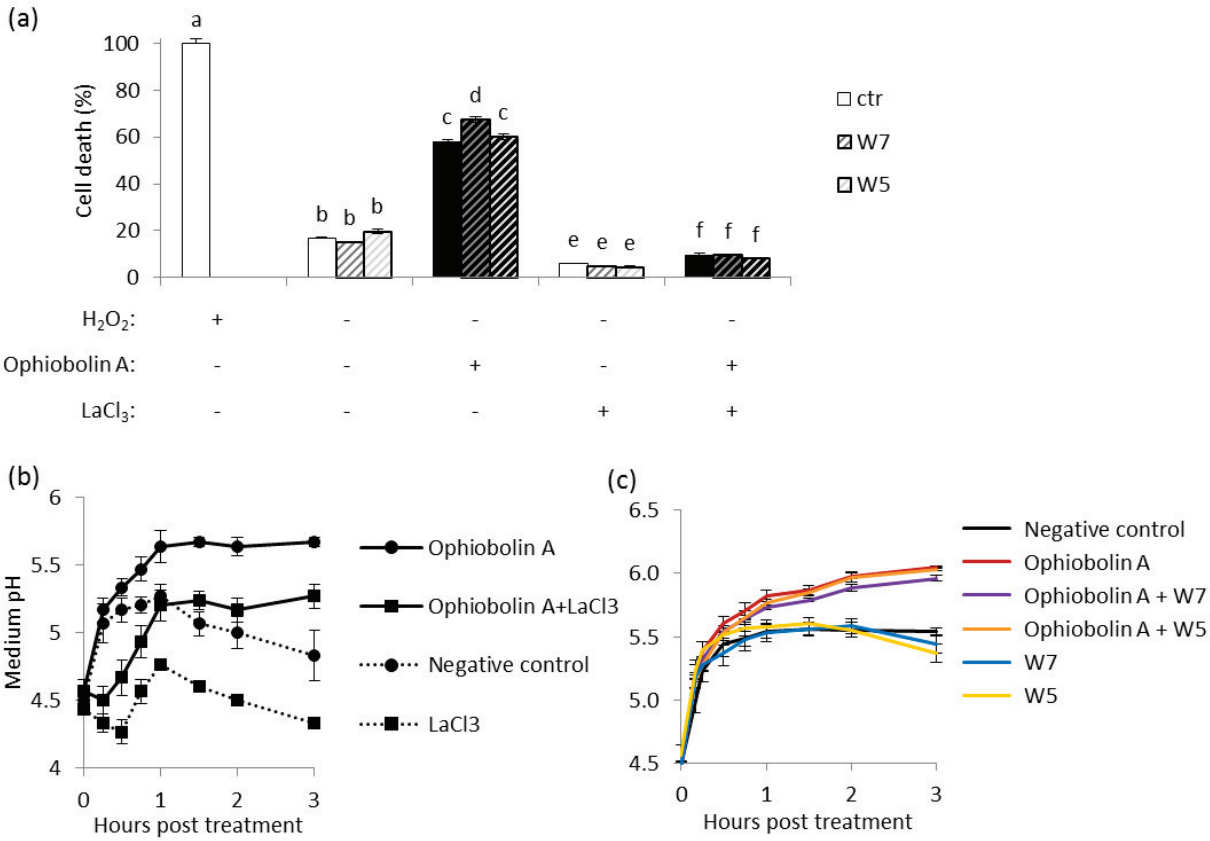


Figure 2.11. Evaluation of cell viability and medium pH of rice (*Oryza sativa*) suspension cells upon treatment with ophiobolin A, to investigate the involvement of Ca^{2+} and calmodulin in ophiobolin A effects. Cell death was determined spectrophotometrically using the Evan's blue assay, pH was monitored during three hours following start of treatment. W7: CaM-antagonist; W5: Inactive structural analogue of W7 **(a)** Cell death at 24 h after treatment with 100 µM W7, 100 µM W5, 100 µM ophiobolin A (ophio), 5 mM $LaCl_3$ or a combination, respectively. Cells treated with 350 mM H_2O_2 were used as a positive control. Results are means (± SE) of the pooled data of three independent biological repeats (n = 9). Statistical analysis by Kruskal-Wallis and Mann-Whitney U multiple comparison non-parametric tests with Holm's sequential Bonferroni correction (α = 0.05). **(b)** and **(c)** Changes in extracellular pH in control cells or $LaCl_3$ (5mM) treated cells after treatment with 100 µM W7, 100 µM W5, 100 µM ophiobolin A or a combination, respectively. Results are means (± SE) of three independent biological repeats.

Discussion

In this study we show unequivocally that ophiobolin A acts as a major virulence factor for *C. miyabeanus*. Its action frame coincides with the early phases of infection, right after penetration of the rice leaf. We identified a *C. miyabeanus* ophiobolin synthase (CmOps1), that catalyses the biosynthesis and cyclization of the C25 terpenoid GFPP to produce the basic ophiobolin 5-8-5 ring structure. When *CmOPS1* was deleted from Cm988, a strain with a high ophiobolin production level, the mutant was no longer able to produce ophiobolin A *in vitro* and *in planta*. This proves that *CmOPS1* is a key gene in ophiobolin biosynthesis.

Ophiobolin-deficiency resulted in a significant reduction of disease severity compared with infection with the highly virulent Cm988 WT strain. Ophiobolin deficient mutants produced fewer lesions, and caused significantly less chlorosis and necrosis than the parental strain. *CmOPS1* deletion had no major effects on fungal growth and conidiation, indicating that lack of ophiobolin A production is the major factor causing the difference in severity of disease symptoms. This was confirmed by complementing the *Cmops1* mutant inoculum with pure ophiobolin A, which restored the level of disease to that caused by the wild-type strain.

The fungus penetrates the rice leaf around 6 h or 12-16 h after spore or mycelium inoculation respectively. This coincides with the first induction of the *CmOPS1* gene expression. It is plausible that *CmOPS1* expression is triggered by an infection- or plant-specific factor. A similar mechanism has been shown for Fumonisin B1 induction by *Fusarium verticillioides,* which needs plant amylopectin as a signal (Bluhm & Woloshuk, 2005). Interesting in this regard is the observed up-regulation of genes involved in the plant mevalonate pathway at 12 hpi (Van Bockhaven *et al.*, 2015b). It is not unlikely that the availability of plant terpene intermediates enhances ophiobolin production. Shlezinger et al (2011) show that during the early phase of infection by the necrotrophic *Botrytis cinerea*, plant and fungus try to kill each other's cells by inducing fungal or plant PCD respectively. They made similar observations for *C. heterostrophus* (Shlezinger *et al.*, 2011). If a similar scenario happens during brown spot disease, ophiobolins may be mostly needed to induce PCD during the early phase, right after penetration of the rice leaf. At 48 h after inoculation the fungus has established itself on the necrotic plant tissue and *CmOPS1* is no longer expressed. Surprisingly, we could no longer detect any ophiobolin A *in planta* after 48 h. It is possible that the compound breaks down rapidly after production, or it could have been actively metabolized by the plant in an attempt to detoxify the compound (Pedras *et al.*, 2001).

The contribution of ophiobolins to disease severity is reflected in the production level of *C. miyabeanus* field strains. The most weakly virulent strain S4 produced almost no ophiobolin A, whereas the intermediately virulent strains produced intermediate amounts of ophiobolin A, but still much lower than the highly virulent strain Cm988. Why *CmOPS1* gene expression and

ophiobolin A production is higher in certain strains is not known. Production of fungal secondary metabolites is regulated at several levels through specific and general transcription factors, often triggered by environmental cues or developmental stages, e.g. asexual sporulation (Calvo *et al.*, 2002; Fox & Howlett, 2008). Strikingly, strains with a low ophiobolin production do not sporulate well in culture, suggesting that global regulator may control both phytotoxin production and sporulation.

Despite the important contribution of ophiobolin A to disease development, ophiobolin-deficient mutants were still able to cause considerable disease symptoms. This is not surprising, as plant-pathogenic fungi typically have multiple virulence strategies (Choquer *et al.*, 2007; Laluk & Mengiste, 2010). In an ophiobolin-negative background, other factors could still be effective and may even be overproduced. Analysis of compounds produced by ophiobolin-deficient strains could elucidate new virulence factors.

Ophiobolin A triggers a signalling cascade associated with plant cell death

Typically, the plant recognizes pathogen attack through the perception of danger signals. These danger signals activate signalling cascades resulting in a specific defense response. A strong defense response is often accompanied by the induction of localized cell death (Boller & Felix, 2009). Danger signals include PAMPs and effectors, but also other pathogen derived factors such as phytotoxins. Phytotoxins can trigger plant defense responses that ultimately lead to PCD (Wang *et al.*, 1996; Desmond *et al.*, 2008; Mase *et al.*, 2013). Whereas in the case of biotrophs localized cell death can be a successful strategy to stop the pathogen, induction of cell death enhances susceptibility to necrotrophs (Govrin & Levine, 2000; Ahn *et al.*, 2005).

Ophiobolin A can trigger PCD when applied to plant cells and might thus induce the plant suicide machinery as a virulence mechanism (Bury *et al.*, 2013). Our results show that ophiobolin A triggers sequential responses mediated by changes in Ca^{2+} homeostasis and characterized by the induction of *OsMPK5* and Os*PAL1* gene expression and activation of cell death-associated genes. It is not clear whether the toxin itself or membrane damage is the trigger for this response.

OsMPK5 and Os*PAL1* gene expression are generally associated with pathogen attack (Xiong & Yang, 2003; Jwa *et al.*, 2006). It is interesting that ophiobolin A alone can also trigger expression of these genes. Especially Os*PAL1* is expressed to a much higher extent when ophiobolin A is present, highlighting its importance in triggering defense responses. *OsNAC4* expression was stimulated both by ophiobolin A treatment *in vitro* and during infection with *C. miyabeanus*. Mutant *Cmops9.1* also resulted in *OsNAC4* induction, but to a much lesser extent, suggesting that other factors apart from ophiobolin A also contribute to *OsNAC4* fold induction. Expression of this transcription factor during disease adds weight to the contention that necrotrophs co-opt similar pathways as their biotrophic counterparts to cause PCD (Oliver & Solomon, 2010; Mengiste, 2012).

Even though plant cell death can be beneficial in first instance (e.g. HR), unlimited cell death will eventually kill the plant (Watanabe & Lam, 2009). In an attempt to contain stress-induced cell death, the plant can upregulate antiapoptotic genes as a secondary defensive act (Sanchez *et al.*, 2000). OsBI, a negative regulator of cell death, is generally up regulated upon pathogen attack and has been implicated in resistance to phytotoxins. Two *AtBI* mutants exhibit accelerated progression of cell death upon infiltration of leaf tissues with a PCD-inducing fungal toxin fumonisin B1 and the PCD-inducing agent tunicamycin (Watanabe & Lam, 2006, 2008). When the Barley *OsBI-1* analog *Mlo* was compromised, the plants displayed higher sensitivity to infiltration of toxic culture filtrate of the necrotroph *Bipolaris sorokiniana* (Kumar *et al.*, 2001). Ophiobolin A also strongly up-regulates *OsBI-1 in vitro*. Surprisingly, this effect is almost gone during pathogen infection. It is likely that *C. miyabeanus* can use an unknown strategy to antagonize *OsBI-1* gene expression to promote infection.

Ophiobolin A induces both Ca^{2+} dependent and Ca^{2+} independent cell death

Ca^{2+} acts as a secondary messenger in numerous defense responses and can activate specific signaling cascades (Levine *et al.*, 1996; Garcia-Brugger *et al.*, 2006; Manzoor *et al.*, 2012). Ca^{2+} signal transduction routes typically involve Ca^{2+} sensors, such as CaM and calcium dependent protein kinases that regulate phosphorylation events and downstream responses (Kudla *et al.*, 2010). Several phytotoxins rely on a Ca^{2+} signature to induce a signaling cascade in plant cells (Kauss *et al.*, 1991; Navarre & Wolpert, 1999; Rasmussen *et al.*, 2004; Tegg *et al.*, 2005). We showed that Ca^{2+} fluxes are needed for a strong induction of plant cell death by ophiobolin A. However, we also demonstrated that ophiobolin A can induce Ca^{2+} independent cell death. This Ca^{2+} independent part is associated with an extracellular pH increase and is probably associated with membrane permeability changes (Chattopadhyay & Samaddar, 1976). Similar changes have been observed in maize seedling roots upon ophiobolin A treatment. This resulted in a K^+ efflux and H^+ influx, accompanied by an alkalinisation of the medium (Tipton *et al.*, 1977; Cocucci *et al.*, 1983). A similar change in conductivity was one of the earliest plant responses observed when rice leaves, roots and coleoptiles were treated with ophiobolin A or after *C. miyabeanus* infection (Chattopadhyay & Samaddar, 1976). Ophiobolin A membrane interaction could well be the trigger for a Ca^{2+} signature, which may ultimately lead to PCD. Further investigation is necessary to clarify the mechanisms of ophiobolin A-induced cell death. Moreover the induction of PCD in rice suspension cells upon treatment with ophiobolin A and during rice brown spot disease should be verified based on the presence of apoptotic hallmarks, such as DNA fragmentation, chromatin condensation and micronuclei formation (Dominguez & Cejudo, 2012; Bury *et al.*, 2013).

Although ophiobolin A has been shown to inhibit CaM *in vitro* by binding CaM irreversibly in a Ca^{2+} dependent manner, CaM inhibition alone cannot explain induction of cell death. However, in an ophiobolin A triggered background, inhibition of CaM by W7 caused more cell death.

Inhibition of CaM by W7 is associated with a small pH decrease. If CaM inhibition by ophiobolin A plays a major role in Ca^{2+} dependent cell death, one would expect that this would partially antagonize the pH increase compared to $LaCl_3$-treated cells. However this is not the case. Although we cannot exclude that ophiobolin A interacts with CaM *in vivo*, from our results it is clear that inhibition of CaM is not strongly associated with the induction of plant cell death by ophiobolin A.

In conclusion, our results show that ophiobolin A-production significantly contributes to disease symptoms caused by *C. miyabeanus*. In addition, we propose a model in which *C. miyabeanus* produces high amounts of ophiobolin A as a virulence factor after penetration of the rice leaf tissue. Production of ophiobolin A lasts for 24 h and causes abundant necrotic lesion formation. Ophiobolin A targets the plant metabolism, possibly via destabilization of the plasma membrane in a Ca^{2+} independent mechanism. Eventually, a Ca^{2+} dependent defense response might be triggered in the plant cell, characterized by *OsPAL1* gene expression and MAPK signalling. This signalling cascade might result in the induction of OsNac4 mediated cell death, contributing to disease development by *C. miyabeanus*.

Materials and Methods

Biological material and general growth conditions

C. miyabeanus strains Cm988 (De Vleesschauwer et al, 2010), *CmOps9.*1 and *Cmop13.1* (*CmOPS1* deletion strain in the Cm988 background) were used. The strains were grown on potato dextrose agar (Difco, BD, Belgium) under dark conditions at 28°C.

The *Oryza sativa* japonica cultivar Nipponbare was grown in commercial potting soil (Universal; Snebbout, Belgium) in a controlled growth chamber under 12 h : 12 h, light : dark at 28°C as described previously (De Vleesschauwer et al, 2010). Rice cell suspension cultures (*japonica* cultivar Kitaake) were grown at 28 °C in the dark on a rotary shaker (100 rpm) and subcultured weekly.

Identification of ophiobolin biosynthesis orthologs

The *A. clavatus AcOS* (ACLA_076850) gene was used to query for homologs in the *C. miyabeanus* ATCC 44560 v1.0 genome sequence in the JGI MycoCosm database (http://genome.jgi-psf.org/Cocmi1/Cocmi1.home.html). Pairwise alignment and consensus sequence calculations were performed using ClustalW in BioEdit v7.1.9. The presence of conserved motifs and domain architecture of the predicted protein was analysed using ScanProSite (de Castro *et al.*, 2006) based on the PROSITE collection of motifs combined with user built patterns.

Fungal transformation and PCR confirmation

The *CmOPS1* gene was deleted using homologous recombination as described by (Bi *et al.*, 2013). The entire selection marker fragment "hygB", including the promoter, was amplified from plasmid pUCATPH using M13R and M13F primers, then fused to the 5' and 3' flanking fragments of the target *CmOPS1* gene. PCR amplification of transformation constructs was carried out with phusion high-fidelity DNA polymerase (Thermo Scientific) following the manufacturer's instructions. Primer sequences used for gene deletion can be found in the Primer List at the end of this thesis.

Transformation of *C. miyabeanus* was carried out as previously described (Inderbitzin *et al.*, 2010), with a slight modification. *C. miyabeanus* was cultured on PDA for 7 days in the dark. For protoplasting, conidia were transferred to 300 ml complete medium (CM) in a 1-l flask and shaken at 100 rpm for 16 h in the dark at 24°C. Protoplasts were harvested and transformed as described previously (Catlett *et al.*, 2003). Candidate transformants were single-conidiated and transferred to PDA containing hygromycin B (100 µg/ml) to confirm sensitivity to the drug, as described previously (Oide *et al.*, 2006). Integration of the constructs at the intended sites was confirmed by diagnostic PCR reactions, as described in (Inderbitzin *et al.*, 2010; Wu *et al.*, 2012). All PCR reactions were conducted with GoTaq (Promega).

Quantification of ophiobolin A using liquid chromatography–tandem-quadrupole mass spectrometry (UPLC-MS/MS).

For *in vitro* toxin production, mutant and WT strains were grown in liquid culture. Three mycelium plugs per strain of *Cmops9.1*, *Cmops13.1* and WT strain Cm988 were grown in 100-ml Erlenmeyer flasks containing 30 ml of Fries medium (Pringle & Braun, 1957) supplemented with 1 ml plant extract, at 28 °C in the dark on a rotary shaker (150 rpm). The plant extract was prepared by extracting 4 g of ground fresh rice leaves with 50 ml double distilled water and filter sterilized using a syringe driven 0.22 µm Millex-HV filter unit (Merck Millipore, Cork, Ireland) before use. After three days, the culture was blended and 1 ml was transferred to fresh medium and incubated for another seven days under the same circumstances. The supernatant was filtered through two layers of cheese cloth, one layer of filter paper (Grade 3hw, Sartorius Stedim Biotech S.A., Germany), and sterilized using a syringe driven 0.45 µm Millex-HV filter unit (Merck Millipore, Cork, Ireland). A 4 ml aliquot of the crude extract was used for toxin extraction.

For *in planta* toxin production, 5 week old rice plants were inoculated with the mutant or WT strain as described below. Infected leaf samples were taken and crushed using liquid N_2. Crushed leaf material was stored at -80 °C until chemical extraction. A total of about 0.5 g leaf material was used for toxin extraction.

The samples (either crude extract or leaf material) were extracted with 10 ml of ethyl acetate:acetonitrile 1:1 (v/v) after addition of 1.8 g $MgSO_4$. The sample was vigorously shaken on an end-over-end shaker (Agitelec, J. Toulemonde & Cie, Paris, France) for 30 min then centrifuged at 4000 g for 15 min. The organic upper phase was evaporated to dryness under a N_2 flow at 40 °C. The dry residue was redissolved in 100 µl of mobile phase A:B 60:40 (v/v) and filter centrifuged in an Ultrafree-MC centrifugal filter unit (Merck Millipore, Bedford, MA, USA) for 5 min at 14,000 g.

A Waters Acquity UPLC system coupled to a Waters Quattro Premier XE tandem-quadrupole mass spectrometer (Waters, Milford, MA, USA) was used to analyze the samples. The column used was a Zorbax Eclipse C18 (100 x 2.1 mm i.d.; 1.8 µm) (Agilent, Santa Clara, CA, U.S.A). Data processing was done with Masslynx software v4.1 (Waters, Milford, MA, USA).

The used UPLC-MS/MS method can be described as follows: the column was kept at 30°C and the injection volume was 10 µl. Mobile phase A consisted of H_2O:methanol 95:5 (v/v) and mobile phase B of methanol:H_2O 95:5 (v/v). To both mobile phases 0.1% formic acid and 1 mM ammonium formate was added to facilitate ionisation. A gradient program was used at a flow rate of 0.4 ml min^{-1}. The gradient started at 99.1% mobile phase A which was held for 0.5 min and next linearly decreased to 0.9% in 19.5 min. An isocratic period of 100% mobile phase B was held for 1 min. Initial column conditions were reached at 24 min using a linear decrease of mobile phase B, and the column was reconditioned for 4 min prior to the following injection. The mass

spectrometer was operated in positive electrospray ionization (ESI+) mode. The capillary voltage was 3.5 kV, and nitrogen was used as the desolvation gas. Source and desolvation temperatures were set at 120 and 300 °C respectively.

Ophiobolin A was identified and quantified using selected reaction monitoring (SRM) mode with the following ion transitions: 401 > 109 (cone 40 V, collision energy 20 eV), 401 > 365.2 (cone 40 V, collision energy 10 eV). Ion transition 401 > 365.2 was used for quantification; ion transition 401 > 109 was used as qualifier. Quanlynx (Waters, Milford, MA, USA) was used for quantification of ophiobolin A in the samples by generating a calibration curve using different concentrations of an ophiobolin A standard (Tebu Bio, Boechout, Belgium).

Toxin Bioassay

Cm988 and *CmOPS1* mutants were grown in liquid culture and the crude extract was filtered as described above. A leaf puncture assay was used as described by (Sugawara *et al.*, 1987), with a droplet of the crude extract of *Cm988*, *Cmops13.1* or pure ophiobolin A (Tebu Bio, Boechout, Belgium) as test solution.

Fungal growth rate and conidiation

A 6 mm diameter plug from a seven day old colony of the respective strain was placed in the center of a PDA plate and incubated at 28 °C in the dark. Fungal growth was measured as the average colony diameter after three or seven days of growth. After 14 days of growth, the conidia from each plate were suspended in 15 ml double distilled water and counted using a Bürker counting chamber. The experiment was repeated once, using three plates per strain.

Infection assays

C. miyabeanus conidia were harvested as described by (Thuan *et al.*, 2006) and resuspended in 0.5% gelatin (type B from bovine skin; Sigma-Aldrich) to a final density of 1 x 10^4 conidia ml^{-1}.

For spore inoculation of leaf pieces, the youngest fully developed leaves of 5-week old rice plants were cut in 7 cm pieces and kept in square Petri dishes lined with moistened tissue. Leaf pieces were spray-inoculated with 0.5 ml conidial suspension per Petri dish. The leaf pieces were kept in the dark at 28 °C and moved to growth chamber conditions after 16 hours. The number of lesions was counted at 48 hpi and disease symptoms were quantified as percentage affected leaf surface at 2, 3, 4, 5 and 6 dpi using APS Assess Software. Statistical analysis was performed using non-parametric Wilcoxon Rank-Sum test ($\alpha = 0.05$).

For infection assays using a mycelial suspension as inoculum, five mycelium plugs per strain were grown in 100-ml Erlenmeyer flasks containing 30 ml of potato dextrose broth (PDB), at 28°C in the dark on a rotary shaker (150 rpm). After 48 hours of growth, the mycelium was collected, blended in 15 ml double distilled water and diluted to 5 x 10^4 CFU ml^{-1} in 0.5% gelatin (type B from bovine skin; Sigma-Aldrich).

Inoculation of intact plants was done as described in (Van Bockhaven *et al.*, 2015a). Disease symptoms were scored at three days after inoculation as percentage affected leaf surface using APS Assess Software. Statistical analysis was performed using non-parametric Wilcoxon Rank-Sum test ($\alpha = 0.05$).

Chemical complementation

The inoculum was complemented with 100 µM pure ophiobolin A. Six leaf pieces per treatment were drop-inoculated with three separate drops of 15 µl of control inoculum or complemented inoculum with Cm988 or *Cmops9.*1 spores respectively. Disease symptoms were scored at 4 dpi as affected leaf area in cm^2 using APS Assess software. Statistical analysis was done as described for infection assays.

RNA Extraction and Quantitative RT-PCR

Each sample for gene expression analysis is composed of leaf pieces of the youngest fully developed leaf of six individual (mock) inoculated/treated plants. Total RNA was extracted from the leaf material using TRIZOL reagent (Invitrogen) and subsequently treated with Turbo DNase (Ambion) to remove genomic DNA. First-strand cDNA was synthesized from 2 µg of total RNA using Multiscribe reverse transcriptase (Applied Biosystems) and random primers following manufacturer's instructions. Quantitative PCR amplifications were conducted in optical 96-well plates with the Mx3005P real-time PCR detection system (Stratagene), using GoTaq qPCR Master Mix containing the CXR reference dye (Promega) to monitor dsDNA synthesis. The expression of each gene was assayed in duplicate in a total volume of 25 µL according to manufacturer's instructions (Promega). The thermal profile used consisted of an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 59 °C for 30 s, and 72 °C for 30 s. To verify amplification specificity, a melting-curve analysis was included according to the thermal profile suggested by the manufacturer (Stratagene).

The amount of *CmOPS1* mRNA was normalized using *CmActin* as internal control for fungal gene expression. Gene expression is relative to expression level at time 0 h. The amount of rice mRNA was normalized using eIF1a as internal control for plant gene expression. Data was calibrated to gene expression level in mock infected leaves at the same time point. Nucleotide sequences of all primers used can be found in the Primer List at the end of this thesis. Statistical analysis of relative expression results was performed using the MxPro software (Stratagene).

pH monitoring and Evan's Blue cell death assay

Extracellular pH changes were monitored with a glass pH electrode in 1 ml treated suspension cells. The pH was measured at fixed time points after treatment start. Cells were shaken at 100 rpm at room temperature. Viability of rice suspension cells was determined by Evan's blue exclusion. A 1% Evan's Blue solution (wt/vol, distilled water) was added to 750 µl treated rice suspension cells to a final concentration of 0.05%. The mixture was incubated for 15 min. The

cells were rinsed three times with distilled water to remove excess dye. 25 mg cells were resuspended in 500 µl of 50% MeOH and 1% Sodium Dodecyl Sulphate (SDS) and incubated at 50 °C for 30 min to release intracellular dye from dead cells. Destained cells were pelleted by centrifugation at 2000 g for 15 min and absorbance of the supernatant was measured at 595 nm. Treatment of cells with 350 mM H_2O_2 caused 100% cell death and was used for calibration.

Acknowledgements

This work was supported by a personal PhD Grant for Strategic Basic Research provided to L.D.B. by the agency for Innovation by Science and Technology (IWT), Belgium. We would like to thank the Department of Energy Joint Genome Institute for making the sequence data of *C. miyabeanus* strain WK1C publicly available on their website (http://www.jgi.doe.gov).

Supplementary figures

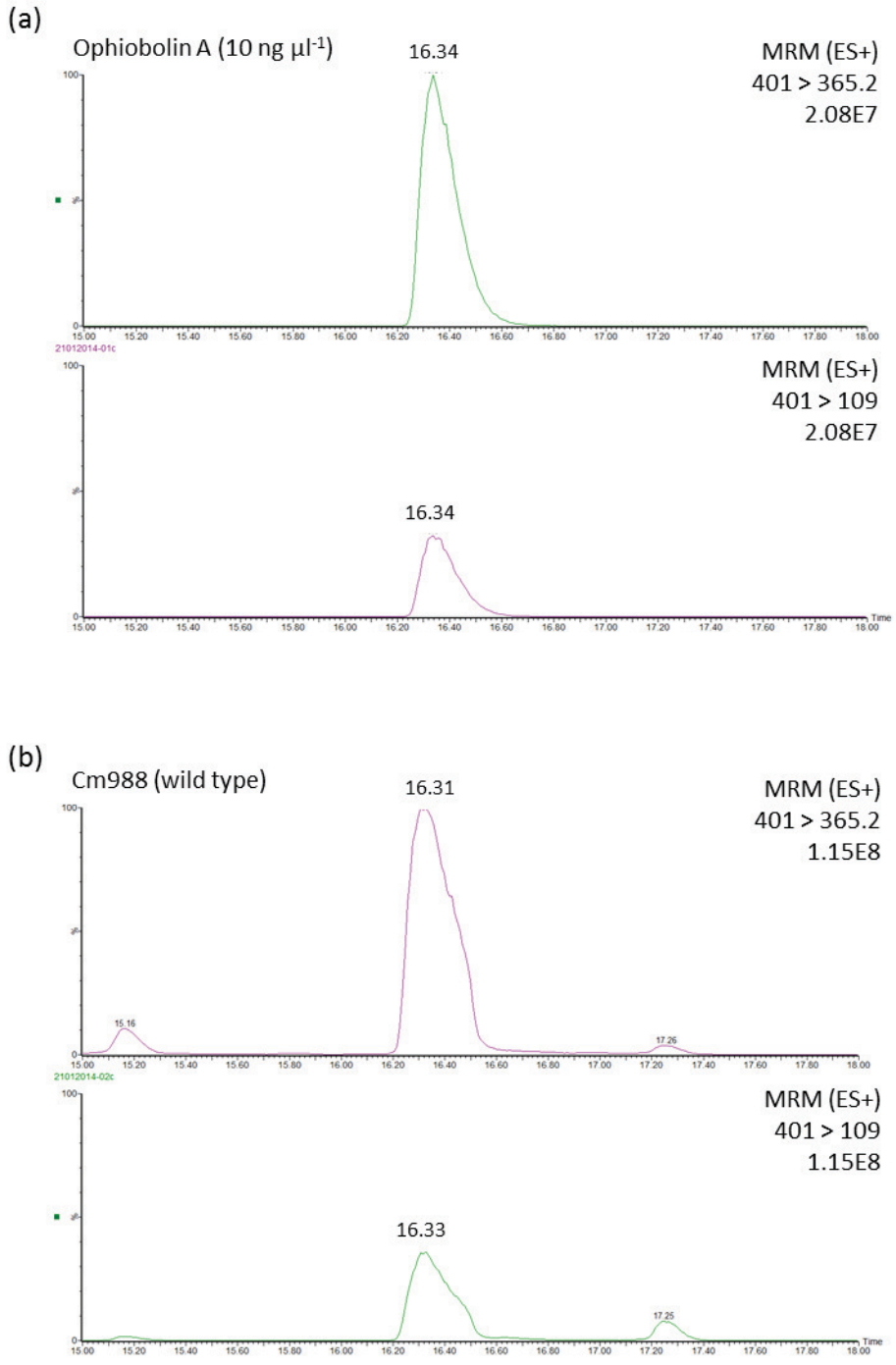


Figure S2.1. **Identification of ophiobolin A using UPLC-MS/MS, based on two characteristic MRM ion transitions: m/z 401 > 365.2 and m/z 401 > 109. (a)** Peaks generated from a pure ophiobolin A standard solution. **(b)** Ophiobolin A detected in liquid culture extract of *Cochliobolus miyabeanus* Cm988, grown in Fries medium for seven days.

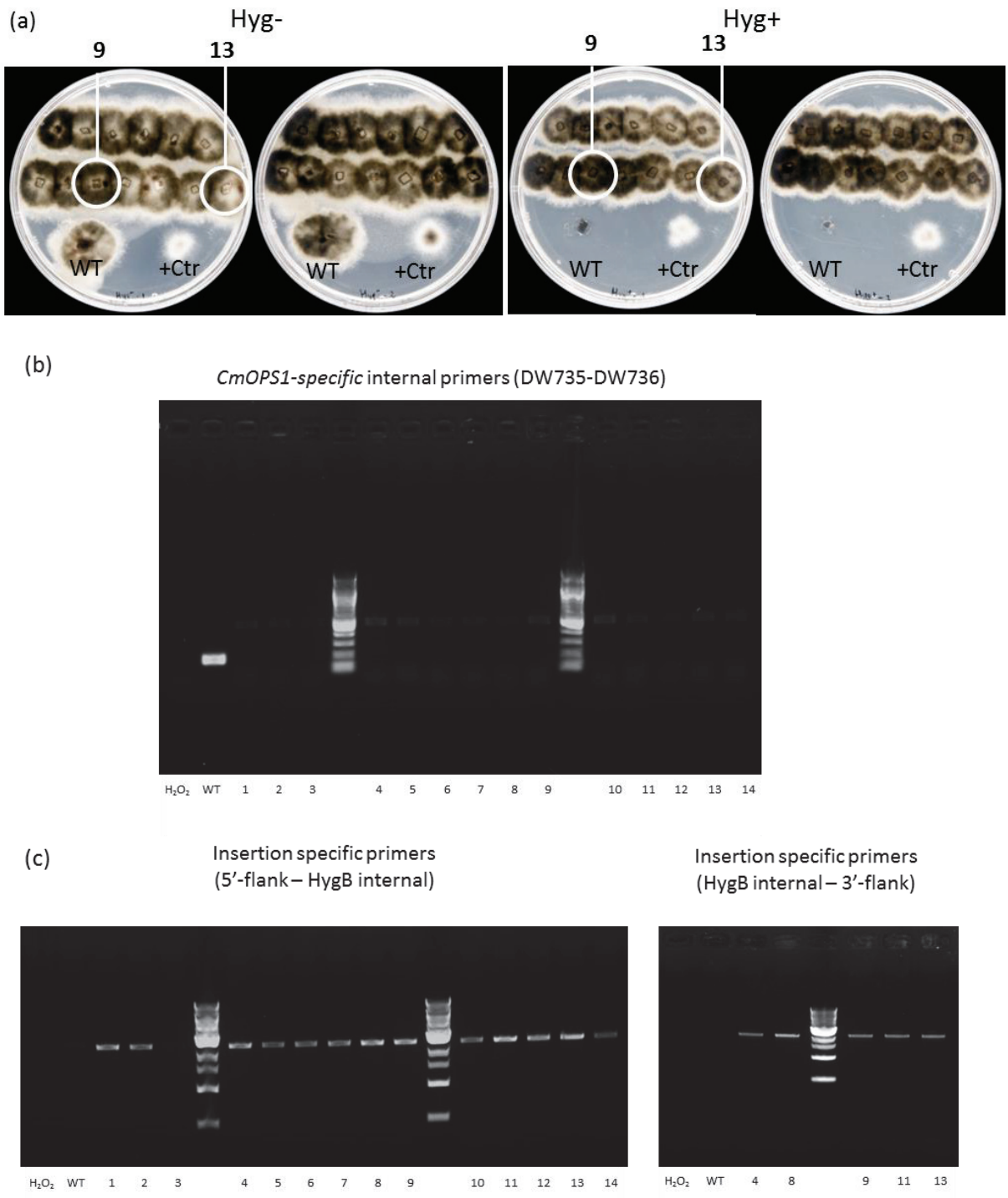


Figure S2.2 Confirmation of successful deletion of *CmOps1* in different candidate transformants (1-14) and specific integration of the transformation construct at the target site. (a) A candidate transformants that grew on HygB-supplemented medium (HygB+) were purified by selection of a single spore and subsequently grown on hygromycin-containing medium to confirm the integration of the HygB resistance gene. A strain carrying the HygB resistance gene and was used as a positive control. The Cm988 wild type (WT) is not able to grow on HygB+ medium. (b) Deletion of the targeted CmNPS3 gene in Cmnps3 was confirmed by PCR amplification using gene specific internal primers on total genomic DNA of the Cm988 wild type (WT) and the selected transformants (1-14). H_2O_2 was used as a negative control. (c) Confirmation of the integration of the transformation construct at the target site based on a PCR reaction with one internal primer for the HygB resistance gene, and one primer specific for the flanking sequence surrounding the targeted gene. 5'-insertion verification primers (DW733and NLC37); 3'-insertion verification primers (NLC38 and DW737).

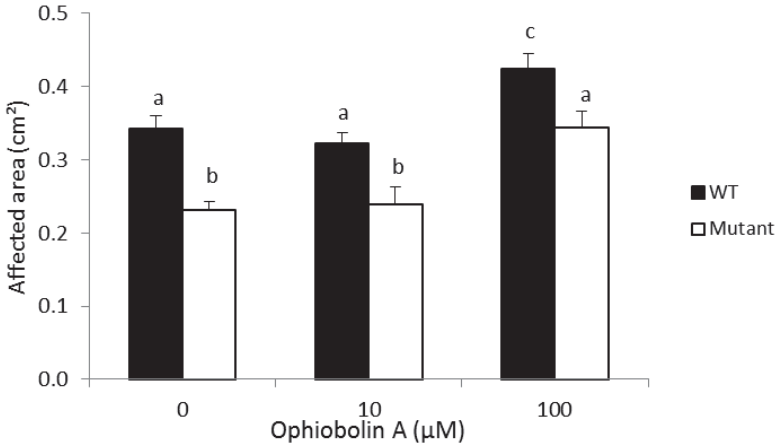


Figure S2.3 Chemical complementation of *Cochliobolus miyabeanus Cmops9.1* mutants with 10 or 100 µM ophiobolin A. Rice (*Oryza sativa*) leaf pieces were drop inoculated with a control inoculum or an ophiobolin A complemented inoculum with wild type and *Cmops9.1* spores respectively. The affected leaf area around the infection sites was measured using APS Assess software at 4 dpi. Results are means (± SE) of one experiment (n=6). Symbols indicate significant differences between treatments within each experiment by Kruskal-Wallis non-parametric test and Mann-Withney U multiple comparison tests after Bonferroni correction ($\alpha = 0.05$).

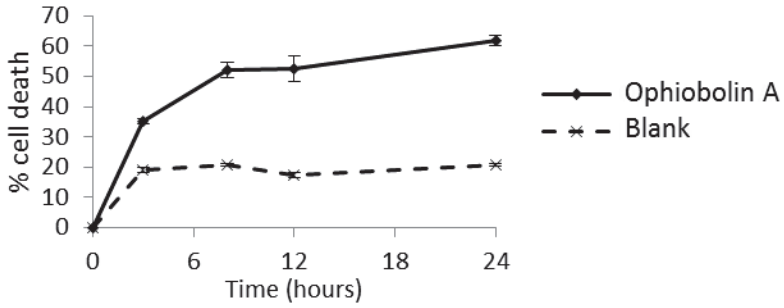


Figure S2.4. Cell death in rice (*Oryza sativa*) suspension cells, induced by 100 µM ophiobolin A. Blank: 1% DMSO solvent control. Dead cells were stained with Evan's blue dye and cell death was measured as absorbance of the supernatant after destaining of dead cells. Treatment with 350 mM H_2O_2 caused 100% cell death and was used for normalization. Data represents mean (±SE) of three biological repeats.

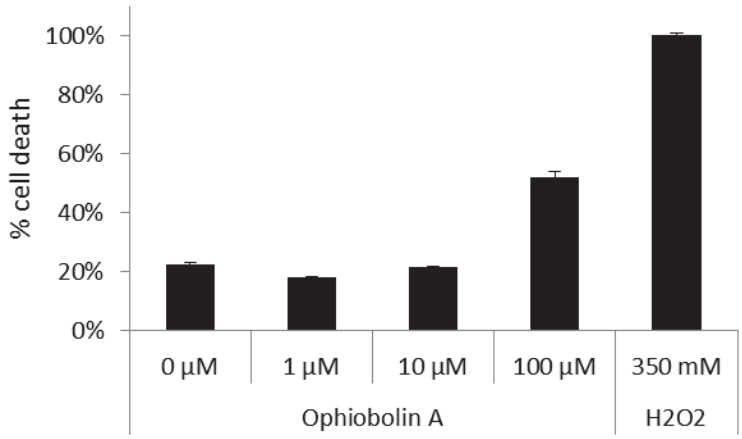


Figure S2.5 Dose-response curve of ophiobolin A-triggered cell death in rice (*Oryza sativa*) suspension cells. Rice cells were treated with the respective concentration of ophiobolin A. Dead cells were stained with Evan's blue dye at 12 hpi and cell death was measured as absorbance of the supernatant after destaining of dead cells. Treatment with 350 mM H_2O_2 caused 100% cell death and was used for normalization. Data represents mean (± SE) of 2 biological repeats.

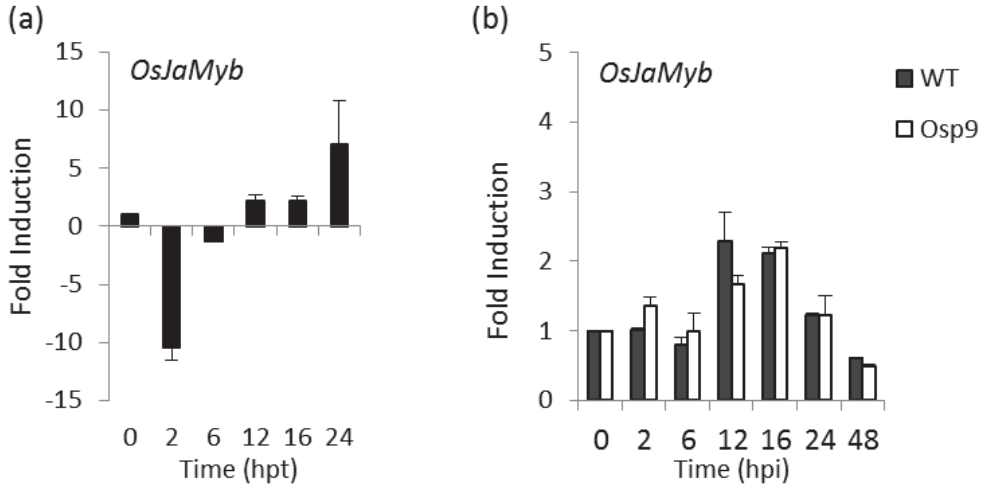


Figure S2.6. **Temporal dynamics of differential expression of *OsJaMyb* in rice (*Oryza sativa*) upon treatment with 100 µM ophiobolin A and upon inoculation of rice leaf pieces by *Cochliobolus miyabeanus* spore suspension (1x10^4 ml^{-1} spores).** Gene expression levels were normalized using *OseIF1a* as an internal reference and expressed relative to control gene expression at each time point. Results represent means (± SE) of the pooled data of two independent experiments (2 biological x 2 technical repeats). The experiment was repeated one more time with results very similar to those presented. **(a)** Fold induction of *OsJAMyb* in rice suspension cells after treatment with 100 µM Ophiobolin A at different time points after treatment. **(b)** Fold induction of *OsJAMyb* in rice leaf pieces at different time points after inoculation with wild type Cm988 or *CmOps9.1* mutant spores.

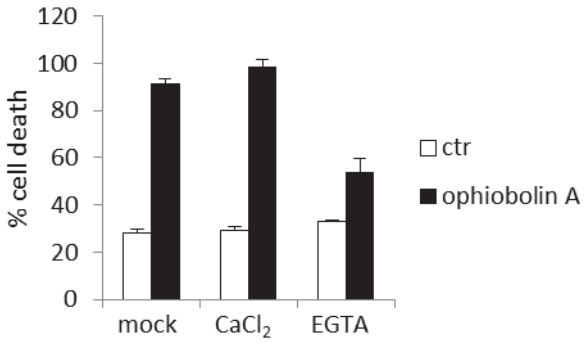


Figure S2.7. **Cell death in rice (*Oryza sativa*) suspension cells, induced by 0 µM (ctr) or 100 µM ophiobolin A in the presence or absence of 5 mM $CaCl_2$ or 5 mM EGTA.** Dead cells were stained with Evan's blue dye and cell death was measured as absorbance of the supernatant after destaining of dead cells. Treatment with 350 mM H_2O_2 caused 100% cell death and was used for normalization. Data represents mean (±SE) of three biological repeats.

Chapter 3

Identification and conservation of an ophiobolin biosynthesis gene cluster

Authors

Lieselotte De Bruyne, David De Vleesschauwer and Monica Höfte

Summary

Most fungal secondary metabolite biosynthesis genes are organized in coexpressed gene clusters. In this chapter we investigated the genomic flanking sequences of the *C. miyabeanus* ophiobolin synthase gene (*CmOPS1*) and identified a putative gene cluster, comprising two specific transcription factors, a P450 monooxygenase, two oxidoreductases and an ABC-transporter. A screening of publicly available fungal genome sequences revealed that homologs of the cluster are conserved in a diverse group of filamentous fungi belonging to different classes of the Pezizomycotina. This conservation suggests that the gene cluster provides an evolutionary advantage to fungi with different lifestyles. The gene cluster probably originated from an ancient ancestor and was transmitted vertically to most species. However, we provide evidence that *Oidiodendron maius* may have horizontally acquired the cluster from a *Coccidioides immitis* ancestor. Future investigations should evaluate the involvement of the different cluster components in ophiobolin biosynthesis.

Author contributions

Lieselotte De Bruyne: *Data acquisition, analysis and interpretation, writing of the chapter.*

David De Vleesschauwer and Monica Höfte: *Critical revision of the chapter*

Introduction

Filamentous fungi produce a plethora of bioactive secondary metabolites, typically polyketides, non-ribosomal peptides or terpenes that are not essential for normal growth and development (Keller *et al.*, 2005). The corresponding biosynthesis genes are typically organized in clusters that can span more than 10 kb and include tailoring enzymes and pathway-specific transcription factors (TF) (Keller *et al.*, 2005; Brakhage, 2013). The backbone biosynthetic NRPS, PKS and TC-encoding genes are responsible for the production of the bare metabolite skeleton structure (Toyomasu *et al.*, 2007; Itoh *et al.*, 2010; Osbourn, 2010; Brakhage, 2013). In most cases, the skeleton is then modified by tailoring enzymes, such as oxidoreductases, methyltransferases, acyltransferases and glycosyltransferases (Kroken *et al.*, 2003; Osbourn, 2010; Condon *et al.*, 2013). Variation in the modifying steps can result in a range of analogous compounds, differing slightly in their chemical structure.

The rate and timing of secondary metabolite production is regulated by a complex network of global and cluster specific TFs (Keller *et al.*, 2005; Brakhage, 2013). About 60% of the clusters analysed today feature at least one specific TF and most of these proteins belong to the Zn_2Cys_6 zinc binuclear cluster domain family. Zn_2Cys_6 TFs are often involved in specific regulation of secondary metabolite clusters, but are able to regulate a broad spectrum of physiological processes, from amino acid metabolism to stress responses (MacPherson *et al.*, 2006; Brakhage, 2013). A typical example of this fungus-unique TF family is AflR that activates most of the genes in the *Aspergillus flavus* aflatoxin biosynthesis cluster (Georgianna & Payne, 2009). Other specific TF may belong to the Cys_2His_6 zinc-finger (e.g. Tri6 in trichothecene production) or the ankyrin family (ToxE in HC-toxin production) (Proctor *et al.*, 1995; Keller *et al.*, 2005; Walton, 2006). Global regulators of secondary metabolism do not belong to a particular cluster and can steer the production of multiple gene clusters often in response to environmental cues such as pH changes (e.g. PacC from *A. nidulans*) or nitrogen use (e.g. AreA in *Giberella* spp.). In iron-depleted conditions, *A. fumigatus* can regulate its siderophore production with the iron-sensing HapX, belonging to the bZIP TF family (Yin & Keller, 2011). LaeA is another type of global TF that can be found in many species, including *C. miyabeanus*. LaeA acts as a master regulator, controlling no fewer than 13 secondary metabolite gene clusters in *A. fumigatus* in a heteromeric velvet complex with VeA and VelB (Tilburn *et al.*, 1995; Tudzynski *et al.*, 1999; Brakhage, 2013). The *C. heterostrophus* orthologs ChLae1 and ChVel1 similarly regulate T-toxin biosynthesis, pathogenicity, super virulence and several other physiological responses in *C. heterostrophus* (Wu *et al., 2012*). Besides biosynthesis and regulatory proteins, gene clusters can comprise transporter proteins in charge of the efflux of secondary metabolites through biological membranes. Often they are indispensable, as build-up of toxic compounds can be detrimental for the producing organism. Two gene families highly represented in clusters are ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) of transporters (Del Sorbo *et al.*, 2000).

The evolutionary mechanisms that create and maintain these co-regulated clusters are unclear, but several possibilities have been suggested, such as strong natural selection or the selfish cluster hypothesis. The latter suggests that the cluster persists not only by providing selective advantage for the producing organism, but also through propagation by horizontal gene transfer (HGT) (Walton, 2000; Khaldi *et al.*, 2008). Indeed, evidence is accumulating that HGT might be a common theme in the evolution of filamentous fungi (Rosewich & Kistler, 2000; Friesen *et al.*, 2006; Khaldi *et al.*, 2008; Akagi *et al.*, 2009; Mehrabi *et al.*, 2011; Campbell *et al.*, 2012). Nonetheless, the evidence for most of the suggested HGT events is not indisputable and often based solely on phylogenetic incongruency with the tree of life or patchy distribution among distantly related species. Although these properties could well indicate involvement of HGT, the possibility of vertical transfer should always be kept in mind (Oliver & Solomon, 2008).

In Chapter 2 we showed that CmOps1 is a TC that catalyzes the production of the isoprenoid ophiobolin skeleton from the mevalonate pathway intermediates farnesyl-pyrophosphate and geranyl-pyrophosphate, via farnesylgeranyl pyrophosphate (Figure 1.4). Today, about 25 different ophiobolin analogues have been described, with a common backbone ring structure, but differing in side chains (Au *et al.*, 2000). Furthermore, ophiobolin production capabilities and *CmOPS1* gene expression can differ significantly between strains, indicating differential regulation of transcription (Chapter 2). These characteristics suggest the existence of a gene cluster. Therefore, we investigated the flanking regions of *CmOPS1* to find helper enzymes and TFs. We identified a putative cluster of seven contiguous regulatory and modifying proteins with a possible role in secondary metabolism. Additionally we performed a phylogenetic analysis, revealing a spotty distribution of the cluster throughout the fungal kingdom and made a hypothesis about its evolutionary origin. To conclude, the possibility of a HGT event is proposed.

Results and discussion

Identification of an ophiobolin synthase gene cluster

The genomic region around *CmOPS1* (*C. miyabeanus* Ophiobolin Synthase) was analysed in the JGI Genome Viewer. *CmOPS1* is located 10 kb from the end of scaffold 46 (Location: scaffold_46:154170-156463). To avoid missing out on genes belonging to the cluster, but not included on the scaffold, we aligned the scaffold with its homologous region in the closely related *C. heterostrophus* genome. Using the downstream flank of *C. heterostrophus* as a template, we were able to identify scaffold 152 as the connecting scaffold (Location: scaffold_152:69543-55000).

The Gene Catalog track in the genome browser on the JGI-MycoCosm website features genes that have been predicted using homology-based and *ab initio* gene predictors, combined with RNAseq data if available (Grigoriev *et al.*, 2014). However, at the moment there is no RNAseq data available for the genes discussed in this chapter. The VISTA tracks use the displayed genomic region of the species under investigation (*C. miyabeanus* in our case) as a reference and illustrates the level of conservation of the corresponding region in closely related species (Frazer *et al.*, 2004; Grigoriev *et al.*, 2014). Six open reading frames (ORFs) were predicted in the region 20 kb upstream of *CmOPS1* (ORF1-ORF6) and another ten ORFs 20 kb downstream (ORF7-ORF16) (Figure 3.1). The InterProScan 5 application was used to predict the function of the putative proteins, based on conserved domains (Jones *et al.*, 2014) (Table 3.1).

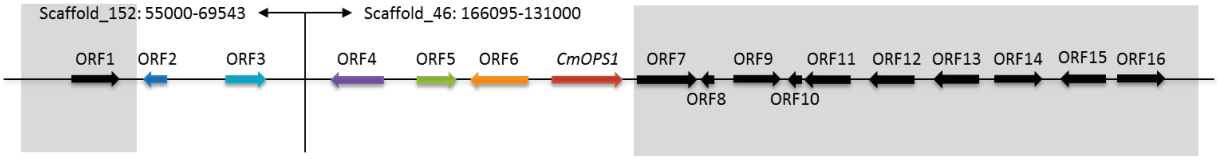


Figure 3.1. Schematic view of the genomic organization of ORFs 10 kb upstream and 10 kb downstream of the *CmOPS1* backbone gene in *Cochliobolus miyabeanus*. Shaded areas indicate a high conservation of the region in closely related species. Illustration is not to scale.

For 6 ORFs (ORF7, ORF8, ORF10, ORF12, ORF15 and ORF16), no functional predictions could be made. The number of predicted genes in a draft genome assembly is often erroneous, as annotation errors can cause over- and under-estimation of gene numbers (Denton *et al.*, 2014). Especially ORF12, ORF15 and ORF16 are suspicious as no homologs could be found. ORF15 and ORF16 have a very short protein sequence (61 and 58 amino acids respectively) and might reflect gene fragments misassembled on the genome, so-called cleaved genes. Furthermore, the gene cluster is overall well preserved in *C. heterostrophus*, lacking only ORF12 and featuring a putative aldehyde dehydrogenase, glutamine synthetase and RNAse P subunit at the location of ORF13, ORF15 and ORF16 respectively (Figure S3.2).

ORF	Genomic Location	protein ID (JGI)	Protein length	Conserved domains[1]	Predicted function	Closest GenBank Homolog , Organism, Predicted function	%C/%ID[2]
ORF1	scaffold_152: 56938-61469	104159	1494	IPR003593 IPR010929 IPR003439	AAA+ ATPase, core CDR ABC transporter ABC transporter-like	XP_003067068.1 *Coccidioides posadasii* multidrug resistance ABC transporter	98/76
ORF2	scaffold_152: 62558-63361	104187	245	IPR016040	NAD(P)-binding	XP_001265655.1 *Neosartorya fischeri* hypothetical protein	99/66
ORF3	scaffold_152: 64695-66146	104202	484	IPR006076	FAD dependent oxidoreductase	XP_001276072.1 *Aspergillus clavatus* FAD dependent oxidoreductase	95/52
ORF4	scaffold_46: 163451-165070	92540	511	IPR001138	Fungal transcriptional regulatory protein, N-terminal Zn(2)-C6 fungal-type DNA-binding domain	XP_003067064.1 *Coccidioides posadasii* Fungal Zn binuclear cluster domain containing protein	98/24
ORF5	scaffold_46: 159835-161059	65946	375	IPR004827	Basic-leucine zipper (bZIP) transcription factor	XP_003067067.1 *Coccidioides posadasii* hypothetical protein	95/25
ORF6	scaffold_46: 157245-159168	92413	525	IPR001128 IPR002403	Cytochrome P450 Cytochrome P450, E-class, group IV	ABY48861.1 *Cochliobolus heterostrophus* putative cytochrome P450 monooxygenase	97/97
ORF7	scaffold_46: 150758-152605	35835	597	NA	NA	XP_002378787 *Aspergillus flavus* conserved hypothetical protein	77/17
ORF8	scaffold_46: 150285-150668	92464	128	NA	NA	CBX93324.1 *Leptosphaeria maculans*	95/69

ORF	Genomic Location	protein ID (JGI)	Protein length	Conserved domains[1]	Predicted function	Closest GenBank Homolog, organism, predicted function	%C/%ID[2]
ORF9	scaffold_46: 148199-149647	4380	446	IPR002523	Mg2+ transporter protein, CorA-like	XP_001935733.1 *Pyrenophora tritici-repentis* Inner membrane Mg transporter mrs2, mitochondrial precursor	81/76
ORF10	scaffold_46: 147521-147956	35832	127	NA	NA	XP_003295322.1 *Pyrenophora teres f. teres* hypothetical protein	100/76
ORF11	scaffold_46: 146023-147256	25430	356	IPR008921 IPR003593	DNA polymerase III clamp loader subunit, C-terminal AAA+ ATPase, core	CBX93322.1 *Leptosphaeria maculans* similar to DNA replication factor C subunit Rfc5	100/96
ORF12	scaffold_46: 144107-144703	92414	199	NA	NA	NA	NA
ORF13	scaffold_46: 140240-142283	92388	647	IPR001312	Hexokinase	XP_001935737.1 *Pyrenophora tritici-repentis* glucokinase GLK1	98/77
ORF14	scaffold_46: 136157-138854	92553	756	IPR016040 IPR013752 IPR008927	NAD(P)-binding Ketopantoate reductase ApbA/PanE, C-terminal 6-phosphogluconate dehydrogenase, C-terminal-like	CBX92946.1 *Leptosphaeria maculans* hypothetical protein	100/52
ORF15	scaffold_46: 132937-133119	92506	61	NA	NA	NA	NA
ORF16	scaffold_46: 132231-132404	92525	58	NA	NA	NA	NA

Table 3.1. List of ORFs in the the genomic region adjacent to *CmOPS1* in the genome of *Cochliobolus miyabeanus* ATCC 44560. ORF1 - ORF6 are located upstream of *CmOPS1*, ORF7 - ORF16 are located downstream of *CmOPS1*. [1]:InterPro ID; [2]: % query coverage and % identity with closest homolog.

Interestingly, although the wider genomic region around *OPS1* is conserved in the closely related *species Pyrenophora tritici-repentis*, *Leptosphaeria maculans* and *Alternaria brassicicola*, a stretch of about 19 kb around *OPS1*, including ORF2 - ORF6, seems to be unique for *Cochliobolus* (Figure S3.2 and S3.3). The predicted functions of these five putative genes are all related to secondary metabolism.

FAD-dependent oxidoreductases like Orf3 are often present in secondary metabolism clusters as tailoring enzymes (Prieto & Woloshuk, 1997; Lorenz *et al.*, 2010). The enzyme could be responsible for the induction of double bonds in ophiobolin analogues. Orf2 has a putative NAD(P) binding domain, which could point to a similar redox activity. Orf4 resembles a typical Zn^{2+}-binding fungal TF with a Zn_2Cys_6 binuclear cluster domain. Orf5 is a putative basic-leucine zipper (bZIP) TF. YAP-like bZIP transcription factors act as redox sensors and can be activated under oxidative stress. They have been implicated in fungal stress responses, secondary metabolism and pathogenicity (Kuge & Jones, 1994; Takahashi *et al.*, 2010; Guo *et al.*, 2011; Yin *et al.*, 2013). The redox-regualted bZIP TF CHAP1 in *Cochliobolus heterostrophus* is activated during early stages of infection and protects against oxidative stress by regulating the expression of antioxidants (Lev *et al.*, 2005). Production of ROS is intrinsic to *C. miyabeanus* infection and could trigger expression of ORF5, which could in turn differentially regulate the ophiobolin gene cluster. Indeed, ophiobolin production is strongly up-regulated after penetration of the plant by *C. miyabeanus*, an event associated with intense interaction between host and pathogen and characterized by the production of H_2O_2 (Kim *et al.*, 2014). Orf6 is a putative monooxygenase CytP450 (CYP), a typical member of fungal secondary metabolite gene clusters (Yu *et al.*, 2004; Ichinose, 2014; Kimura *et al.*, 2014). CYPs are haem-containing monooxygenases that catalyse a wide variety of reactions by inserting one atom of molecular oxygen into an organic substrate (Lah *et al.*, 2011). Ophiobolin A and several other ophiobolin analogues feature hydroxyl and other oxygen containing side groups, which could depend on Orf6-like monooxygenases. Another interesting enzyme can be found upstream of the *Cochliobolus*-unique region: ORF1 encodes a putative ABC transporter-like AAA+ ATPase and is also conserved in closely related genomes (Figure S3.2 and S3.3). ABC transporters can play a key role in the efflux of secondary metabolites (Wolfger *et al.*, 2001). As ophiobolins have clear anti-fungal characteristics, the existence of a secretion mechanism seems opportune.

The putative proteins encoded in the downstream conserved flank are less common in fungal secondary metabolism. Orf9 resembles a membrane Mg^{2+} transporter enzyme. In bacteria, Mg^{2+} transporters can be associated with virulence, and some virulence-controlling systems even regulate expression of Mg^{2+} transporters (e.g. the *Salmonella* PhoP/PhoQ system) (Groisman et al. 2013). There is no evidence for a similar mechanism in fungi. Orf11 is another protein with an AAA+ ATPase domain and is likely involved in DNA replication as a homolog of a DNA replication factor C subunit Rfc5 from *Leptosphaeria maculans*. Further downstream, we found a

homolog of a *P. tritici-repentis* glucokinase (Orf13) and a putative NAD(P)-binding ketopantoate reductase, resembling the *Salmonella* ApbA/PanE (Orf14).

Involvement of these last four putative proteins in an ophiobolin metabolic pathway is questionable. But the close proximity of ORF1 - ORF6 to *CmOPS1* and their respective predicted functions suggest a likely implication in ophiobolin biosynthesis. True involvement would be reflected in a gene expression pattern correlating with ophiobolin production. Future investigations should comprise co-expression analyses, combined with gene knock-out experiments. To shed light on the functionality of the modifying enzymes, chemical structure analysis is prerequisite to determine a possible change in altered side chains caused by specific gene knock-outs.

The genomic region spanning the putative gene cluster is conserved within the *Cochliobolus* genus. However, sequence similarity is lower in *C. sativus* and *C. carbonum*. When we compared the active domain conservation of Ops1 homologs among five *Cochliobolus* species, we noticed that only *C. heterostrophus* features the exact same amino acid sequence as *C. miyabeanus* (Figure S3.1). The sequences in *C. sativus*, *C. carbonum* and *C. victoriae* have been compromised on several positions, some of which in key positions. These substitutions might have turned the ophiobolin synthase inactive. Indeed, ophiobolin production has only been reported for *C. heterostrophus* (Sugawara *et al.*, 1987; Shen *et al.*, 1999). The loss of gene function in some of the *Cochliobolus* species could explain the faster rate of evolution in this genomic region, although overall sequence similarity is still at least 93% (Table S3.1). Nevertheless, failure to detect ophiobolin does not necessarily mean that is not produced in conditions different from those tested.

Conservation across fungal kingdom

Apart from *Cochliobolus* spp., ophiobolin production has also been described for *Aspergillus clavatus* and *Aspergillus ustus*, suggesting that the ophiobolin gene (cluster) is not limited to *Cochliobolus* spp. (Liu *et al.*, 2011; Chiba *et al.*, 2013). To determine the extent of this distribution, we looked for homologs among the genomes in JGI MycoCosm. Ops1 belongs to a terpene cyclase gene family also including fusicoccadiene synthase. Chiba et al. (2013) suggested that both proteins originated from a common ancestor. They are 46% identical, 58% similar and have a very similar protein structure. To avoid selecting more distant homologs, we used stringent selection criteria. Only homologs with at least 60% identity, a score higher than 2000 and E-value = 0 were withheld. Surprisingly, closely related homologs of Ops1 are present in five additional species belonging to three different fungal classes, Leotiomycetes (*Oidiodendron maius*), Sordariomycetes (*Beauveria bassiana* and *Anthostoma avocetta*) and Eurotiomycetes (*Aspergillus clavatus* and *Coccidioides immitis*). We selected additional homologs from genbank for a phylogenetic analysis based on the Ops1 protein sequence (Figure 3.2).

The phylogenetic tree constructed from all the selected homologs showed a clade with 100% bootstrap support around CmOps1, including homologs from the species selected from JGI MycoCosm, as well as from one additional species retrieved from genbank: *Coccidioides possadasi*. For the latter, the genome sequence is not publicly available; hence we could not investigate its genome for the presence of an *OPS1* gene cluster. For the other species included in the Ops1 clade, the genomic region around the Ops1 homologs was subsequently screened for the presence of more putative cluster components in near vicinity of the gene (Figure 3.3b).

Orf6, encoding a P450 was present in all species in the same genomic orientation as in the *Cochliobolus* gene cluster. Orthologs of Orf3 were present in all clusters, except for *B. bassiana* and Orf4 was present in all clusters except for *A. clavatus*. When gene-by-gene phylogenetic trees were constructed with additional homologs, it is clear that genes from the five selected species always group together with the *C. miyabeanus* homolog, supported by a bootstrap value of at least 60% (Figure 3.4). Surprisingly, the conserved Orf1, encoding a putative ABC transporter is also present in *B. bassiana*, *C. immitis* and *A. clavatus*, but not in *O. maius* or *A. avocetta*.

Orf5 (encoding a putative bZIP TF) deviates from this trend. Although a putative bZIP TF is present in *A. clavatus*, *C. immitis* and *O. maius*, the protein does not group together with Orf5 from *Cochliobolus* sp (Figure 3.5). Because *O. maius* and *C. immitis* form a clade with 83% bootstrap support, we designated the protein Orf5.2. The *A. clavatus* Orf5 homolog did not group together with any of the other putative Orf5 proteins, we designated the protein Orf5.3. Finally, the Orf5 homolog of *C. heterostrophus* and *C. miyabeanus* clustered together with *B. bassiana*, and was designated Orf5.1. ORF5 can take three possible positions in the gene cluster, independent of the version. The lower similarity scores of Orf5 compared with other cluster components could indicate a higher evolutionary rate, possibly because of a loss of functionality. Overall, phylogenetic analysis of the gene cluster strongly indicates a common origin, but also shows a reshuffling of the genes into two different topologies (Figure 3.3b).

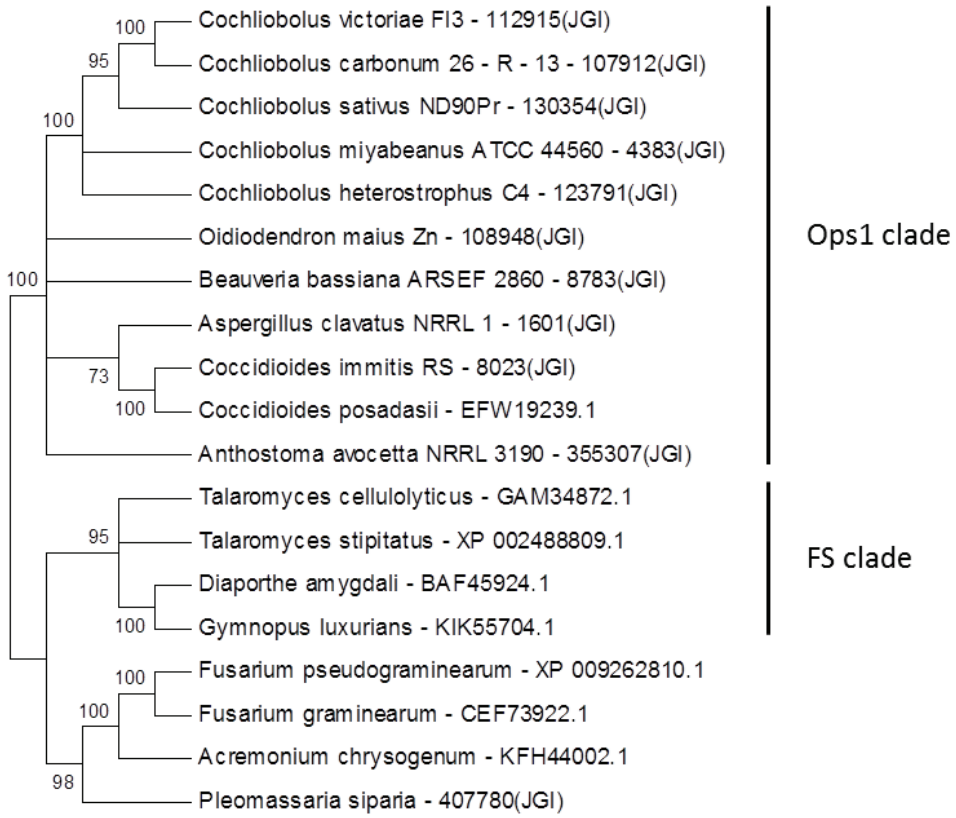


Figure 3.2. Phylogenetic analysis of Ops1 homologs. The unrooted phylogenetic tree is a bootstrap consensus tree after maximum likelihood analysis (1000 bootstraps, cut-off at 50% bootstrap support). The tree is based on Ops1 protein sequence data, after MUSCLE alignment and after elimination of poorly aligned positions and divergent regions using the GBlocks server. Sequences were derived from GenBank or JGI and the according database IDs are shown. OPS: Ophiobolin synthase; FS: Fusicoccadiene Synthase.

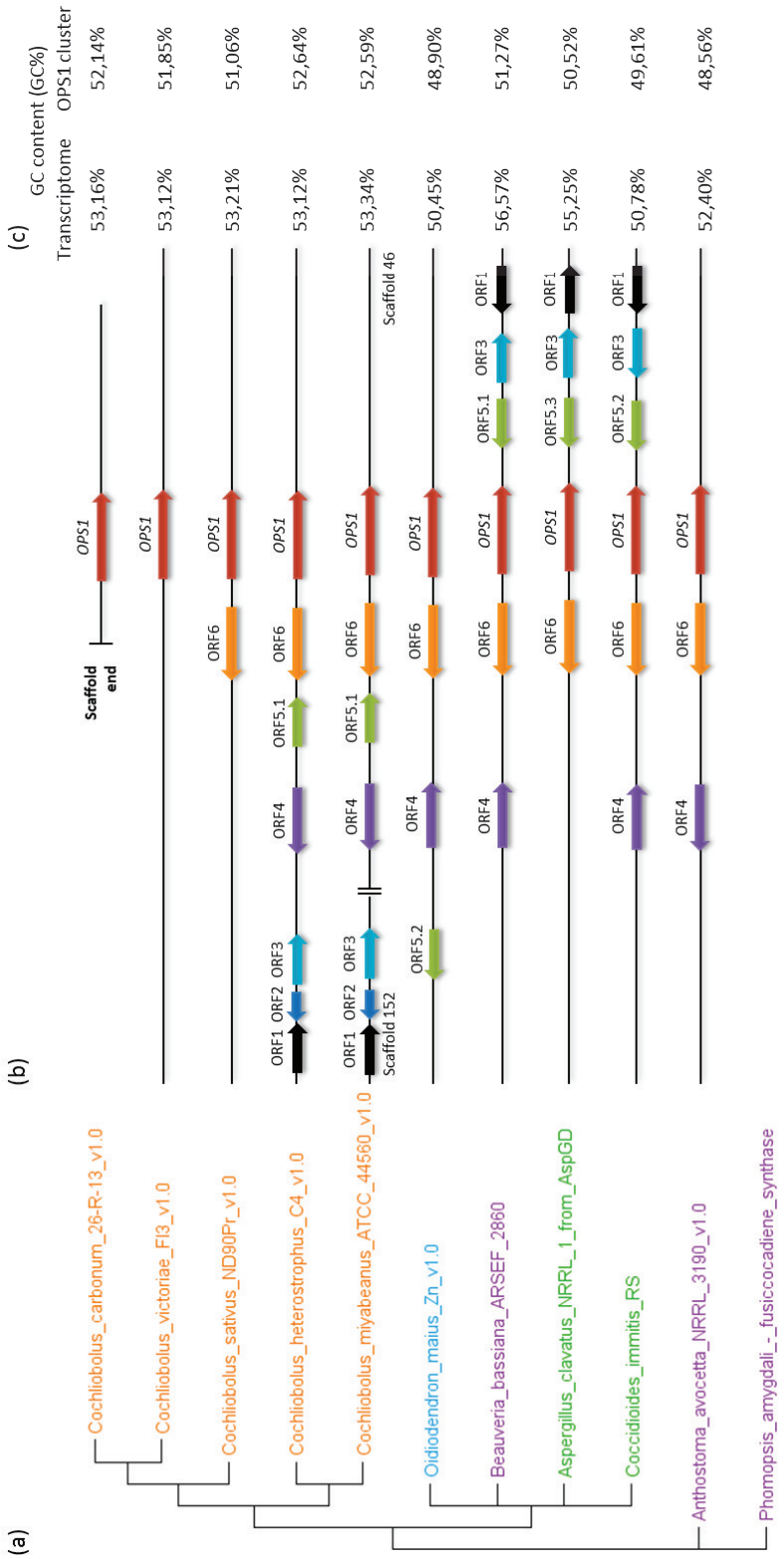


Figure 3.3. Comparison of topology and GC-content of putative ophiobolin biosynthesis gene clusters. (a) The unrooted phylogenetic tree is a bootstrap consensus tree after maximum likelihood analysis (1000 bootstraps, cut-off at 50% bootstrap support). The tree is based on Ops1 protein sequence data, after MUSCLE alignment and after elimination of poorly aligned positions and divergent regions using the GBlocks server. Same colors group species from the same fungal class: Orange: Dothideomycetes Purple: Sordariomycetes, Green: Eurotiomycetes, Blue: Leotiomycetes.**(b)** Visualisation of topology of orthologous *Ops1* gene clusters. Homologous genes have same colors, independent of species colors. **(c)** Comparison of GC-content of the species transcriptome and the *OPS1* gene cluster, respectively.

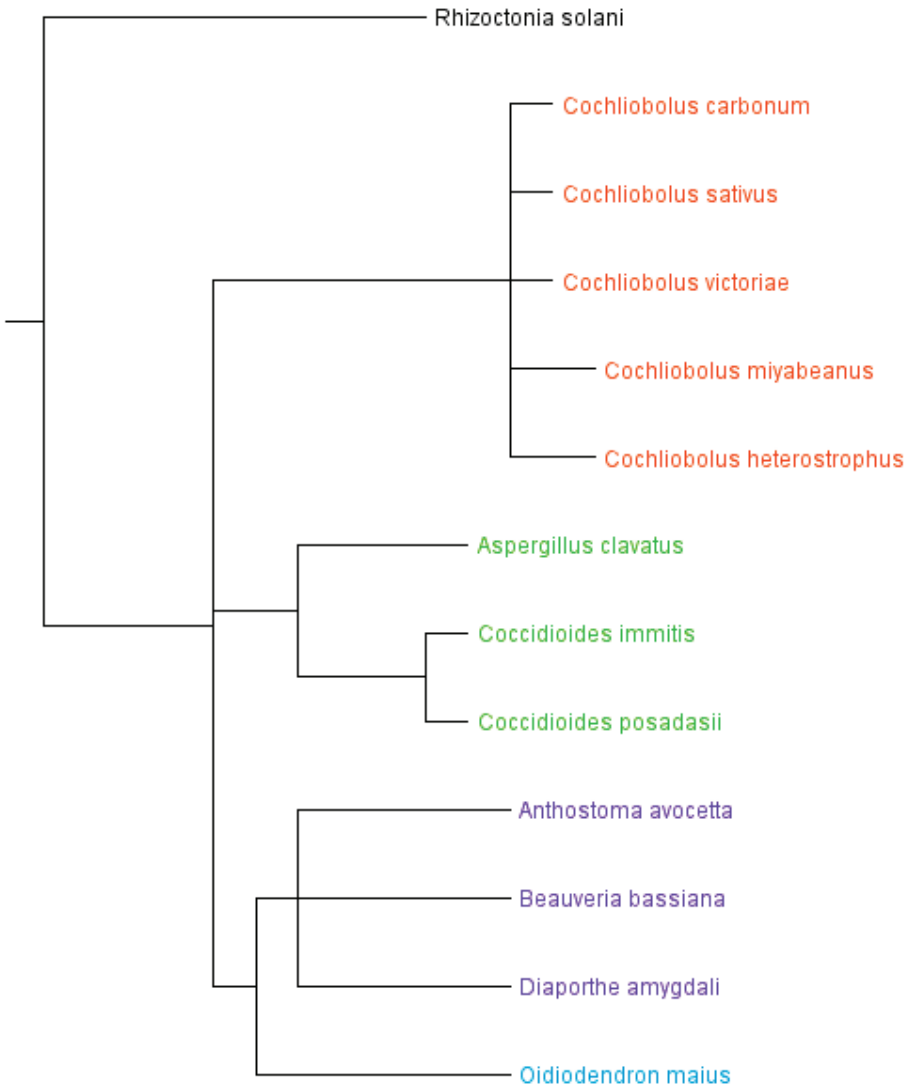
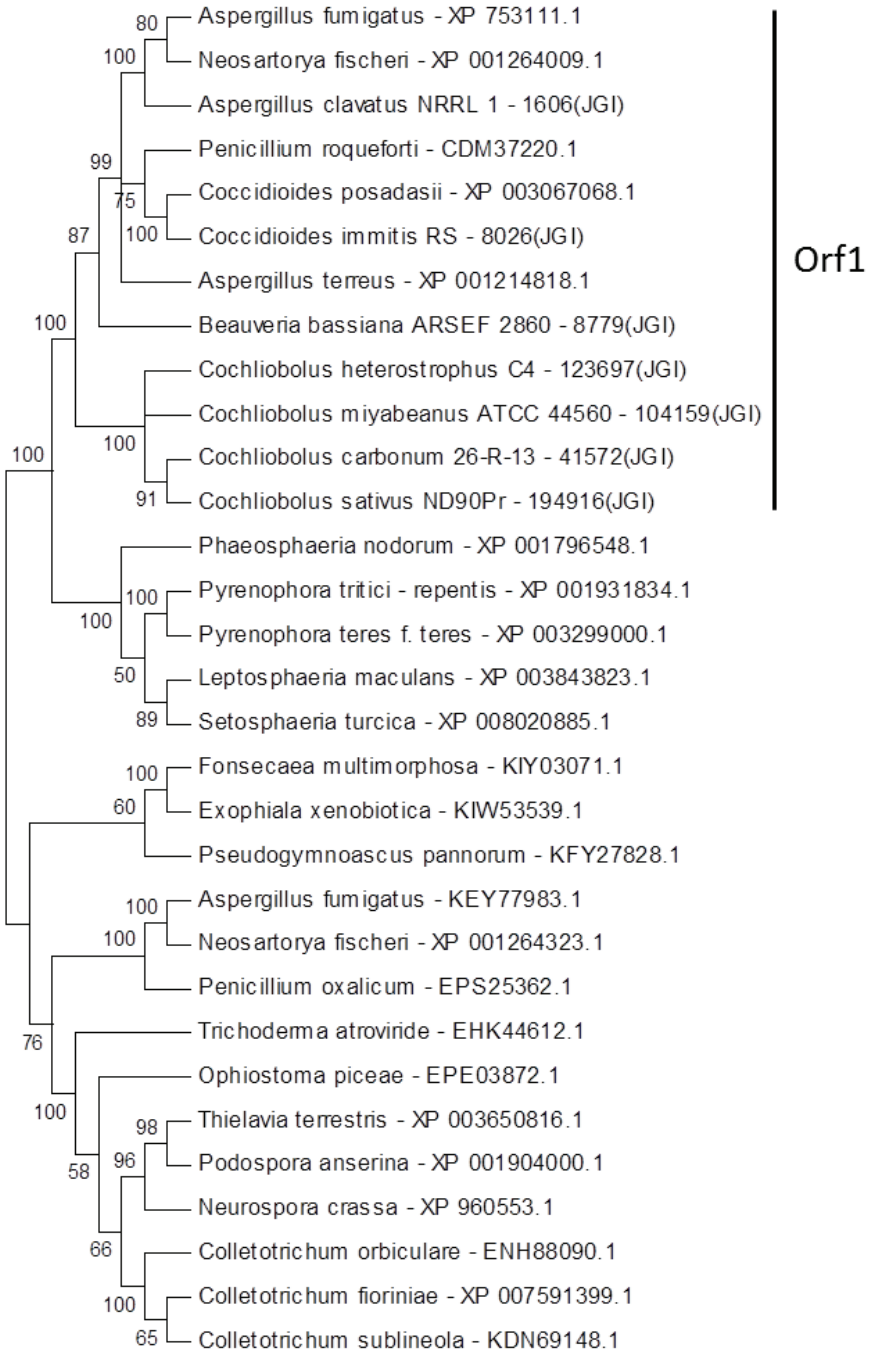


Figure 3.4. Representation of the phylogenetic relationship between species carrying a putative ophiobolin biosynthesis gene cluster. The phylogenetic tree is based on NCBI taxonomy, *Rhizoctonia solani* is used as an outgroup. Orange: Dothideomycetes, Green: Eurotiomycetes, Purple: Sordariomycetes, Blue: Leotiomycetes. The same color code was used in Figure 3.3.

(a)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Neosartorya fischeri - XP 001264009.1
Aspergillus clavatus NRRL 1 - 1606(JGI)
Penicillium roqueforti - CDM37220.1
Coccidioides posadasii - XP 003067068.1
Coccidioides immitis RS - 8026(JGI)
Aspergillus terreus - XP 001214818.1
Beauveria bassiana ARSEF 2860 - 8779(JGI)
Cochliobolus heterostrophus C4 - 123697(JGI)
Cochliobolus miyabeanus ATCC 44560 - 104159(JGI)
Cochliobolus carbonum 26-R-13 - 41572(JGI)
Cochliobolus sativus ND90Pr - 194916(JGI)
Orf1
Phaeosphaeria nodorum - XP 001796548.1
Pyrenophora tritici - repentis - XP 001931834.1
Pyrenophora teres f. teres - XP 003299000.1
Leptosphaeria maculans - XP 003843823.1
Setosphaeria turcica - XP 008020885.1
Fonsecaea multimorphosa - KIY03071.1
Exophiala xenobiotica - KIW53539.1
Pseudogymnoascus pannorum - KFY27828.1
Aspergillus fumigatus - KEY77983.1
Neosartorya fischeri - XP 001264323.1
Penicillium oxalicum - EPS25362.1
Trichoderma atroviride - EHK44612.1
Ophiostoma piceae - EPE03872.1
Thielavia terrestris - XP 003650816.1
Podospora anserina - XP 001904000.1
Neurospora crassa - XP 960553.1
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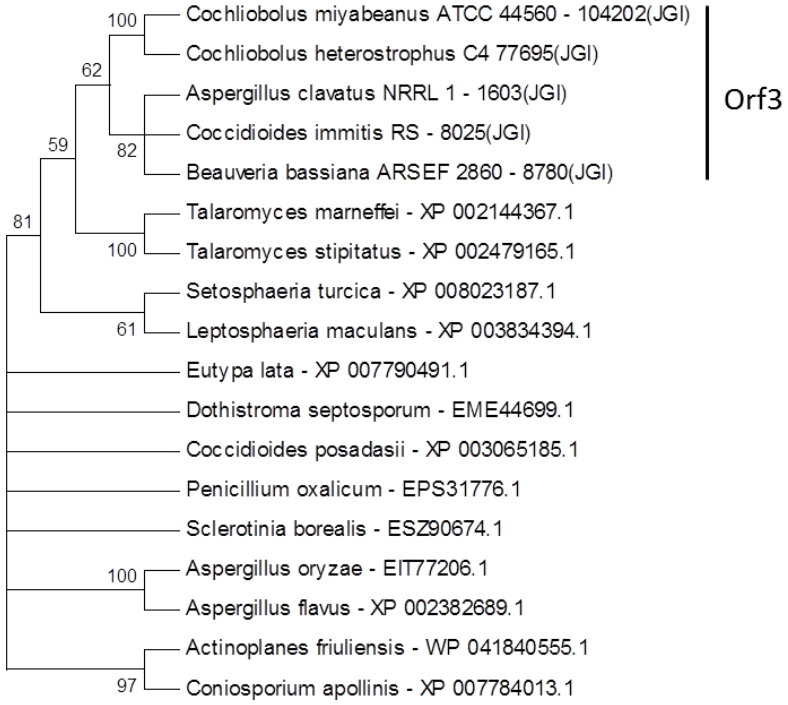
(b)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Beauveria bassiana ARSEF 2860 - 8780(JGI)
Talaromyces marneffei - XP 002144367.1
Talaromyces stipitatus - XP 002479165.1
Setosphaeria turcica - XP 008023187.1
Leptosphaeria maculans - XP 003834394.1
Eutypa lata - XP 007790491.1
Dothistroma septosporum - EME44699.1
Coccidioides posadasii - XP 003065185.1
Penicillium oxalicum - EPS31776.1
Sclerotinia borealis - ESZ90674.1
Aspergillus oryzae - EIT77206.1
Aspergillus flavus - XP 002382689.1
Actinoplanes friuliensis - WP 041840555.1
Coniosporium apollinis - XP 007784013.1
100
62
59
82
81
100
61
100
97
Orf3

(c)


Coccidioides immitis RS - 8021(JGI)
Coccidioides posadasii - XP 003067064.1
Oidiodendron maius Zn - 109798(JGI)
Anthostoma avocetta NRRL 3190 - 523982(JGI)
Cochliobolus miyabeanus ATCC 44560 - 35839(JGI)
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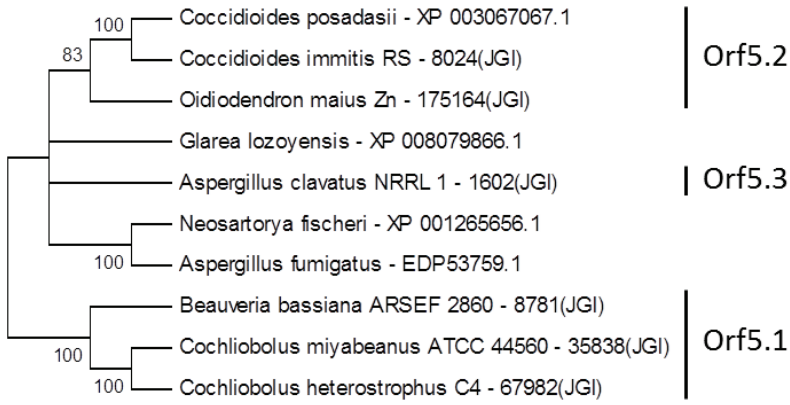
Beauveria bassiana ARSEF 2860 - 8785(JGI)
100
92
54
100
Orf4

(d)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Neosartorya fischeri - XP 001265656.1
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Beauveria bassiana ARSEF 2860 - 8781(JGI)
Cochliobolus miyabeanus ATCC 44560 - 35838(JGI)
Cochliobolus heterostrophus C4 - 67982(JGI)
100
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100
100
Orf5.2
Orf5.3
Orf5.1

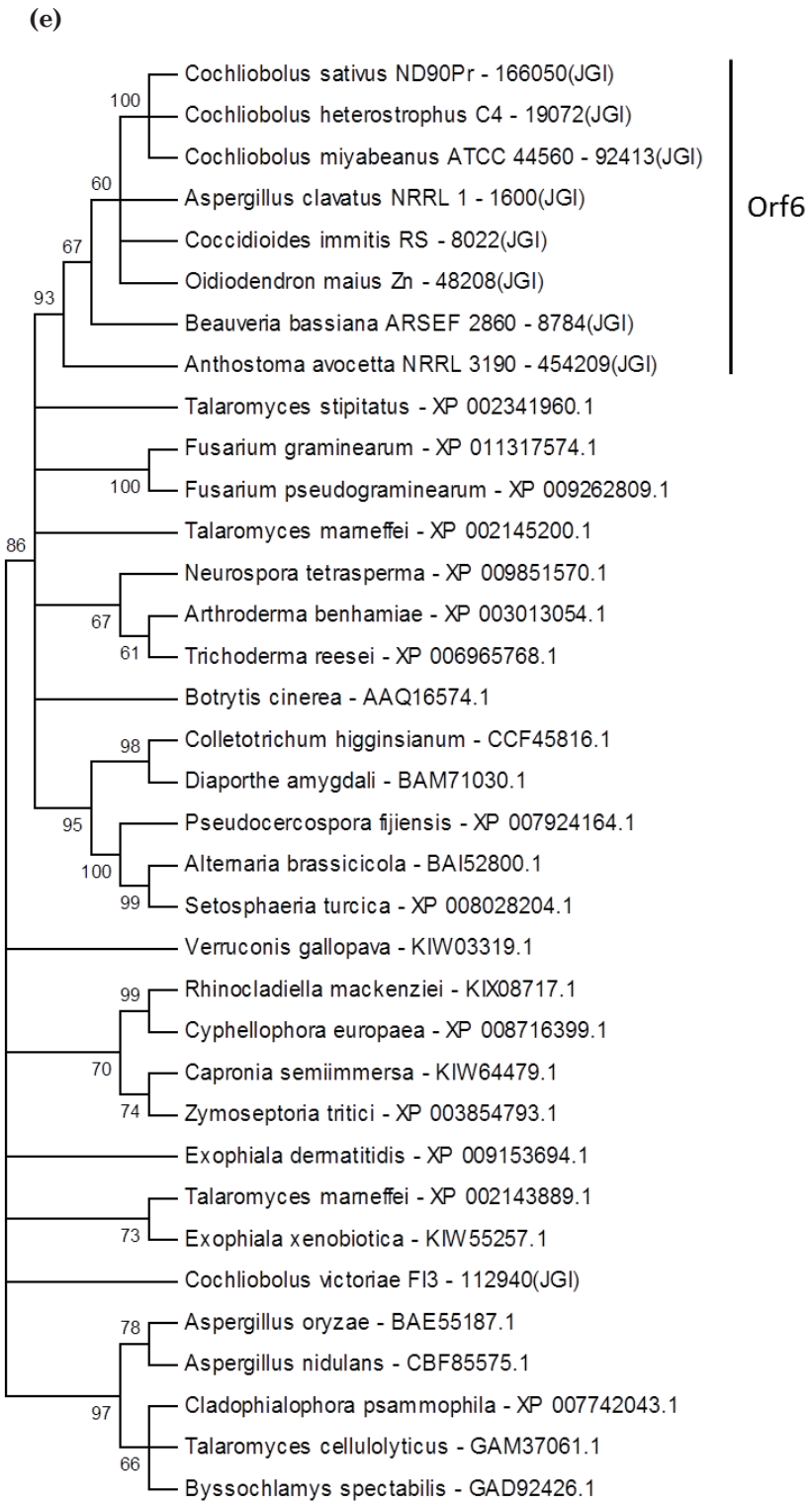

Figure 3.5. Phylogenetic analysis of homologs of Orf1 – Orf6. The unrooted phylogenetic tree represents the bootstrap consensus tree after maximum likelihood analysis (1000 bootstraps, cut-off at 50% bootstrap support). The tree is based on protein sequence data, after MUSCLE alignment and after elimination of poorly aligned positions and divergent regions using the GBlocks server. Sequences were derived from GenBank or JGI and the according database IDs are shown. **(a)** Orf1 homologs **(b)** Orf3 homologs **(c)** Orf4 homologs **(d)** Orf5 homologs **(e)** Orf6 homologs

Evolutionary origin of the gene cluster

Because the *OPS1* gene cluster was found only in *Cochliobolus* spp. and five additional distantly related species and the phylogenetic tree based on Ops1 does not entirely follow the expected tree of life (Figure 3.4), we wondered if any HGT events could have played a role in the evolutionary history. Interestingly, all species featuring the *OPS1* cluster belong to the Pezizomycotina, a subphylum of the ascomycota in which HGT has been shown to be more prevalent (Marcet-Houben & Gabaldón, 2009; Oliver & Solomon, 2010; Wisecaver *et al.*, 2014).

A deviation of the gene cluster GC-content from overall transcriptome GC-content can be a strong indication of horizontal integration of a gene cluster (Campbell *et al.*, 2012). Interestingly, the GC% is very similar in all homologous *OPS1* clusters and ranges from 49% to 53% (Figure 3.3c). Although, this is in line with the transcriptome GC-content for most of the species, the transcriptomes of *B. bassiana* and *A. clavatus* diverge from this rule with respectively 57% and 55% GC-content in their transcriptome, suggesting a different evolutionary rate or a foreign origin of the gene cluster in these species.

The OPS1 cluster is embedded in a region with a very low overall conservation in all species. Although conserved genes can be found in the flanking regions, none of the cluster components have homologs in closely related species (Figure S3.2, S3.3, S3.4, S3.5, S3.6, S3.7 and S3.8), except for Orf1 and Orf5. Orf1 is a putative ABC transporter, and is present in the cluster of *Cochliobolus* spp., *B. bassiana*, *C. immitis* and *A. clavatus*, but also in their close relatives. This suggests that the cluster may have been inherited vertically, leaving the ABC transporter as a relic in related lineages. As mentioned earlier, Orf5 is a special case and clusters into three clades, also reflected in much lower similarity scores than calculated for the other cluster components (Table S3.5). Especially Orf5.3 seems to have evolved at a much faster pace and may have changed functionality. Surprisingly, although *O. maius* and *C. immitis* are distantly related from each other, they feature the same Orf5.2 variant and display a relatively high similarity score.

A. avocetta, *B. bassiana* and *P. amygdali* all belong to the Sordariomycetes. *P. amygdali* does not carry an *OPS1* gene cluster, but features a fusicoccadiene synthase, which might be distantly related to *CmOPS1*. An ancient duplication event could have resulted in both paralogs, of which one was vertically transmitted and retained in a gene cluster in only a few species like *A. avocetta* and *B. bassiana*. The presence of Orf1 in both classes suggests that the parental gene originated in an ancient ancestor of both Sordariomycetes and Dothideomycetes. The loss of the putative ABC transporter in *A. avocetta* could indicate that the cluster has lost its functionality or may have pivoted to the production of a less toxic compound.

A. clavatus and *C. immitis* are closely related species and the presence of a highly similar Ops1 homolog in *C. posadassii* suggest vertical gene transfer within this lineage as well.

Furthermore, the presence of the putative ABC transporter in both species as well as in related species even indicates inheritance from the same ancient ancestor of the Sordariomycetes and Dothideomycetes. However, the diverging GC-content of the gene cluster in *A. clavatus* is enigmatic and could indicate that the cluster may have a different origin than the rest of the transcriptome.

So far all evidence points to vertical gene transfer of the ophiobolin biosynthesis cluster, while *O. maius* seems to offer an exception to the rule. The lack of homologs among other Leotiomycetes and the absence of the ABC transporter suggest the possibility of a foreign origin of the gene cluster. Moreover, the Leotimycetes originated from the Sordariomycetes, but the gene cluster in *O. maius* is much more similar to *A. clavatus* and *C. immitis* than to any other gene cluster. Based on high similarity scores of 85% for Orf4 and Orf6 respectively, and 80% for Ops1 and the occurrence of the Orf5.2 bZIP variant in the *O. maius* gene cluster, we propose that *O. maius* may have horizontally acquired the cluster from a *C. immitis* ancestor. In comparison, the very recent lateral transfer of ToxA in *P. tritici-repensis* resulted in more than 90% similarity with the originating *S. nodorum* cluster (Friesen *et al.*, 2006). The efflux of the secondary metabolite in the absence of an ABC transporter in *O. maius*, could have been taken over by an alternative transporter already present at the side of integration. The upstream flanks of the *O. maius* ophiobolin gene cluster features a gene encoding for a putative major facilitator superfamily (MFS) transporter protein (Figure S3.8).

Conclusion and future perspectives

In conclusion, we characterized a cluster of seven putative proteins involved in the ophiobolin biosynthesis pathway, including a terpene cyclase backbone gene, two specific regulators, a P450 monooxygenase, 2 oxidoreductases and an ABC-type transporter. The specific regulators could provide a clue about the differential expression level of *CmOPS1* during infection and in different field strains. The existence of multiple modifying enzymes can explain the occurrence of multiple analogues within and between ophiobolin producing strains. Finally, the putative ABC transporter (Orf1) is a homolog of a multidrug resistance transporter protein in *C. posadassii* (Figure 3.5a; XP_003067068.1) and is likely involved in the secretion of ophiobolins from fungal cells.

Phylogenetic analysis of the gene cluster shows a patchy distribution among species belonging to the Sordariomycetes, Dothideomycetes, Eurotiomycetes and Leotiomycetes. The most likely explanation for this distribution is vertical gene transfer from a common ancient ancestor of all these fungal classes. Only for *O. maius*, the only known Leothiomycete carrying the cluster, HGT provides a more likely explanation. Although caution should be exercised, as the genome sequences of only 13 Leotiomycetes have been deposited in the JGI database, opposed to 80

Dothideomycetes, 57 Eurotiomycetes and 59 Sordariomycetes. The publication of additional Leothiomycete genomes could provide more insight into the conservation of the ophiobolin cluster among this fungal class.

Moreover, the postulation of a HGT event in this chapter is based on limited evidence and additional proof is certainly needed. Because the GC-content of the overall transcriptome of *O. maius* and *C. immitis* is very similar (Figure 3.3c), divergence of GC-content between the cluster and the transcriptome cannot be used as a criterion in this case. However, codon usage patterns can differ substantially between fungal species and could therefore be used to detect foreign genomic sequences (Oliver & Solomon, 2008; Fitzpatrick, 2012). An advantage of this technique is that it can be applied to a single genome. Additional evidence could be gained from the analysis of different fungal genotypes (Oliver & Solomon, 2008). Therefore, the occurrence and conservation of the *OPS1* cluster in different *O. maius* isolates, preferably with a diverse geographical origin, should be analysed. If the gene cluster has been horizontally acquired, it is likely to be missing from certain strains, although the cluster may have become fixed in the population if the event is ancient and if the cluster provides the fungus with a strong evolutionary benefit (Friesen *et al.*, 2006; Oliver & Solomon, 2008).

HGT has been shown to contribute to the evolution of fungal genomes and metabolic pathways, in addition to other evolutionary processes such as gene duplication, recombination and loss (Rosewich & Kistler, 2000; Friesen *et al.*, 2006; Khaldi *et al.*, 2008; Akagi *et al.*, 2009; Marcet-Houben & Gabaldón, 2009; Oliver & Solomon, 2010; Mehrabi *et al.*, 2011; Campbell *et al.*, 2012; Wisecaver *et al.*, 2014). The horizontal acquisition of gene clusters may allow fungi to adapt to new environments. In the case of phytotoxic gene clusters, fungi can develop a broader host range and could eventually cause new plant diseases (Friesen *et al.*, 2006; Mehrabi *et al.*, 2011). Given the broad bioactivity spectrum of ophiobolins, including antimicrobial activity, it is plausible that *O. maius* has gained an evolutionary advantage over its competitors by acquiring the gene cluster. The selfish cluster theory hypothesizes that horizontal transfer not only provides selective advantage to the receiving pathogen, but also serves the persistence of the cluster during evolution (Walton, 2000). Although even very large clusters, such as the 23 gene sterigmatocystin cluster, can be entirely transferred between fungi (from *Aspergillus nidulans* to *Podospora anserine* in this case) while keeping their functionality (Slot & Rokas, 2011), in some cases genes may be lost from the cluster resulting in novel metabolism (Campbell *et al.*, 2012). For example, the transfer of the bikaverin gene cluster from a *Fusarium* sp. to *Botrytis cinerea* resulted in inactivation of the entire cluster, except for one regulatory gene *bik4*. It is postulated that *bik4* may have become involved in non-bekaverin processes in *B. cinerea* and contributes to novel regulation of metabolism (Campbell *et al.*, 2012). Differential gene loss within a cluster can also happen during fungal evolution (Turgeon *et al.*, 2008; Gabaldon & Koonin, 2013; Wisecaver *et al.*, 2014). Comparison of the *Ops1* orthologous clusters shows clear differences in size. It is therefore likely that functionality of the cluster may differ accordingly.

One cluster in the phylogenetic tree based on Ops1 homologs groups *P. amygdali* fussicoccadiene synthase together with homologs from Sordariomycetes, Eurotiomycetes and Dothideomycetes (Figure 3.3). This suggests that an ancient duplication event resulting in the *OPS1* and *FS* ancestor genes might have happened in an ancestor of those three lineages, very early in the evolution of Pezizomycotina. The occurrence of the ophiobolin gene cluster in species with divergent life styles is easily understood (Table 3.2). Ophiobolins are not only phytotoxic and advantageous for plant pathogens like *C. miyabeanus* and *C. heterostrophus*, but also exhibit antifungal, antibacterial and nematocidal bioactivity and can induce apoptosis in animal cells (Singh *et al.*, 1991; Li *et al.*, 1995; Fujiwara *et al.*, 2000; Krizsán *et al.*, 2010). Therefore the acquisition and maintenance of the *OPS1* gene cluster can provide a selective advantage to a wide range of fungi. The high similarity of the clusters in distantly related lineages indicates that a strong selective pressure keeps the gene cluster in check. Nonetheless, there is no biological evidence for cluster functionality, except for *C. miyabeanus*, *C. heterostrophus* and *A. clavatus*. Chemical analysis should reveal whether the gene clusters generate the same or new metabolites, or have lost functionality.

Organism name	Class	Life style
Cochliobolus miyabeanus	Dothideomycetes	Plant pathogen
Cochliobolus heterostrophus	Dothideomycetes	Plant pathogen
Cochliobolus sativus	Dothideomycetes	Plant pathogen
Cochliobolus victoriae	Dothideomycetes	Plant pathogen
Cochliobolus carbonum	Dothideomycetes	Plant pathogen
Oidiodendron maius	Leotiomycetes	Endomycorhiza on Ericoids
Coccidioides immitis	Eurotiomycetes	Animal pathogen
Aspergillus clavatus	Eurotiomycetes	Occasional human/animal pathogen
Beauveria bassiana	Sordariomycetes	Entomopathogen
Anthostoma avocetta	Sordariomycetes	Associated with trees

Table 3.2. List of species carrying a putative ophiobolin biosynthesis cluster and their respective lifestyles

Although ophiobolin production by plant pathogenic fungi is unfavourable within agricultural systems, the apoptosis-inducing bioactivity in animal cell lines makes it a promising compound in the quest for new cancer treatments (Fujiwara *et al.*, 2000; Bladt *et al.*, 2013; Bury *et al.*, 2013). Knowledge about the biosynthesis gene cluster and transcriptional regulation could translate into efficient metabolic engineering strategies to improve *in vitro* production.

Materials and methods

Prediction of the ophiobolin synthase gene cluster

The putative ophiobolin biosynthesis cluster was discovered by screening the flanking regions of *CmOPS1* in the *Cochliobolus miyabeanus* ATCC 44560 v1 (strain WK1C) genome sequence on the JGI MycoCosm website for open reading frames (ORFs). ORFs were chosen from the gene catalog track, which groups all predicted ORFs based on manual and automatic curation. Functional predictions for each gene were generated by the MycoCosm website, based on the detection of InterPro conserved domains.

Conservation of the cluster among fungal species

All fungal genomes submitted to the JGI MycoCosm database were screened for the presence of ophiobolin gene cluster components. First, a BlastP search was performed with CmOps1 as a template. Species with homologs featuring 60% amino acid identity, score >2000 and E-value = 0 were withheld. The active domains of the CmOps1 homologs were aligned to compare conservation of key positions using ClustalW in BioEdit v7.1.9. Subsequently the genomes of each of the selected species were screened for additional cluster components in the near vicinity of the *CmOPS1* homolog using BlastP with the putative cluster proteins from the *OPS1* gene cluster in *C. miyabeanus* as template. Genes were considered clustered if they were less than 7 genes apart on a scaffold.

Phylogenetic analysis

Additional homologs of each of the putative cluster components were retrieved from GenBank by BlastP. Proteins with an E-value < $1E10^{-4}$, > 70% query coverage and > 40% similarity were selected and aligned using MUSCLE (Multiple Sequence Alignment) (http://www.ebi.ac.uk/Tools/msa/muscle/). The aligned sequence was subsequently trimmed using the Gblocks server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) with at least 30% of the original positions remaining. Maximum likelihood analysis was performed on the trimmed alignment using Mega (version 6.06), with 1000 bootstrap replications and using the Jones-Taylor-Thornton (JTT) model for amino acid substitutions. The optimal tree was determined based on the Nearest-Neighbor-Interchange (NNI) heuristic method. A consensus tree was made based on at least 50% bootstrap support.

Determination of protein sequence similarities (supplementary tables)

Protein sequence similarities were calculated for MUSCLE aligned protein sequences using the SIAS server (http://imed.med.ucm.es/Tools/sias.html).

Supplementary figures

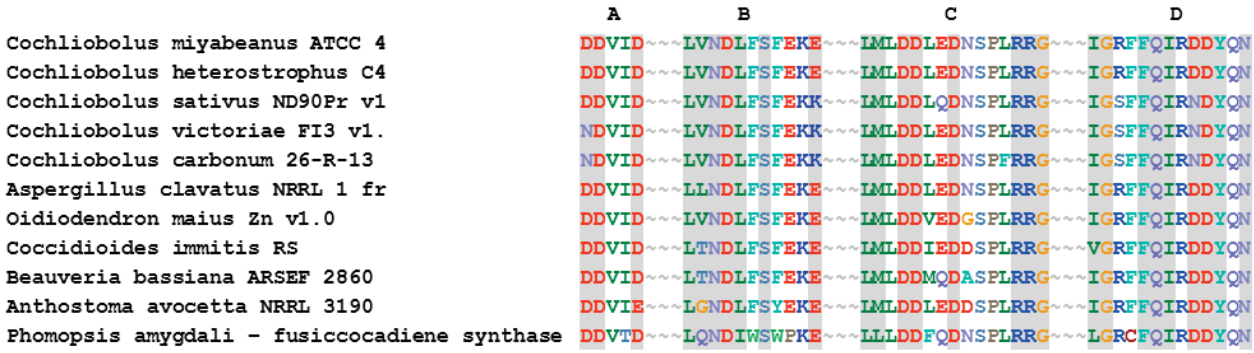

Figure S3.1. Conservation of active domains in putative Ops1 homologs. Shaded positions mark key positions in the motifs A-D.

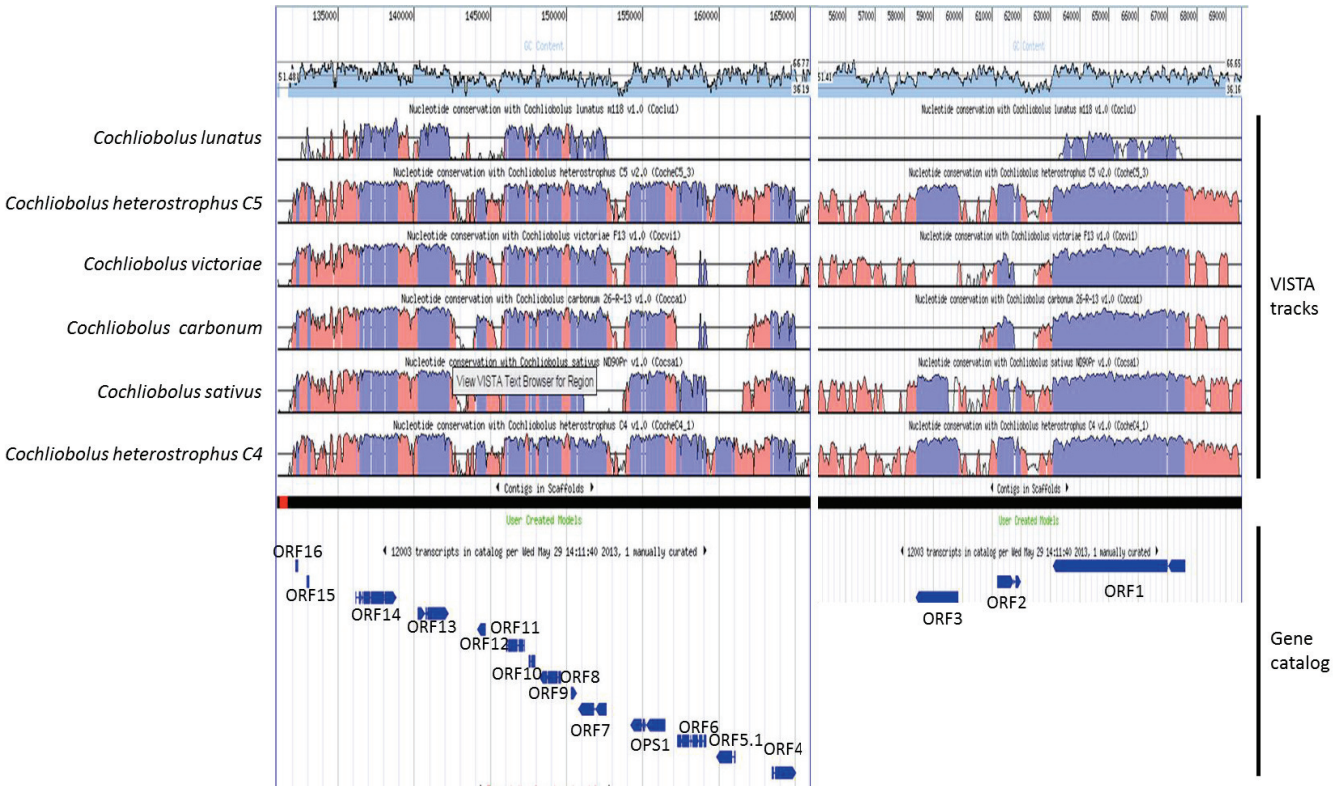

Figure S3.2. Conservation of the genomic region harbouring the *OPS1* gene cluster in *Cochliobolus miyabeanus* strain WK1C. Left side of the picture: Scaffold 46: 131000-166095, right side of the picture: Scaffold 152: 69543-55000 (reverse strand). The VISTA tracks illustrate the similarity of the reference genome with the corresponding region in closely related species (Frazer *et al.*, 2004; Grigoriev *et al.*, 2014). Blue: coding sequence, Pink: non-coding sequence. The Gene Catalog features predicted genes (Grigoriev *et al.*, 2014).

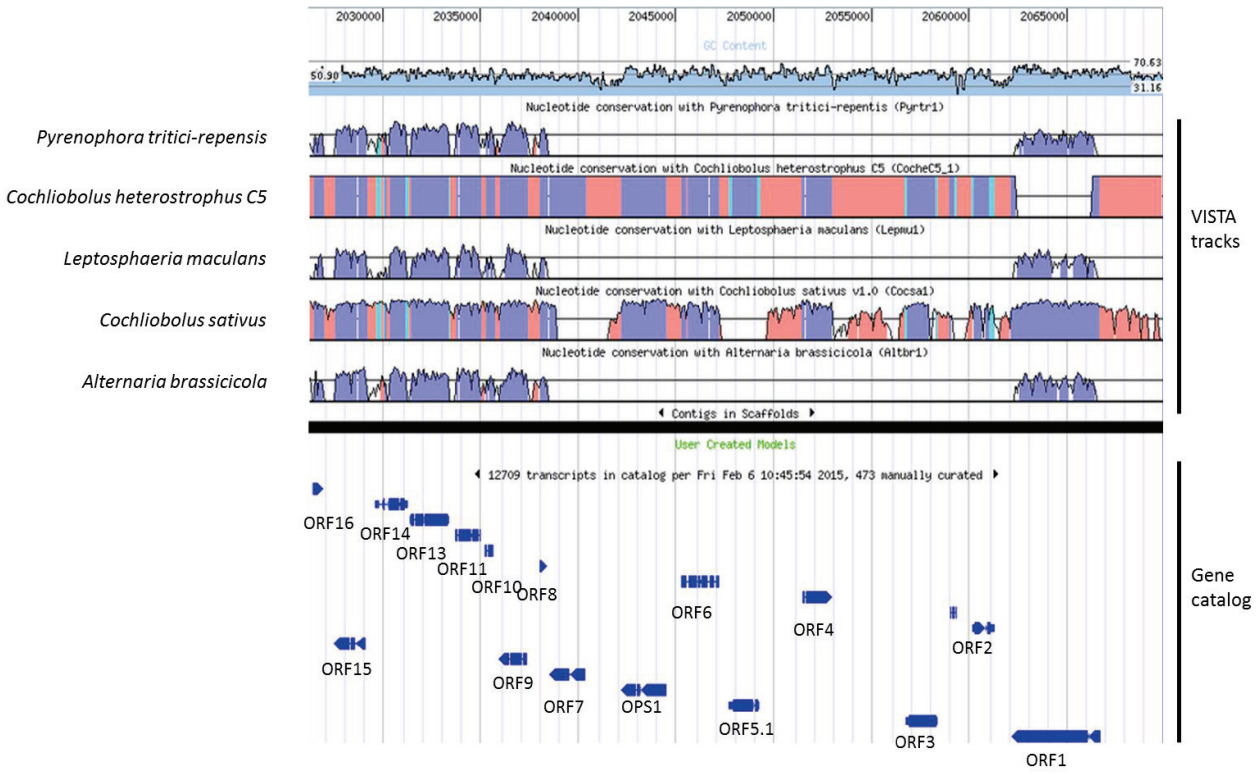

Figure S3.3. Conservation of the genomic region harbouring the *OPS1* gene cluster in *Cochliobolus heterostrophus* strain C4 scaffold 1:2026200-2069960. The VISTA tracks illustrate the similarity of the reference genome with the corresponding region in closely related species (Frazer *et al.*, 2004; Grigoriev *et al.*, 2014). Blue: coding sequence, Pink: non-coding sequence. The Gene Catalog features predicted genes (Grigoriev *et al.*, 2014).

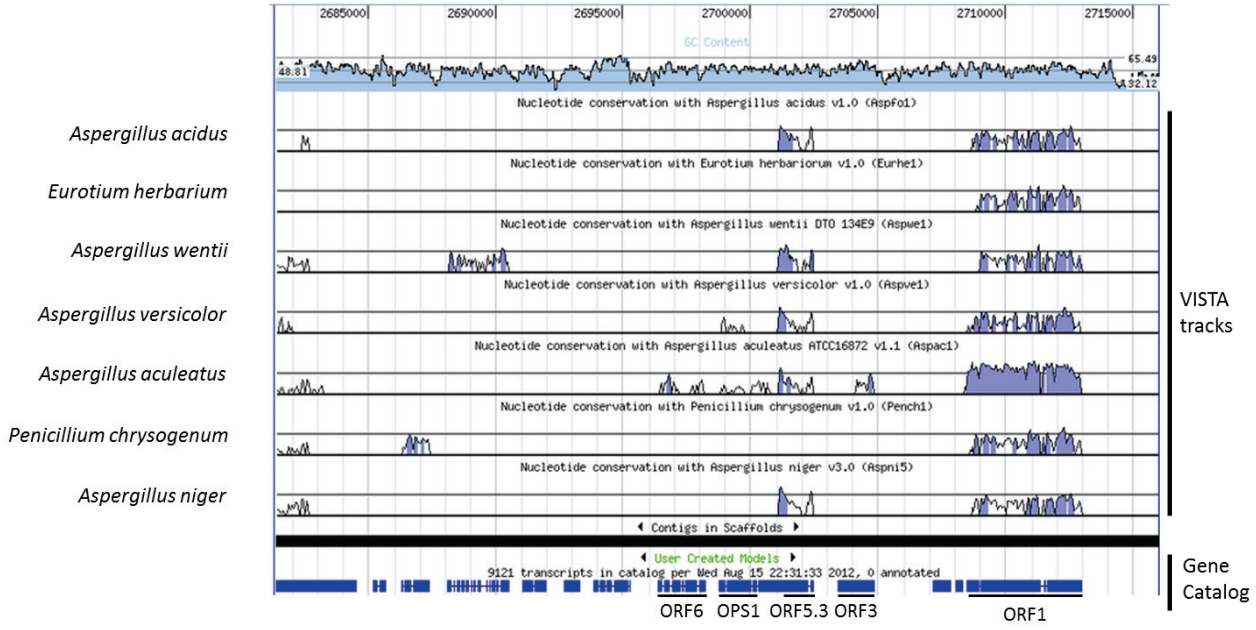

Figure S3.4. Conservation of the genomic region harbouring the *OPS1* gene cluster in *Aspergillus clavatus* NRRL 1 v1.0 scaffold 1099423829791:2681391-2716063. The VISTA tracks illustrate the similarity of the reference genome with the corresponding region in closely related species (Frazer *et al.*, 2004; Grigoriev *et al.*, 2014). Blue: coding sequence, Pink: non-coding sequence. The Gene Catalog track features predicted genes (Grigoriev *et al.*, 2014).

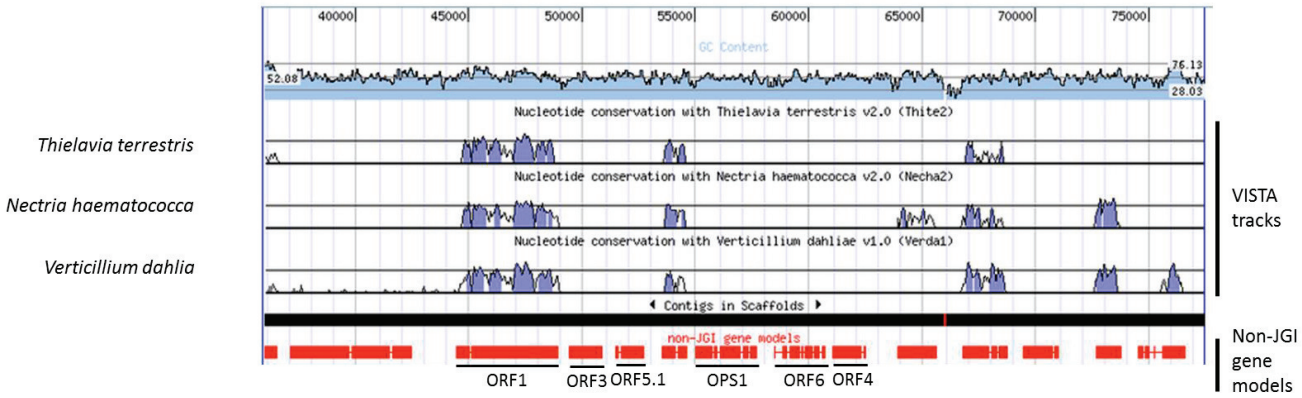

Figure S3.5. Conservation of the genomic region harbouring the *OPS1* gene cluster in *Beauveria bassiana* scaffold_00040:36000-77500. The VISTA tracks illustrate the similarity of the reference genome with the corresponding region in closely related species (Frazer *et al.*, 2004; Grigoriev *et al.*, 2014). Blue: coding sequence, Pink: non-coding sequence. The Non-JGI gene models track represents a non-redundant set of genes predicted by different automated gene-finding methods.

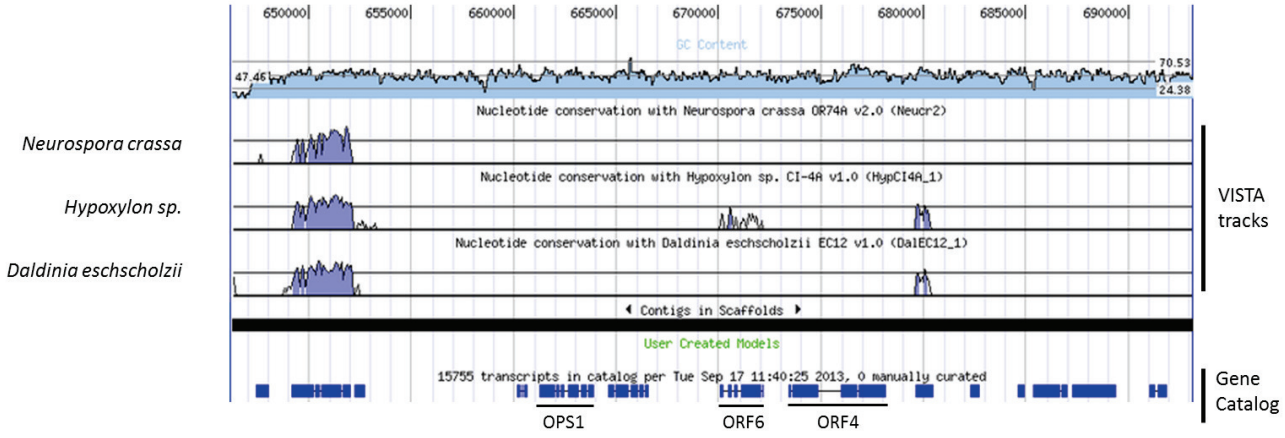

Figure S3.6. Conservation of the genomic region harbouring the *OPS1* gene cluster in *Anthostoma avocetta* scaffold_26:646267-693182. The VISTA tracks illustrate the similarity of the reference genome with the corresponding region in closely related species (Frazer *et al.*, 2004; Grigoriev *et al.*, 2014). Blue: coding sequence, Pink: non-coding sequence. The Gene Catalog track features predicted genes (Grigoriev *et al.*, 2014).

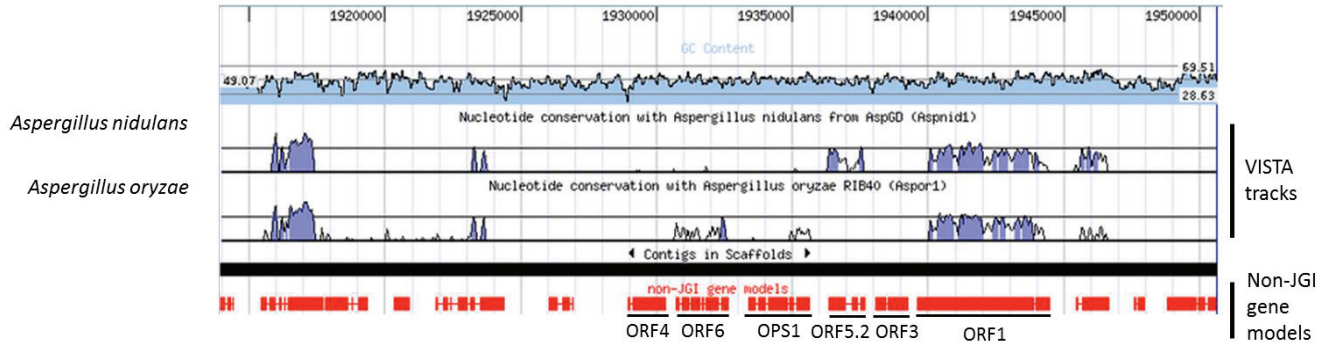

Figure S3.7. Conservation of the genomic region harbouring the *OPS1* gene cluster in *Coccidioides immitis* Supercontig_5:1913936-1950654. The VISTA tracks illustrate the similarity of the reference genome with the corresponding region in closely related species

(Frazer *et al.*, 2004; Grigoriev *et al.*, 2014). Blue: coding sequence, Pink: non-coding sequence. The Non-JGI gene models track represents a non-redundant set of genes predicted by different automated gene-finding methods.

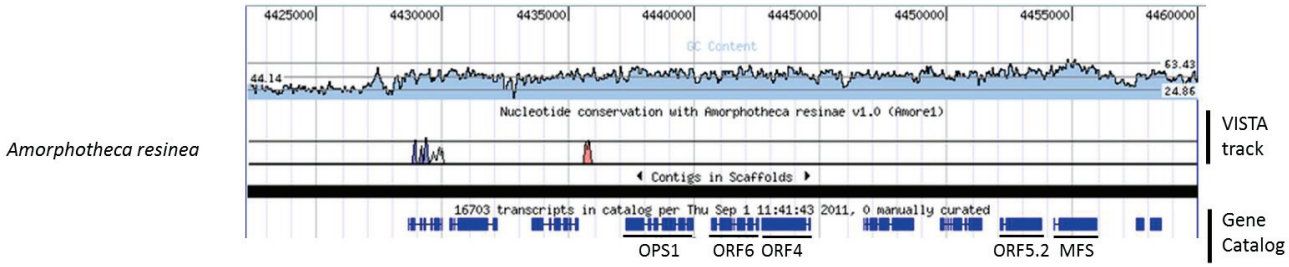

Figure S3.8. Conservation of the genomic region harbouring the *OPS1* gene cluster in *Oidiodendron maius* scaffold_1:4422280-4460000. The VISTA track illustrates the similarity of the reference genome with the corresponding region in *Amorphoteca resinea* (Frazer *et al.*, 2004; Grigoriev *et al.*, 2014). Blue: coding sequence, Pink: non-coding sequence. The Non-JGI gene models track represents a non-redundant set of genes predicted by different automated gene-finding methods

Supplementary tables

	C. carbonum	*C. victoriae*	*C. sativus*	*C. hetero-strophus*	*C. miya - beanus*	*P. amygdali*	*B. bassiana*	*A. avocetta*	*A. clavatus*	*C. immitis*	*O. maius*
C. carbonum	100.0										
C. victoriae	99.2	100.0									
C. sativus	93.2	93.4	100.0								
C. hetero-strophus	94.1	94.0	96.5	100.0							
C. miya - beanus	94.3	94.3	96.7	99.3	100.0						
P. amygdali	55.9	56.1	57.1	57.8	58.0	100.0					
B. bassiana	60.2	60.1	61.8	62.4	62.2	46.7	100.0				
A. avocetta	71.5	71.5	72.6	74.3	74.2	60.9	61.3	100.0			
A. clavatus	75.0	74.8	76.5	77.7	77.7	56.9	61.3	64.7	100.0		
C. immitis	67.2	67.1	68.4	69.4	69.2	53.2	56.7	60.2	75.2	100.0	
O. maius	76.4	76.1	77.7	78.5	78.6	57.8	61.6	68.0	80.9	79.5	100.0

Table S3.1. Percentage protein sequence similarities between Ops1 homologs. Grey areas illustrate similarities of less than 60%. *P. amygdali*: Fusicoccadiene synthase protein sequence.

C. miyabeanus	100				
C. heterostrophus	98.01	100			
B. bassiana	80.14	79.81	100		
C. immitis	83.38	83.18	83.52	100	
A. clavatus	82.72	82.19	82.66	89.41	100
	C. miyabeanus	***C. heterostrophus***	***B. bassiana***	***C. immitis***	***A. clavatus***

Table S3.2. Percentage protein sequence similarities between Orf1 homologs

C. heterostrophus	100.0				
C. miyabeanus	96.1	100.0			
B. bassiana	66.9	65.6	100.0		
A. clavatus	67.1	67.3	73.9	100.0	
C. immitis	56.6	56.4	63.8	61.9	100.0
	C. heterostrophus	***C. miyabeanus***	***B. bassiana***	***A. clavatus***	***C. immitis***

Table S3.3. Percentage protein sequence similarities between Orf3 homologs. Grey areas illustrate similarities of less than 60%.

C. miyabeanus	100.0				
C. heterostrophus	96.3	100.0			
B. bassiana	74.0	75.9	100.0		
C. immitis	77.6	78.5	78.5	100.0	
O. maius	77.0	78.5	78.5	84.9	100.0
	C. miyabeanus	***C. heterostrophus***	***B. bassiana***	***C. immitis***	***O. maius***

Table S3.4. Percentage protein sequence similarities between Orf4 homologs

C. miyabeanus Orf5.1	100.00					
C. heterostrophus Orf5.1	94.6	100.0				
B. bassiana Orf5.1	57.6	58.0	100.0			
A. clavatus Orf5.3	48.1	48.1	45.2	100.0		
C. immitis Orf5.2	55.0	55.2	50.9	56.3	100.0	
O. maius Orf5.2	56.3	56.5	53.2	59.3	72.5	100.0
	C. miyabeanus Orf5.1	***C. heterostrophus Orf5.1***	***B. bassiana Orf5.1***	***A. clavatus Orf5.3***	***C. immitis Orf5.2***	***O. maius Orf5.2***

Table S3.5. Percentage protein sequence similarities between Orf5 homologs. Grey areas illustrate similarities of less than 60%.

C. miyabeanus	100.0						
C. heterostrophus	99.5	100.0					
B. bassiana	76.8	77.2	100.0				
A. avocetta	75.1	75.3	74.8	100.0			
A. clavatus	80.6	80.7	79.7	76.7	100.0		
C. immitis	79.2	79.4	77.3	78.4	81.9	100.0	
O. maius	80.7	80.9	81.4	79.0	84.7	85.0	100.0
	C. miyabeanus	***C. heterostrophus***	***B. bassiana***	***A. avocetta***	***A. clavatus***	***C. immitis***	***O. maius***

Table S3.6. Percentage protein sequence similarities between Orf6 homologs

Chapter 4

Comparative chemical screening and genetic analysis reveal tentoxin as a new virulence factor in *Cochliobolus miyabeanus*

Authors

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Summary

Apart from ophiobolin A and NPS6, no virulence factors have been described for *C. miyabeanus*. Therefore we set out to identify new compounds with a role in disease development. In this chapter we describe for the first time the production of tentoxin by *C. miyabeanus* as a virulence factor during brown spot disease. We compared chemical compounds produced by *C. miyabeanus* strains differing in virulence ability using ultra-high performance liquid chromatography (UHPLC) coupled to a high resolution Orbitrap mass spectrometry (HRMS). The production of tentoxin by a highly virulent strain was revealed by principal component analysis of the detected ions and confirmed by UHPLC coupled to tandem-quadrupole mass spectrometry (MS/MS). The corresponding NRPS, CmNps3, was identified by *in silico* genome analysis and confirmed by gene deletion. Infection tests with wild type and *Cmnps3* mutants showed that tentoxin acts as a virulence factor and is correlated with chlorosis development during the second phase of infection. Although rice has previously been classified as a tentoxin-insensitive species, our data demonstrate that tentoxin-production by *C. miyabeanus* affects symptom development.

Author contributions

Lieselotte De Bruyne: *Study conception and design, genome analysis, fungal transformations, data acquisition in all experiments, data analysis and interpretation, writing of the chapter.*

Christof Van Poucke**:** *Analysis of UHPLC-Orbitrap-MS data, Assistance with UPLC-MS/MS analysis, critical revision of the chapter*

Jose Diana Di Mavungu: *Development of the UHPLC-Orbitrap-MS method, Critical revision of the chapter*

Nur Ain Izzati Mohd Zainudin: *Transformation of* C. miyabeanus *strain WK1C, Critical revision of the chapter*

Lynn Van Haecke, David De Vleesschauwer, B. Gillian Turgeon, Sarah De Saeger and Monica Höfte: *Critical revision of the chapter*

Introduction

Cochliobolus miyabeanus is a filamentous ascomycete belonging to the Pleosporales order of the class Dothideomycetes. Many species belonging to this order are notorious for the production of phytotoxins (Yoder, 1980; Panaccione, 1993; Wolpert *et al.*, 2002; Condon *et al.*, 2013; Stergiopoulos *et al.*, 2013). Phytotoxins can either be host-selective (HST) or non-selective (non-HST) and are mainly small secondary metabolites like terpenes, nonribosomal peptides, polyketides, alkaloids or metabolites of mixed origin that in most cases lead to the induction of cell death. HSTs often act as pathogenicity determinants and determine the host range of a pathogen (Berestetskiy, 2008; Stergiopoulos *et al.*, 2013). Although the existence of a *C. miyabeanus* HST has been proposed, such a compound has never been characterized (Vidhyasekaran *et al.*, 1986). *C. miyabeanus* seems to rely on an array of non-selective virulence factors, which is reflected in its host range (Chakrabarti, 2001).

C. miyabeanus produces multiple ophiobolins, a family of sesterterpenoid non-host specific phytotoxins (Orsenigo, 1957; Nakamura & Ishibashi, 1958; Yun *et al.*, 1988; Xiao *et al.*, 1991). The highly bioactive analogue ophiobolin A contributes significantly and quantitatively to disease symptoms caused by *C. miyabeanus*, as shown in Chapter 2. Besides, NPS6, a NRPS involved in the biosynthesis of extracellular siderophores, acts as a virulence factor of *C. miyabeanus*. Deletion of *NPS6* results in reduced virulence and hypersensitivity to H_2O_2 (Oide *et al.*, 2006). Although so far no additional virulence factors have been characterized for *C. miyabeanus*, their existence is suggested by the remaining level of disease caused by ophiobolin-deficient mutants. This idea is also supported by the identification of eleven non-ribosomal peptide synthetase (NPS) and 21 polyketide synthase (PKS) genes in the *C. miyabeanus* genome, although none are unique for the species (Condon *et al.*, 2013). Small peptides and polyketides produced via multimodular megasynthases (NRPSs and PKSs respectively) comprise a group of secondary metabolites with diverse bioactivity, including phytotoxicity (Bushley & Turgeon, 2010; Condon *et al.*, 2013; Stergiopoulos *et al.*, 2013). They are good candidates for putative virulence factors.

In an attempt to identify unknown virulence factors produced by *C. miyabeanus* during brown spot disease, we compared chemical compounds produced by a highly virulent strain with less virulent strains. Hereto we used ultra-high performance liquid chromatography, coupled to a high resolution Orbitrap Mass spectrometry (UHPLC-Orbitrap HRMS). A compound characteristic for the most virulent strain was selected and analysed for the involvement in disease development. Additionally, a genetic analysis was performed to identify genes involved in the biosynthesis of the selected compound.

Results

UHPLC-Orbitrap-HRMS analysis of liquid culture filtrates

We compared metabolites produced by four *C. miyabeanus* strains with variable virulence abilities in a chemical screening. The highly virulent strain Cm988 and the less virulent strains WK1C and G513 were grown in liquid Fries medium, known to stimulate toxin production *in vitro*. Non-inoculated medium was used as a control, to distinguish between medium components and fungal secretions. Metabolites were extracted from the crude culture filtrate using acetonitrile:ethylacetate in an attempt to collect as many compounds as possible. The extracted metabolites were screened using liquid chromatography coupled to Orbitrap mass spectrometry (UHPLC-Orbitrap-HRMS) in the positive (ESI^+) and negative (ESI^-) electrospray ionisation mode. Different metabolites were separated into frames based on retention times and accurate mass data generated by Orbitrap-HRMS, using Sieve™ software. The 5000 frames with the highest detection level in ESI^+ mode were selected for analysis. A principal component analysis (PCA) analysis was performed on the integrated intensities of the selected compounds using SPSS Statistics 21 software to identify compounds that correlate with the virulence level of the investigated strains.

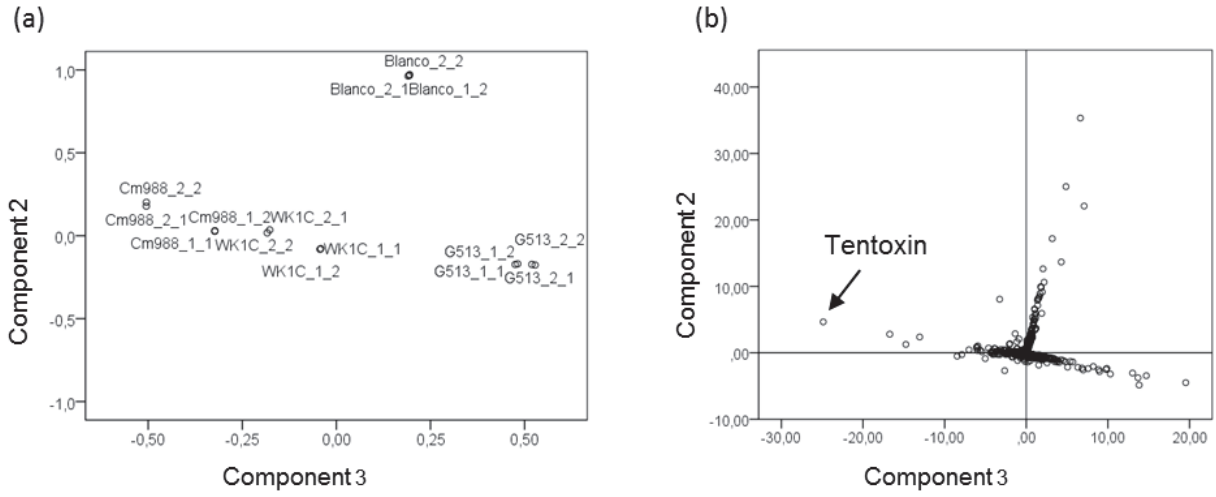

Figure 4.1. Score (a) and loading (b) plots representing how 5000 ions detected in a liquid culture filtrate correlate with different *Cochliobolus miyabeanus* strains after separation according to principal components 2 and 3. The highly virulent strain Cm988, and the less virulent strains WK1C and G513 were grown in liquid Fries medium. The acetonitrile:ethylacetate extract of the filter sterilized liquid culture filtrate was analysed using UHPLC-Orbitrap-HRMS. The 5000 ions with the highest integrated peak intensities were selected and analysed using principal component analysis. The principal components are based on the ions that represent the highest variance between samples from different *Cochliobolus miyabeanus* strains.The ion corresponding to tentoxin is characteristic for the highly virulent strain Cm988.

The first three calculated principal components could explain 92% of the total variance in the dataset (Table S4.1). PC1 and PC2 can divide well between control and fungal samples, making a separation between medium compounds and metabolites. PC3 can distinguish between strains differing in virulence level and could provide insight in possible virulence factors. A combination of PC2 and PC3 gave the best separation of the different strains, according to virulence level (Figure S4.1). One ion correlated strongly with PC3 and was characteristic for the most virulent strain (Figure 4.1).

This compound was selected as a candidate virulence factor and further analysed. The compound eluted at 14.12 min and the Orbitrap measured a m/z of 415.2329 for the precursor ion generated in ESI^+ mode, corresponding to a chemical compound with a MW of 414.50 and a monoisotopic mass of 414.2267. Based on the Orbitrap accurate mass data, an elemental composition of $C_{22}H_{30}N_4O_4$ was proposed with a mass error Δppm of 2.68. A second minor peak with the same mass characteristics and a Δppm = 1.65 eluted 1.27 min earlier (at 12.85 min). A KEGG database search via the Chemspider website (www.chemspider.be) based on the elemental composition returned tentoxin (C08441) as the only known metabolite in the KEGG metabolic database with the same elemental composition (Table 1.1). The compound was also detected by UHPLC-Orbitrap-HRMS analysis in ESI^- mode (data not shown).

Confirmation of tentoxin based on UPLC-MS/MS analysis

To confirm the identity of the selected compound, we compared the retention time and fragmentation pattern to that of a commercial tentoxin reference standard using a UPLC-MS/MS system. The retention time and multiple reaction monitoring (MRM) spectra were indistinguishable. Both compounds had a retention time of 12.79 min and precursor ion of m/z 413.3 in negative mode. The difference of 1.33 min in retention time compared to the peak in the UHPLC-Orbitrap system (14.12 min) is due to a different configuration of the UPLC system.

Tentoxin was fragmented into the deprotonated dipeptides $[M-H]^-$ Leu-Me(Δ)Phe and $[M-H]^-$ Gly-MeAla with m/z 271.2 and m/z 141.0 respectively (Liu & Rychlik, 2013). Hence we used MRM mode with ion transitions 413.3>271.2 and 413.3>141.0 in ESI^- mode to analyse our samples. The m/z 413.3 $[M-H]^-$ ion in the sample produced the same fragment ions as tentoxin and their integrated peak intensity ratio ([m/z 271.2] / [m/z 141.0]) was 0.5 for both the standard and the sample (Figure 4.2). Similar to our observations in the UHPLC-Orbitrap-HRMS system, a second smaller peak with the same chemical characteristics was detected at 11.57 min, 1.22 min before the main peak. We believe that this peak might represent the tentoxin isomer, isotentoxin (Liebermann *et al.*, 1996; Horiuchi *et al.*, 2003).

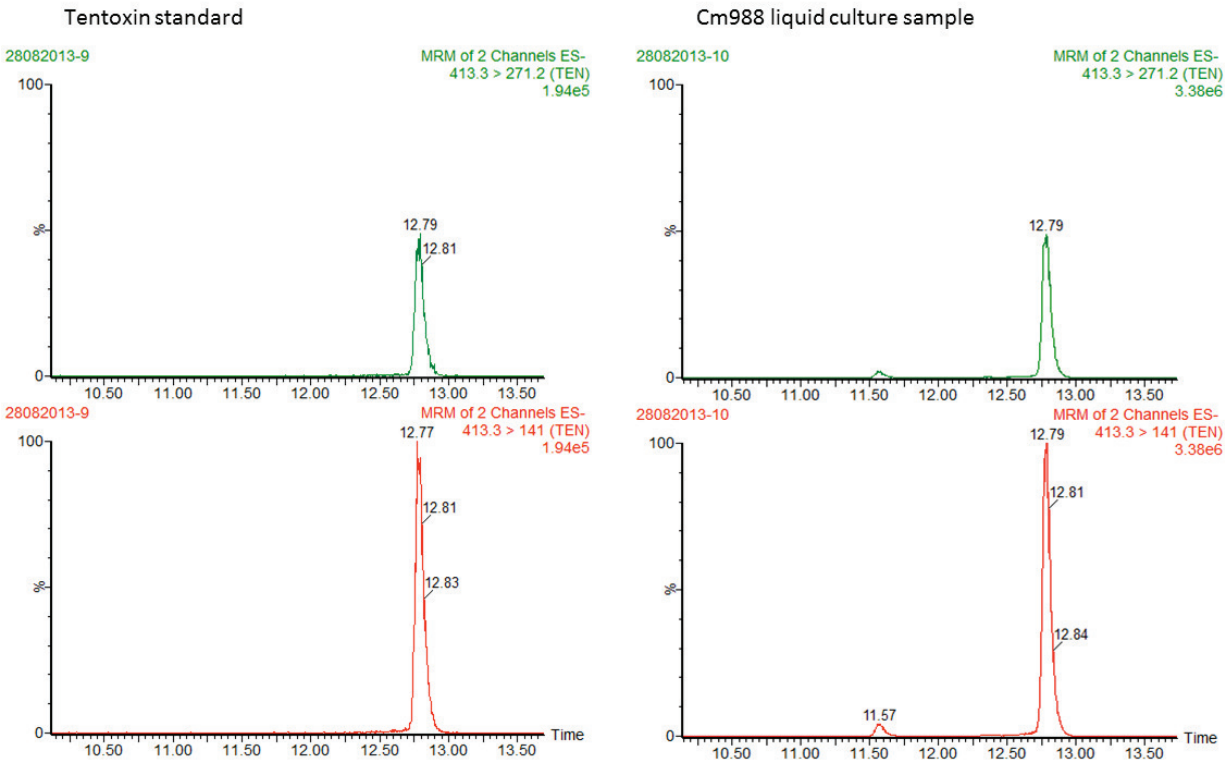

Figure 4.2. Verification of the identity of the selected compound from *Cochliobolus miyabeanus* based on retention time and the peak intensity ratio of two MRM ion transitions characteristic for tentoxin: m/z 413.3 > 271.2 and m/z 413.3 > 141.0. The left side of the figure shows the peaks by a pure tentoxin standard, the right side of the picture shows the compound detected in a liquid culture extract of Cm988, grown in liquid Fries medium for seven days.

Tentoxin synthase gene identification and deletion.

Tentoxin is a cyclic tetrapeptide with structure cyclo(Gly-L-MeAla-L-Leu-MePhe[(Z)Δ]) and is produced by a non-ribosomal peptide synthetase (NRPS) (Table 1.1). The corresponding NRPS has not been identified before, but based on the peptide structure is probably constructed of four modules, featuring two methylation domains. The genome of *C. miyabeanus* features eleven NRPS encoding genes (Condon *et al.*, 2013). The web based application antiSMASH was used to identify the different modules in the predicted NRPS genes.

We used the NRPSPredictor2 for fungal sequences to predict the likely physico-chemical properties of the monomers of the resulting NRP based on a support vector machine (SVM) method and compared them to the Stachelhaus code. The *C. miyabeanus* NPS3 homologue n8391 from *C. miyabeanus* strain WK1C (designated CmNps3) with JGI protein ID 98843 met the structural requirements (Figure 4.3). CmNps3 is 5158 amino acids long, corresponding to a 574.7 kDa protein. The encoding gene is 15.477 kb long and does not feature alternative splicing sites. Of all NRPS-encoding genes detected in the *C. miyabeanus* genome, *CmNPS3* is the most likely candidate involved in tentoxin production, although the monomer predictions were not completely consistent with the known tentoxin structure (Table 4.1).

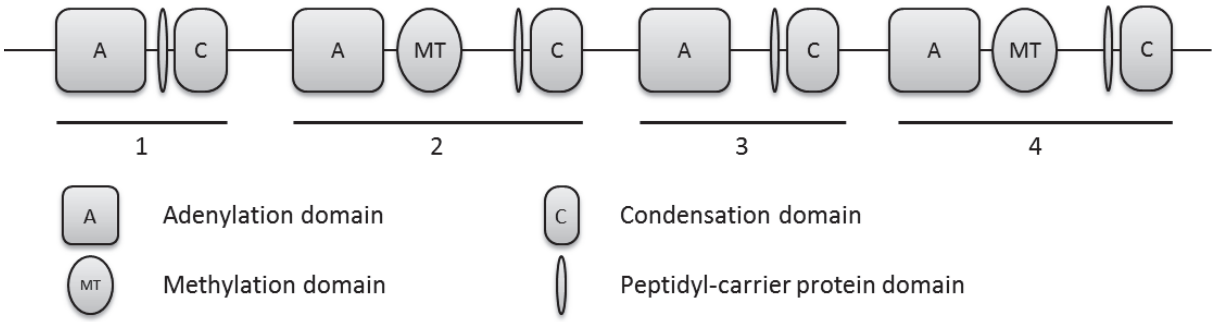

Figure 4.3. Module and domain architecture of the non-ribosomal peptide synthetase CmNps3, responsible for tentoxin biosynthesis in *Cochliobolus miyabeanus*. Modules (1-4) and corresponding domains were predicted using antiSMASH.

The SVM based method predicted that the first two monomers are hydrophobic-aliphatic amino acids with SVM scores of 0.70 and 1.12 respectively. The second module features a methylation domain. Therefore, these monomers could correspond to Gly and MeAla in tentoxin. Prediction of the last two monomers is less consistent with the tentoxin sequence. Whereas tentoxin features Leu on position 3, NRPSPredictor2 predicts a hydrophobic-aromatic monomer for this module, but the low SVM score (score = 0.06) could indicate an inconsistent prediction. Although the last module harbours a methylation domain corresponding to MePhe in tentoxin, NRPSPredictor2 predicts a hydrophobic-aliphatic monomer, with a high score of 1.07. Based on the Stachelhaus code, the monomer sequence would be Gly – MeVal – Phe – MePhe. Gly was confirmed as the first monomer with 90% identity to a known Stachelhaus code. As Stachelhaus predictions at 70% or lower are less reliable, we cannot trust the last three monomer predictions (Rausch *et al.*, 2005). Because this NRPS was still the best candidate for a tentoxin synthase in *C.miyabeanus*, it was selected for further analysis.

A-domain	Modification	NRPSPredictor2[a] Prediction and Score	Stachelhaus code Prediction and Score	Tentoxin
1	-	Hydrophobic – aliphatic 0.70	Gly – 90%	Gly
2	Methylation	Hydrophobic – aliphatic 1.12	Val – 70%	Me-Ala
3	-	Hydrophobic – aromatic 0.06	Phe – 70%	Leu
4	Methylation	Hydrophobic – aliphatic 1.07	Phe – 60%	Me-Phe

Table 4.1. Monomer specificity predictions of CmNps3 adenylation domains, compared to actual tentoxin monomers. [a] Rausch et al. (2005)

To determine the phenotype of *CmNPS3*, the gene was deleted from two *C. miyabeanus* field strains, WK1C and Cm988, using homologous recombination in a protoplast transformation protocol. The candidate transformants were purified by single spore selection and exchange of the *CmNPS3* gene for the Hygromycin B resistance gene was confirmed by PCR-verification and fungal growth on HygB+ medium (Figure S4.2). Two independent, confirmed knock-out mutants in the Cm988 background (*Cm988ΔCmnps3.1* and *Cm988ΔCmnps3.2)* and one confirmed mutant in the WK1C background (*WK1CΔCmnps3*) were selected for phenotyping.

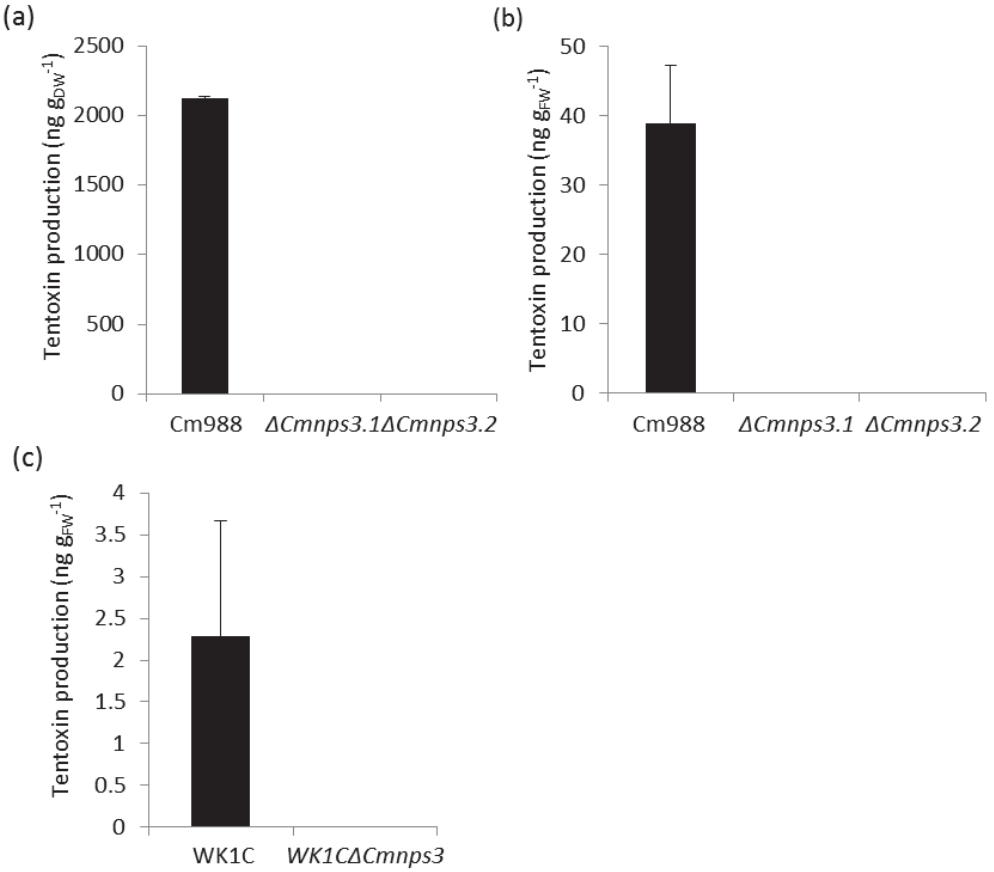

Figure 4.4. Quantification of tentoxin production by *Cochliobolus miyabeanus* strains Cm988 and WK1C wild type (WT) and respective *Cmnps3* mutants, using UPLC-MS/MS. (a) Tentoxin production in liquid culture. WT and *Cm988ΔCmnps3* mutants were grown in liquid Fries medium for seven days, tentoxin was extracted from the culture medium, quantified using UPLC-MS/MS and divided by total fungal dry weight. **(b)** Tentoxin production *in planta*. Rice (*Oryza sativa*) leaf pieces were spray inoculated with a spore solution ($1x10^4$ spores/ml) containing Cm988 WT and *Cm988ΔCmnps3* mutant spores, respectively. Tentoxin was extracted from the infected leaf tissue at 48 hpi, quantified using UPLC-MS/MS and divided by total leaf fresh weight. **(c)** Tentoxin production *in planta*. Rice leaf pieces were inoculated with a mycelial suspension ($5x10^4$ CFU/ml) containing WK1C WT and *WK1CΔCmnps3* mycelium respectively. Tentoxin was extracted from infected leaf pieces at 48 hpi, quantified using UPLC-MS/MS and divided by total leaf fresh weight. Results are means ± SE of at least 3 biological repeats.

The production of tentoxin by the wild type (WT) and mutant strains was measured using UPLC-MS/MS (Figure 4.4). Cm988 WT strain produced tentoxin *in vitro* and *in planta*, whereas WK1C only produced tentoxin *in planta*. After seven days of growth in Fries medium, we extracted an average of 2121.2 ± 19.1 ng tentoxin g^{-1} DW_{fungus} from the WT Cm988 cultures, while

tentoxin production by both independent mutant strains was completely abolished. Similarly, *Cmnps3* mutants of both WT strains could no longer produce tentoxin *in planta* at 48 hpi, whereas WT Cm988 produced an average of 38.8 ± 8.4 ng g^{-1} FW_{leaf} after spore inoculation and WK1C produced 2.28 ± 1.4 ng tentoxin g^{-1} FW_{leaf} after mycelium inoculation. These results confirm the involvement of CmNps3 in tentoxin biosynthesis.

Fungal growth rate and conidiation

To determine whether CmNps3 has a role in normal fungal development, we investigated the effect of *CmNps3* deletion on fungal growth and conidiation (Figure 4.5). Only the tentoxin knock-out strain *Cm988ΔCmnps3.2* grew slightly, but significantly slower than compared with WT Cm988 ($p < 0.05$). The growth rate of the other knock-out strains did not differ significantly from the respective WT. Furthermore, both *Cm988ΔCmnps3* mutants produced the same number of spores as the parental strain during 14 days of growth on PDA. The WK1C strain sporulates very poorly under lab conditions.

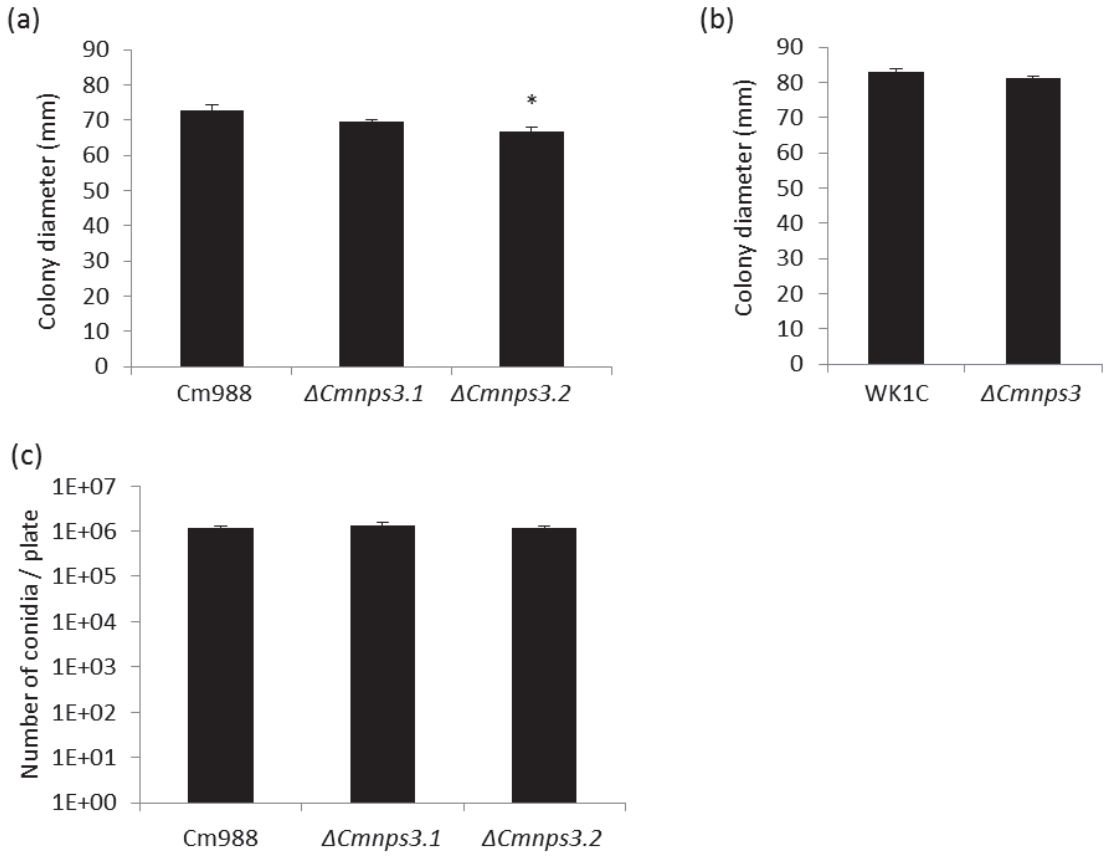

Figure 4.5. Effect of *CmNPS3* deletion on fungal growth rate and conidiation of *Cochliobolus miyabeanus*. (a) Growth rate of WT Cm988 and *Cm988ΔCmnps3* mutants, measured as the colony diameter after seven days of growth on potato dextrose agar (PDA) medium. **(b)** Growth rate of WT WK1C and *WK1CΔCmnps3* measured as the colony diameter after seven days of growth on complete medium with xylose (CMX) medium. **(c)** Conidiation of WT Cm988 and *Cm988ΔCmnps3* mutants measured as the number of spores produced on PDA medium after 14 days of growth. Results are means (± SE) of the pooled data of two independent experiments (n = 6). * indicate significant differences with WT by Wilcoxon Rank-Sum non-parametric test ($p < 0.05$).

Tentoxin production is related to severity of disease symptoms

In order to find out whether tentoxin might play a role during brown spot disease development, we compared the number of lesions and the total affected leaf surface caused by infection with WT and tentoxin-deficient strains (Figure 4.6). Plants were inoculated with a spore suspension of Cm988 and *Cm988ΔCmnps3* and with a mycelium suspension of WK1C and *WK1CΔCmnps3.1,* respectively. Because of this difference in inoculation method, the results cannot be compared between strains. The average number of lesions that had appeared at 48 hpi was not significantly different between Cm988 and *Cm988ΔCmnps3.1* (53 ± 6 and 50 ± 7 respectively, $p > 0.05$). However, the amount of leaf surface affected differed significantly. Whereas Cm988 caused on average damage to 28.7 ± 5.4 % of the leaf surface three days after spore inoculation, only 12.9 ± 2.2 % of the leaf surface was affected by *Cm988ΔCmnps3.1* infection. This difference is mainly attributable to the development of chlorosis during the WT infection, which was mostly absent during infection with tentoxin-deficient mutants. No significant differences were observed in number of lesions (80 ± 16 and 81 ± 10 respectively, $p > 0.05$), or in affected leaf area (23.5 ± 5.4 % and 21.7 ± 3.0 % respectively, $p > 0.05$) during infection by the WK1C WT or *WK1CΔCmnps3* mutant respectively.

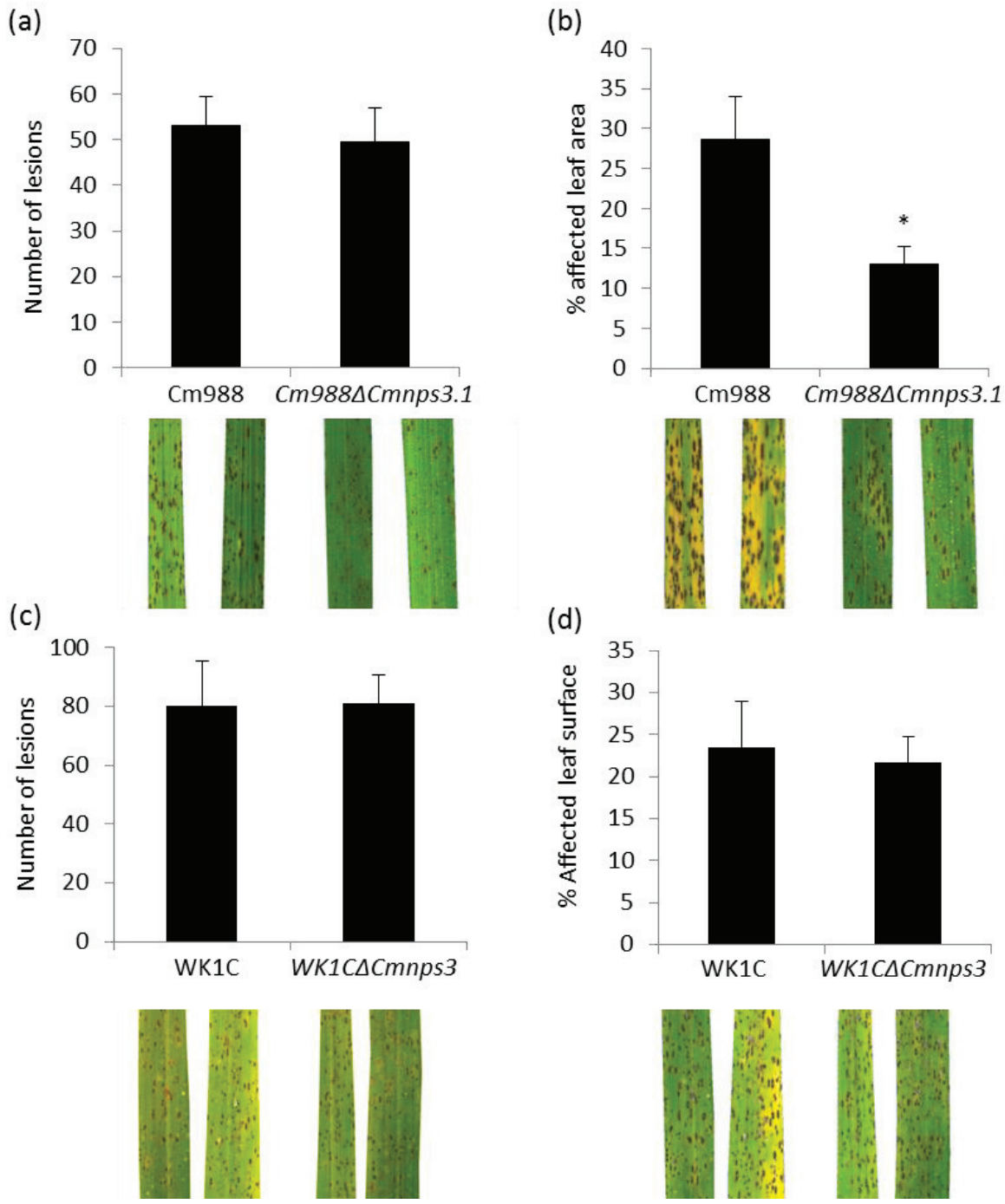

Figure 4.6. *Cochliobolus miyabeanus* infection assays on rice (*Oryza sativa*) leaf pieces. (a) and **(b)** Leaf pieces of five week old rice plants were spray inoculated with a spore suspension (1 x 10^4 conidia ml^{-1}) of *C. miyabeanus* Cm988 wild type (WT) or *Cm988ΔCmnps3.1* respectively. Lesions were counted per leaf piece at 48 hpi **(a)** and the percentage affected leaf area was measured at 72 hpi **(b)**. **(c)** and **(d)** Leaf pieces of five week old plants were spray inoculated with a mycelial suspension (5x10^4 CFU/ml) of WK1C WT or *WK1CΔCmnps3* mutant respectively. Lesions were counted per leaf piece at 48 hpi **(c)** and the percentage affected leaf area was measured at 72 hpi **(d)**. Pictures show representative symptoms at the respective time points. Results are means ± SE, * indicate significant difference with WT by Wilcoxon Rank-Sum non-parametric test ($p < 0.05$).

To test the effect of pure tentoxin on mature rice leaves, we punctured leaf pieces and applied two concentrations of pure tentoxin (Figure 4.7). The control leaves and leaves treated with 50

µM tentoxin did not show any symptoms. However, treatment with 500 µM tentoxin caused a small necrotic ring around the puncture wound in 33 % of the treatments. The other 66 % of the leaf pieces did not show any symptoms.

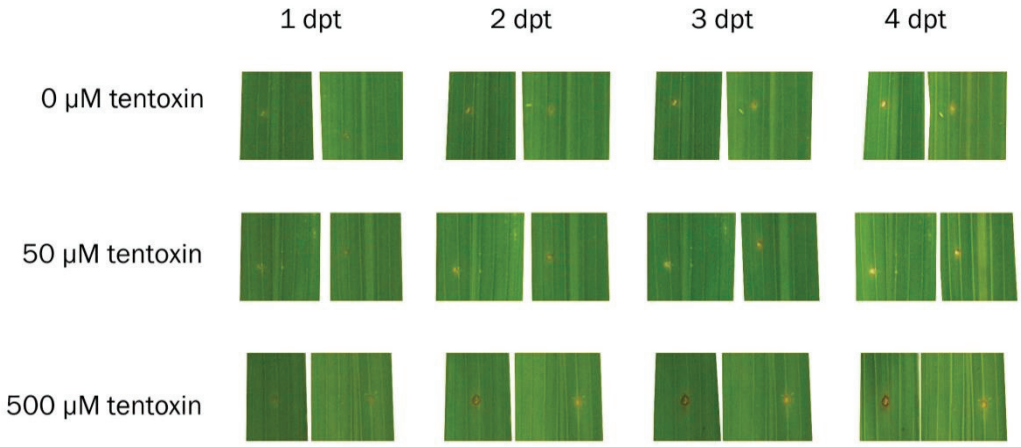

Figure 4.7. Effect of pure tentoxin on mature rice (*Oryza sativa*) leaves. Leaf pieces of 5 week old plants were punctured and treated with 10 µl of pure tentoxin at the indicated concentration. Symptoms were monitored during 4 days. Pictures represent the same replicate at four different time points after treatment. dpt: days post treatment

Furthermore, we quantified the amount of tentoxin produced during infection by field strains with different virulence abilities (Fonteyne, 2011) (Figure 4.8). All strains started to produce tentoxin at 16 hpi, and production increased while the disease progressed. Interestingly, the highly virulent Cm988 strain produced very high amounts of tentoxin. Tentoxin production by less virulent strains G513 and WK1C, and the very weak strain S4 was about 100 times lower and not significantly different from each other. These results suggest that tentoxin is not necessary for pathogenicity and initiation of the infection, but contributes to disease severity during later phases.

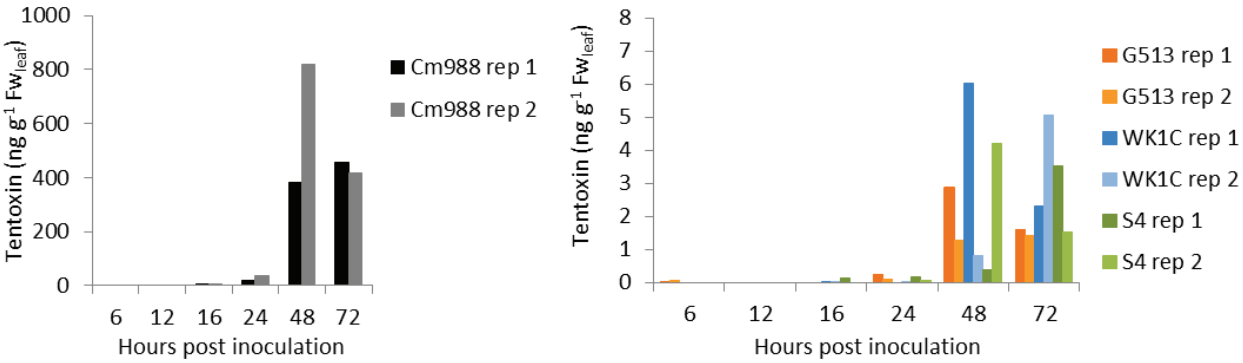

Figure 4.8. Tentoxin production during infection of rice (*Oryza sativa*) leaves is correlated with the virulence level of different *Cochliobolus miyabeanus* wild type strains. Tentoxin was extracted from infected leaf pieces at the indicated time points after mycelium-inoculation and quantified using UPLC-MS/MS. Results of two independent biological replicates (rep) are presented.

Tentoxin synthase gene conservation

CmNPS3 is an ortholog of *C. heterostrophus NPS3* (*ChNPS3*) (Turgeon *et al.*, 2008; Condon *et al.*, 2013). Comparison of the two genomic sequences (*C. miyabeanus* strain WK1C and *C. heterostrophus* strain C4) reveals the presence of many SNPs. Furthermore, an intron spanning 51 bp in the adenylation (A) domain of the third module of *ChNPS3* causes a loss of 17 amino acids in ChNps3 compared to CmNps3. These changes could indicate change or loss of function of ChNps3, although overall protein similarity is still 95%. Therefore we screened *C. heterostrophus* strain C4 for tentoxin production *in vitro* and *in planta* during infection of Maize leaves. We could not detect any tentoxin (data not shown), suggesting that ChNps3 function could indeed be different from CmNps3. Because tentoxin was previously reported in *Alternaria alternata*, we also screened the genome of *Alternaria alternata* strain SRC1lrK2f for orthologs. We found a NRPS with 85% similarity to CmNps3: AaNps3 (JGI protein ID 1065195) (Table 4.2). Like *ChNPS3, AaNPS3* features an intron in the A-domain of the third module resulting in loss of 13 amino acids, but at a different site than *ChNps3*. Functional analysis of the gene should shed light on a possible involvement in tentoxin production by *A. alternata*.

AaNps3	100%		
CmNps3	85%	100%	
ChNps3	85%	95%	100%
	AaNps3	**CmNps3**	**ChNps3**

Table 4.2. Protein sequence similarities between Nps3 homologs of *A. alternata*, *C. miyabeanus* and *C. heterostrophus*.

Discussion

The aim of our study was to characterize new disease-associated metabolites in *C. miyabeanus* using a comparative metabolomics approach. We show that the non-HST tentoxin is a virulence factor in *C. miyabeanus*. To our knowledge, this is the first report of tentoxin production by a non-*Alternaria* plant pathogen. Tentoxin biosynthesis was induced at 24 h post inoculation and is correlated with chlorosis development. This finding reinforces the hypothesis that *C. miyabeanus* produces additional virulence factors besides ophiobolins and NPS6 and relies mainly on non-selective phytotoxins.

Although tentoxin production by *Alternaria* has been known for a long time, the NRPS responsible for its synthesis has not been characterized before. NRPS are multimodular megasynthases that catalyse the biosynthesis of small peptides independent of the ribosomes (Stachelhaus *et al.*, 1999). Each module represents a single step in the chain elongation and is composed of separate domains catalysing the selection, activation and modification of its amino acid substrate (Stein *et al.*, 1996). Monomer specificity is conferred by the A-domain. Based on the amino acids lining the substrate-binding pocket, predictions can be made about substrate specificity. Several algorithms have been developed to aid monomer prediction of each module. We used antiSMASH to predict the modular backbone and monomer specificity of *C. miyabeanus* NRPSs, based on the stachelhaus code and NRPSpredictor2 (Rausch *et al.*, 2005; Medema *et al.*, 2011; Röttig *et al.*, 2011). One NRPS, n8391, stood out because of the tetramodular structure, including methylation domains in the 2nd and 4th module. Because *n8391* is an ortholog of *C. heterostrophus NPS3*, we designated the enzyme CmNps3. Deletion of *CmNPS3* from two *C. miyabeanus* strains confirmed its involvement in the biosynthesis of tentoxin.

The first two predicted monomers of CmNPS3 were consistent with the known amino acid sequence of tentoxin (Gly, MeAla, Leu, MePhe), but the last two monomers were not. It is not clear whether the A-domain specificity prediction is erroneous or whether the biosynthesis of tentoxin does not follow the colinearity rule. The currently available amount of training data for NRPS predicition algorithms is relatively low; there is still a large amount of sequences for which prediction is uncertain or impossible. Especially for fungal sequences, the predictions are not straight forward. There are indications that eukaryotic A domains might have developed alternative substrate-binding patterns, but most of the characterized A-domains that serve as a basis for specificity predictions have a bacterial origin (Rausch *et al.*, 2005; Stack *et al.*, 2007; Bushley & Turgeon, 2010). Besides, eukaryotic NRPS elongation does not always follow the colinearity rule. It is thus not surprising to find discrepancies between expected and predicted monomer specificity. Furthermore, it is not clear whether the peptide elongation process proceeds stepwise starting from glycine, or by binding two dipeptides Gly-MeAla and Leu-MePhe (Ramm *et al.*, 1994). The latter case would explain the switched positions of the predicted monomers, but

cannot explain the methylation of an aliphatic amino acid. To resolve this, one should confirm module specificity by an ATP-PPi-exchange reaction (Lee & Lipmann, 1975; Phelan *et al.*, 2009).

CmNPS3 is an ortholog of *C. heterostrophus NPS3* (*ChNPS3*) and is also conserved in *C. sativus* (Lee *et al.*, 2005; Condon *et al.*, 2013). Although other sequenced *Cochliobolus* spp. do not have a full *NPS3* ortholog, they all have additional NRPS proteins constructed from separate NPS1/NPS3 modules. The seemingly volatile nature of NPS1/NPS3 modules could lead to new proteins with new functionalities, and eventually new virulence factors (Bushley & Turgeon, 2010; Condon *et al.*, 2013). This would imply that tentoxin is only one possible NRPS product, resulting from a specific combination of four modules. Mining of the genome of the tentoxin producer *Alternaria alternata* revealed the presence of another *NPS3* ortholog (*AaNPS3*) that could be responsible for tentoxin production in this genus. Although the same monomer specificity is predicted for ChNps3, AaNps3 and CmNps3, the functionality of the *ChNPS3* gene can be questioned. The *C. heterostrophus NPS3* region shows many SNPs (only 90% sequence identity) when compared to *CmNPS3*. Furthermore, part of the DNA sequence changed to an intron, which has resulted in the loss of a stretch of 17 amino acids in the A-domain of the third module. *C. heterostrophus* strains C4 showed minimal expression of *ChNPS3* after 24h of culture in a N-poor medium (Lee *et al.*, 2005). We could not detect any tentoxin present in the supernatant under these conditions, nor during infection of Maize leaves (data not shown). Although AaNps3 in the genome of the *A. alternata* strain SRC1lrK2f also lacks 13 amino acids in the third A-domain compared to CmNps3, the known production of tentoxin by *Alternaria alternata* suggests that *AaNPS3* has the same functionality. Therefore the capacity of strain SRC1lrK2f to produce tentoxin should be verified, in addition to functional analysis of *AaNPS3*.

Several phytotoxic effects have been reported for tentoxin, but the main mode of action of tentoxin is the induction of chlorosis via inhibition of photophosphorylation in sensitive species. *In vitro*, tentoxin binds to the F1 subunit of chloroplast ATP-synthase of sensitive species, thereby blocking ATP synthesis (Steele *et al.*, 1976; Groth, 2002; Pavlova *et al.*, 2004; Meiss *et al.*, 2008). Although ATP-synthase inhibition alone cannot explain the chlorosis effects, it could deplete ATP necessary for the incorporation of polyphenol oxidase (PPO) into thylakoids (Sommer *et al.*, 1994; Holland *et al.*, 1997). Loss of plastidic PPO can stop the electron flow, leading to the overenergization of thylakoid membranes and production of reactive oxygen species, which ultimately results in chlorosis (Vaughn and Duke 1984, 1981; Holland et al. 1997). Other phytotoxic effects include interference with membrane functions responsible for ion transport and stomatal closure (Durbin *et al.*, 1973; Heitz *et al.*, 1986; Erdei & Klotz, 1988; Klotz, 1988; Klotz & Erdei, 1988). Interestingly, photophosphorylation was also inhibited in chloroplasts of insensitive plant species by high concentrations of tentoxin, independent of ATP-synthase inhibition, suggesting the existence of additional tentoxin effects on energy transfer (Steele *et al.*, 1976). Although rice seedlings were classified as insensitive to chlorosis effects of tentoxin (Durbin & Uchytil, 1977), our own study shows a correlation between tentoxin production and chlorosis

during *C. miyabeanus* infection of fully developed rice leaves of five week old plants. Further investigation should shed a light on the tentoxin sensitivity of rice, in particular the involvement of photophosphorylation. Nevertheless, tentoxin does not seem to exert a clear direct phytotoxic effect on mature rice leaves and even a very high concentration of 500 µM tentoxin only resulted in small necrotic symptoms in 33% of the times. Therefore, it is plausible that tentoxin-production has an indirect effect on chlorosis development.

Chlorosis development during the second phase of brown spot shows a high biological variability. The amount of tentoxin measured for different biological replicates is similarly highly variable. When *CmNPS3* was deleted from Cm988, the variability in disease symptoms decreased considerably. It is not clear why tentoxin production fluctuates, but induction of tentoxin at 24h after inoculation suggests the need for an extracellular stimulus. This might be an infection specific trigger originating from plant or fungus, the strength of which could correlate with the amount of tentoxin produced. Furthermore, NRPS are generally organized in gene clusters, which can include specific transcription factors. Both these specific TF as well as global TF can regulate the expression of secondary metabolism genes (Yu & Keller, 2005). Further research should shed light on how tentoxin biosynthesis is triggered and regulated.

During *C. miyabeanus* infection, the rice physiology is subjected to radical changes characterized by a drop in photosynthetic processes and remobilization of nitrogen, characterized by the induction of glutamine synthetases (Van Bockhaven *et al.*, 2015b). Fungi rely almost exclusively on the plant for their N-source (Seifi *et al.*, 2013a). The availability of amino acids necessary to produce tentoxin (Gly, Ala, Leu and Phe), as well as a methionine source for the methylation reactions, could determine the level of tentoxin produced. Hence, tentoxin production may be directly correlated to the severity of the infection and the change in plant metabolism during the first 24 h. This hypothesis is further supported by the observation that mutants that lack ophiobolins also produce significantly less tentoxin *in planta* (Preliminary results). This coud explain the biological variability of tentoxin produced during disease.

Furthermore, apart from possible direct phytotoxic effects, the mere use of plant amino acids for the production of tentoxin could enhance disease, as valuable N-sources are drained away from the plant and are lost for recycling to other plant parts. Moreover, some of the tentoxin-specific amino acids are needed in plant defense related pathways. Phenylalanine is a precursor for phenolic compounds that can protect the plant against *C. miyabeanus* (Shabana *et al.*, 2008; Van Bockhaven *et al.*, 2015a). Glycine is decarboxylated to serine during photorespiration (Figure 6.2). The HST Victorin from *C. victoriae* can induce senescence in oats by targeting glycine decarboxylase in the photorespiration (Navarre & Wolpert, 1999). Hence, it seems plausible that inhibiting photorespiration through drainage of glycine to tentoxin could have a similar effect. This would support the hypothesis that tentoxin should be made *in planta* in order to have an effect and would also explain the insensitivity of rice seedlings to tentoxin treatment.

Alternatively, it is also possible that tentoxin only causes symptoms under certain circumstances, e.g. when the plant is already stressed. An indirect effect of tentoxin through interference with plant physiology would imply the need for high tentoxin-production in order to drain considerable amounts of amino acids. This could explain why deletion of *CmNPS3* only reduced disease symptoms upon infection by Cm988, as this strain produces 100-fold more tentoxin than WK1C.

Tentoxin has mainly been studied as a mycotoxin produced by *A. alternata*, often in the context of food contamination. The risk to human health has not been extensively investigated and no *in vivo* genotoxicity or carcinogenicity data are available today, but tentoxin is non-mutagenic in bacteria. Although tentoxin is considered unlikely to be a human health concern (dietary exposures are more than 4-fold lower than the threshold of toxicological concern), detection strategies have been developed to detect tentoxin in food matrices (European Food Safety Authority (EFSA), 2011; Walravens *et al.*, 2014). Tentoxin can be detected in many commercial food samples, including rice (Liu & Rychlik, 2013; Walravens *et al.*, 2014). Often the presence of tentoxin is used as an indicator for the presence of *Alternaria* spp. Our results show that at least in the case of contaminated rice samples, the presence of tentoxin might be correlated with the presence of *Cochliobolus* spp..

To summarize we show that *C. miyabeanus* also produces the non-selective toxin tentoxin as a virulence strategy. This supports the idea that broad host range necrotrophs rely on an array of non-selective virulence factors. Each factor typically targets a general plant target or supresses defense responses, adding quantitatively to overall virulence and/or disease severity. Identification of new virulence factors can complement breeding efforts as it provides additional tools to screen for more resistant genotypes.

Materials and Methods

Biological material and growth conditions

C. miyabeanus wild type strains Cm988 (De Vleesschauwer et al, 2010) and WK1C (Condon et al, 2013) were used. *CmNPS3* (*n8391*) was deleted from each strain, resulting in tentoxin-deficient mutants *Cm988ΔCmnps3.1, Cm988ΔCmnps3.2* and *WK1CΔCmnps3* respectively. Cm988, *Cm988Δnps3.1* and *Cm988ΔCmnps3.2* were grown on potato dextrose agar (PDA) under dark conditions at 28°C and WK1C and *WK1CΔCmnps3* were grown on complete medium with xylose (CMX) (Inderbitzin et al., 2010) under constant light at 23°C for optimal conidiation.

The *Oryza sativa* japonica cultivar Nipponbare was grown in commercial potting soil in a growth room under 12 h light/12 h dark at 28°C as described previously (De Vleesschauwer et al, 2010).

Sample preparation for chemical analysis

Liquid culture extracts were collected as follows. Three mycelium plugs per strain were grown in 100-ml Erlenmeyer flasks containing 30 ml of Fries medium (Pringle and Braun, 1957) supplemented with 0.1% yeast extract and 1ml plant extract, at 28 °C in the dark on a rotary shaker (150 rpm). The plant extract was prepared by extracting 4g of grounded fresh rice leaves with 50 ml double distilled water and filter sterilized using a syringe driven 0.22 µm Millex-HV filter unit (Merck Millipore, Cork, Ireland) before use. After three days, the culture was blended and 1 ml was transferred to fresh medium and incubated for another seven days under the same circumstances. The supernatant was filtered through two layers of cheese cloth, one layer of filter paper (Grade 3hw, Sartorius Stedim Biotech S.A., Germany), and sterilized using a syringe driven 0.45 µm Millex-HV filter unit (Merck Millipore, Cork, Ireland). The mycelium was collected from the filter and dried at 80 °C for 16 hr before measuring dry weight. The total volume of the crude culture filtrate was measured and a 4 ml aliquot were used for metabolite extraction.

Samples for *in planta* tentoxin quantification were prepared as follows. Leaf pieces were inoculated as described for the infection assays. Infected leaf samples were crushed using liquid N_2 and stored at -80 °C until chemical extraction. A total of approximately 0.5 g leaf material was used for toxin extraction.

The samples (either crude extract or leaf material) were extracted with 10 ml of ethyl acetate:acetonitril 1:1 (v/v) after addition of 1.8 g $MgSO_4$. The sample was vigorously shaken on an end-over-end shaker (Agitelec, J. Toulemonde & Cie, Paris, France) for 30 min then centrifuged at 4000 g for 15 min. The organic upper phase was evaporated to dryness under a N_2 flow at 40 °C. The dry residue was redissolved in 100 µl of mobile phase A:B 60:40 (v/v) and filter centrifuged in an Ultrafree-MC centrifugal filter unit (Merck Millipore, Bedford, MA, USA) for 5 min at 14000 g.

UHPLC-Orbitrap HRMS conditions

An Accela™ High Speed LC (UHPLC) system (Thermo Fisher Scientific, San José, CA, USA) coupled to an Orbitrap Exactive™ mass analyser (Thermo Fisher Scientific) was used to analyse the samples. The Orbitrap was equipped with an electrospray ionisation (ESI) interface, which was operated in alternating positive and negative ionisation mode. Spray voltage was set at 4.5 kV; sheath, auxiliary and sweep gas flow rate at 45, 10 and 5 arbitrary units, respectively; capillary and heater temperature at 250 °C; capillary, tube lens and skimmer voltage at 45 (-45), 125 (-125) and 22 (-23) respectively for positive (negative) mode. The instrument was operated in full-scan mode at a resolution of 100,000 full width at half maximum height (FWHM) with an m/z scan range of 100 - 1,000. The maximum injection time was 250 ms and the automatic gain control (AGC) target was set at ultimate mass accuracy (5×10^5 ions).

The column used was a Zorbax Eclipse C18 (2.1 × 100 mm i.d., 1.8 µm) (Agilent, Santa Clara, CA, USA). Mobile phase A consisted of H_2O:methanol 95:5 (v/v) and mobile phase B of methanol:H_2O 95:5 (v/v). To facilitate ionisation 0.1% formic acid and 1 mM ammonium formate were added to both mobile phases. The gradient elution programme for UHPLC-Orbitrap HRMS analyses was applied as follows: 0–0.5 min: 0% B, 0.5– 20 min: 0–99.1% B, 20–21 min: 99.1% B, 21–24 min: 99.1–0% B, 24–28 min: 0% B. The flow rate was 0.4 ml min^{-1}. The column temperature was set at 30 °C and the temperature of the autosampler was 10 °C.

Instrument control and data processing were carried out using Xcalibur™ 2.1 (Thermo Fisher Scientific). Sieve™ software (Thermo Fisher Scientific) was used for peak picking according to the following settings: positive charge, m/z range of 100-1,000 Da, an m/z width of 10 ppm, a retention time range of 5.0-25.0 min, a peak intensity threshold of 105,000 arbitrary units, a maximum peak width of 0.5 min and a maximum number of 5,000 frames. The peak intensities of the 5,000 selected frames were further analysed using principal component analysis in SPSS Statistics 21.

UPLC-MS/MS conditions

We confirmed tentoxin through analysis of a pure standard and its corresponding fragmentation pattern. A Waters Acquity UPLC system coupled to a Waters Quattro Premier XE tandem-quadrupole mass spectrometer (Waters, Milford, MA, USA) was used to confirm the identity of tentoxin based on fragmentation patterns of a pure commercial tentoxin standard (Biopure Referenzsubstanzen GmbH, Austria). The column used was a Zorbax Eclipse C18 column (100 x 2.1 mm i.d.; 1.8 µm) (Agilent, Santa Clara, CA, USA). Data processing was done with Masslynx software v4.1 (Waters, Milford, MA, USA). The LC conditions were the same as described for the AccelaTM High Speed LC (UPLC) system described under "UPLC-Orbitrap MS conditions".

The mass spectrometer was operated in the negative electrospray ionization (ESI^-) mode. The capillary voltage was 3.5 kV, and nitrogen was used as the desolvation gas. Source and desolvation temperatures were set at 120 and 300°C respectively. Tentoxin was analysed using selected reaction monitoring (MRM) mode with the following ion transitions: 413.3 > 271.2 (cone 44 V, collision energy 18 eV), 413.3 > 141.0 (cone 44 V, collision energy 24 eV). Ion transition 413.3 > 141.0 was used for quantification; ion transition 413.3 > 271.2 was used as qualifier.

Quanlynx (Waters, Milford, MA, USA) was used for quantification of tentoxin in the samples by generating a calibration curve using different concentrations of a tentoxin standard. Tentoxin production per g mycelium dry weight or per g leaf fresh weight was calculated for *in vitro* or *in planta* production respectively.

Gene identification and gene knock-out

The NRPSs present in the *C. miyabeanus* genome, as identified by Condon et al (2013) were screened using the program antiSMASH (Medema *et al.*, 2011). One candidate tentoxin synthase was chosen (NODE 8391 (Condon *et al.*, 2013)), JGI protein ID 98843).

The selected gene was deleted using homologous recombination as described by (Bi *et al.*, 2013). The entire selection marker fragment 'hygB', including the promoter, was amplified from plasmid pUCATPH using M13R and M13F primers, and fused to the 5' and 3' flanking fragments of the target *CmNPS3* gene. PCR amplification of transformation constructs was carried out with phusion high-fidelity DNA polymerase (Thermo Scientific) following the manufacturer's instructions.

Transformation of *C. miyabeanus* was carried out as previously described (Inderbitzin *et al.*, 2010), with a slight modification. The Cm988 strain was cultured on PDA for seven days in the dark and WK1C on CMX for 14 days under continuous light. For protoplasting, conidia were transferred to 300 ml complete medium (CM) in a 1-liter flask and shaken at 100 rpm for 16 h in the dark at 24°C. Protoplasts were harvested and transformed as described previously (Catlett *et al.*, 2003). Candidate transformants were single-conidiated and transferred to PDA or CMX containing hygromycin B (100 μg ml^{-1}) to confirm sensitivity to the drug, as described previously (Oide et al. 2006). Integration of the constructs at the intended sites was confirmed by diagnostic PCR reactions, as described previously (Inderbitzin *et al.*, 2010; Wu *et al.*, 2012). All PCR reactions were conducted with GoTaq (Promega). Confirmed transformants were selected and scanned for tentoxin production using UPLC-MS/MS. Primer sequences used for gene deletion can be found in the Primer List at the end of this thesis.

Fungal growth rate and conidiation

A 6 mm diameter plug from a seven day old colony of the respective strain was placed in the center of a PDA plate and incubated at 28°C in the dark. Fungal growth was measured as the average colony diameter after three or seven days of growth. After seven days of growth, the conidia from each plate were suspended in 15 ml of double distilled water and counted using a Bürker counting chamber. The experiment was repeated once, using three PDA plates per strain.

Infection assays

Disease severity was assessed using leaf piece infection assays. For Cm988 and *Cm988Δnps3.1* inoculum preparation, conidia were harvested as described by (Thuan *et al.*, 2006) and resuspended in 0.02% Tween20 to a final density of $1x10^4$ conidia ml^{-1}.

For all other strains (and also for Cm988 in the infection assay were tentoxin production by different field strains is compared) a mycelial suspension was used as inoculum. Five mycelium plugs per strain were grown in 100-ml Erlenmeyer flasks containing 30 ml of potato dextrose

broth (PDB), at 28°C in the dark on a rotary shaker (150 rpm). After 48 h of growth, the mycelium was collected and blended in 15 ml double distilled water. The resulting mycelial suspension was counted and diluted to $5x10^4$ CFU ml^{-1} in 0.02% Tween20.

The youngest fully developed leaves of 5-week old rice plants were cut in 7-cm pieces and kept in square petri dishes (9 x 9 cm, Greiner bio one), lined with moistened tissue. Leaf pieces were spray-inoculated with 0.5 ml inoculum per petri dish. The leaf pieces were kept in the dark at 28 °C and moved to growth chamber conditions after 16 h. The number of lesions was counted at 48 hpi using APS Assess Software. Disease symptoms were quantified as percentage affected leaf surface at 3 dpi using APS Assess Software. Statistical analysis was performed using non-parametric Wilcoxon Rank-Sum test ($\alpha = 0.05$).

Toxicity test

Tentoxin (Sigma-Aldrich, Diegem, Belgium) was dissolved in 10% ethanol to a final stock concentration of 1.2 mM. The youngest fully developed leaves of 5-week old rice plants were cut in 7-cm pieces and kept in square petri dishes (9 x 9 cm, Greiner bio one), lined with moistened tissue. Each leaf piece was punctured at three different places and treated with 10 µl of tentoxin solution at the indicated concentrations. Symptom development was monitored from1 until 4 dpi.

Supplementary data

Component	Initial Eigenvalues		
	Total	% of Variance	Cumulative %
1	8.902	59.348	59.348
2	3.013	20.087	79.435
3	1.905	12.703	92.138

Extraction Method: Principal Component Analysis.

Table S4.1. Representation of the total variance explained by the first three principal components. Principal component analysis was performed on the integrated intensities of the 5000 most abundant ions detected in a culture filtrate of three different *Cochliobolus miyabeanus* wild type strains grown in liquid Fries medium. Analysis was performed in SPSS Statistics 21.

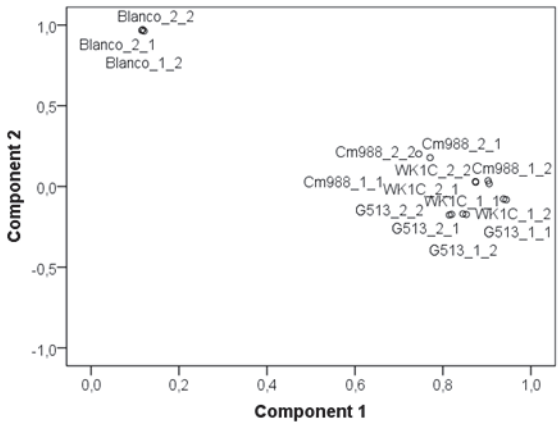

Figure S4.1. Score plot representing how different strains are separated based on principal components 1 and 2. The highly virulent strain Cm988, and the less virulent strains WK1C and G513 were grown in liquid Fries medium. The acetonitrile:ethylacetate extract of the filter sterilized liquid culture filtrate was analysed using UHPLC-Orbitrap-HRMS. The 5000 ions with the highest integrated peak intensities were selected and analysed using principal component analysis. The principal components are based on the compounds that represent the highest variance between treatments.

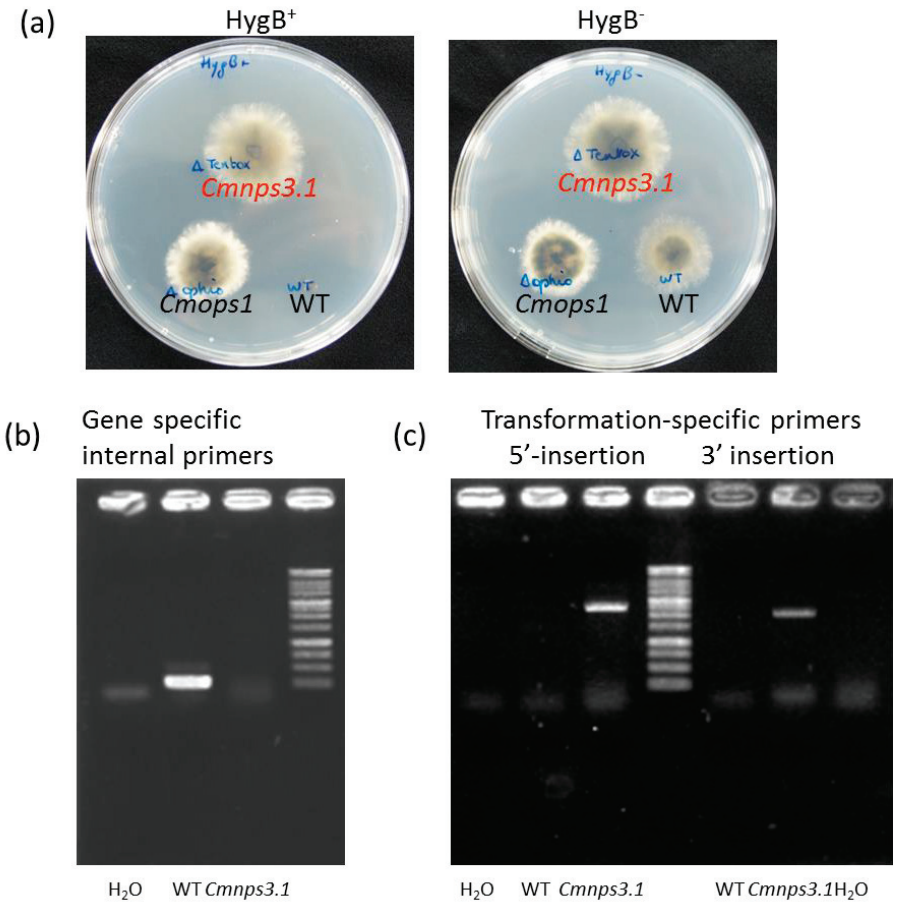

Figure S4.2 Illustration of the strategy used to confirm successful deletion of the target gene and specific integration of the transformation construct at the target site. Pictures represent verification data of the *Cm988ΔCmnps3*.1 mutant, the same procedure was used to succesfully verify the other *Cmnps3*.1 mutants. (a) A candidate transformant was purified by selection of a single spore and subsequently grown on hygromycin-containing medium to confirm the integration of the HygB resistance gene. *Cmops9.1* was used as a positive control. The Cm988 wild type (WT) is not able to grow on the hygromycin B-containing medium (HygB+). (b) Deletion of the targeted *CmNPS3* gene in *Cmnps3.1* was confirmed by PCR amplification using gene specific internal primers, CmNODE8391_amp1F1 and CmNODE8391_amp1R1, on total genomic DNA of the Cm988 wild type (WT) and the selected transformant (Cmnps3.1). H_2O_2 was used as a negative control. (c) Confirmation of the integration of the transformationconstruct at the target site based on a PCR reaction with one internal primer for the transformation construct, and one primer specific for the flanking sequence surrounding the targeted gene. 5'-insertion verification primers: 8391_UpVer1 and NLC37; 3'-insertion verification primers: NLC38 and 8391_DownVer1.

Chapter 5

Ethylene acts as a central node in brown spot disease susceptibility

Authors

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Summary

Plant responses to pathogen attack are regulated by a blend of plant hormones that can enhance resistance or susceptibility depending on the specific plant-pathogen interaction. Ethylene is a clear susceptibility enhancing factor in the case of rice-*C. miyabeanus* interactions. Gene expression and proteome analysis show that ethylene biosynthesis is induced upon infection. Therefore it seems plausible that the fungus hijacks the plant ethylene metabolism to enhance disease. In this chapter we investigated how *C. miyabeanus* could interact with plant ethylene metabolism and we focused on the interplay between ethylene and phytotoxins. We show that *C. miyabeanus* can produce ethylene by itself via two distinct pathways, the EFE (ethylene forming enzyme) pathway and the KMBA (α-keto-γ-methylthiobutyric acid) pathway. Real-time ethylene measurements during disease show a remarkable ethylene production pattern, featuring two distinct ethylene emission peaks, each coinciding with a wave of phytotoxin production. Our efforts to find a trigger for the first ethylene peak showed that ophiobolin A is not responsible for ethylene production, but needs ethylene to cause symptoms. The involvement of fungal ethylene in the induction of plant ethylene biosynthesis remains inconclusive. Fungal ethylene may also act as a toxin synergist or enhance the plant ethylene response. During the second ethylene burst, the rate of tentoxin production is associated with ethylene emissions and the onset of senescence and chlorosis. During this phase it is plausible that fungal ethylene acts autocatalytically to enhance the already started senescence process. The exact function of fungal ethylene during disease warrants additional research.

Author contributions

Lieselotte De Bruyne: *Study conception and design, fungal transformations, data acquisition, analysis and interpretation, writing of the chapter.*

Jonas Van Bockhaven: *Study conception, critical discussion of the experiments*

Bram Van de Poel: *Assistance with LPAD data acquisition and analysis, critical discussion of the experiments.*

Simona Cristescu: *Assistance with LPAD data acquisition and analysis*

***Christof Van Poucke and Sarah De Saeger*:** *Assistance with UPLC-MS/MS analysis*

Dominique Van der Straeten: *Critical discussion of the experiments.*

Monica Höfte and David De Vleesschauwer: *Critical discussion of the experiments, critical revision of the chapter*

Introduction

The evolutionary arms race between plants and pathogens has provided plants with a sophisticated immune system that allows early recognition of pathogen attack and subsequent induction of an immune response. Central to this immune response is a specific blend of cross-communicating hormones that steer the eventual defense signaling (Anderson *et al.*, 2004; Verhage *et al.*, 2010; Pieterse *et al.*, 2012a). Besides the archetypal defense related hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET), other hormones like abscisic acid, gibberellins, auxins, cytokinins and brassinosteroids are also implicated in the regulation of plant immunity (Robert-Seilaniantz *et al.*, 2011; Yang *et al.*, 2013; De Vleesschauwer *et al.*, 2013, 2014; De Bruyne *et al.*, 2014). Depending on the pathogen encountered, hormones can enhance resistance or promote susceptibility. It is therefore utterly important for the plant to activate the appropriate defense response (Mengiste, 2012).

Recent insights in the role of ethylene during plant immune responses reveal a complex picture in which ethylene can reduce or enhance resistance depending on the plant-pathogen interaction. Previously it was believed that ethylene acts synergistically with JA to enhance resistance to necrotrophs, whereas it would induce susceptibility toward biotrophs by antagonizing SA-defense signaling (van Loon *et al.*, 2006; Adie *et al.*, 2007). This is indeed the case in some pathosystems. Arabidopsis mutants impacted in ethylene reception or signaling became more susceptible to the necrotrophs *Erwinia carotovora* and *Botrytis cinerea* and at the same time ethylene insensitive mutants were more resistant to the biotroph *P. syringae* (Van der Ent & Pieterse, 2012). Similarly, ethylene induces resistance to *B. cinerea* in tomato (*Solanum lycopersicum*) (Díaz *et al.*, 2002). However it has become clear that ethylene can also induce susceptibility towards necrotrophs in certain plant-pathogen interaction. In Arabidopsis, ethylene renders plants more susceptible towards *Ralstonia solanacearum* (Hirsch *et al.*, 2002; Pantelides *et al.*, 2010). In rice, ethylene induces resistance to *Magnaporthe oryzae*, while at the same time enhancing susceptibility to *Xanthomonas oryzae* pv. *oryzae* (Iwai *et al.*, 2006; Shen *et al.*, 2011). Interestingly, many of the necrotrophs to which ethylene enhances susceptibility, are notorious phytotoxin producers, suggesting that ethylene may act as a toxin synergist. Ethylene is involved in susceptibility to *Alternaria alternata* f. sp. *lycopersici* and *Fusarium oxysporum* f. sp. *lycopersici* in tomato and to *Verticillium dahliae* in Arabidopsis and tomato (Cronshaw & Pegg, 1976; Hirsch *et al.*, 2002; Pantelides *et al.*, 2010, 2013). The link between ethylene and phytotoxin production is clear in the case of *Fusarium moniliforme* and *A. alternata* f.sp. *lycopersici*. Their respective phytotoxins fumonisin B1 and AAL-toxin are known PCD-inducers that rely on an active ethylene signaling pathway to exert their effect (Moore *et al.*, 1999; Asai *et al.*, 2000; Hoeberichts & Woltering, 2003; Gechev *et al.*, 2004). Interestingly, some phytotoxins can induce ethylene production by themselves. For example AAL-toxin-induced cell death is associated with an early induction of the ethylene-pathway (Gechev *et al.*, 2004). Furthermore, a range of species,

including *Fusarium oxysporum*, *Verticillium dahliae* and *Sclerotinia sclerotiorum* produce necrosis-and ethylene-inducing proteins (NEP) (Bae *et al.*, 2006; Bashi *et al.*, 2010; Zhou *et al.*, 2012). Similarly, the senescence-inducing phytotoxin victorin seems to trigger ethylene biosynthesis (Wolpert *et al.*, 2002).

The ambiguous effects of plant hormones in different pathosystems provides a target for pathogens who tap into the hormonal crosstalk to redirect the signaling towards the production of a susceptibility-promoting blend (Arshad & Frankenberger JR., 1992; Robert-Seilaniantz *et al.*, 2007; Denancé *et al.*, 2013; Kazan & Lyons, 2014). Clear examples are *X. campestris* pv. *vesicatoria* produced AvrBs3 effectors that induce auxin responsive genes and the PstDC3000 effector AvrPtoB that triggers accumulation of abscisic acid, promoting bacterial growth (Marois *et al.*, 2002; Robert-Seilaniantz *et al.*, 2007). Some pathogens go one step further and produce hormones or mimics thereof themselves. A well-known example is the production of the JA-mimic coronatine by *P. syringae* in an attempt to activate JA signaling instead of the effective SA-dependent defenses (Denancé *et al.*, 2013). Furthermore, *A. tumefaciens* produces auxins analogs and *B. cinerea* is able to produce abscisic acid, a hormone that can enhance susceptibility in tomato by repressing SA-defense responses (Audenaert *et al.*, 2002; Denancé *et al.*, 2013). As more plant pathogens are being reported to rely on functional hormone mimics or produce plant hormones themselves, hijacking defense hormone signaling networks as a virulence strategy is emerging to be a common theme (Robert-Seilaniantz *et al.*, 2007; Kazan & Lyons, 2014).

Several micro-organisms are able to produce ethylene, some of which are known plant pathogens. In fungi three ethylene biosynthesis pathways have been characterized (Figure 1.7). Ethylene can be synthesized from methionine via either 1-aminocyclopropane-1-carboxylic acid (ACC) or via α-keto-γ-methylthiobutyric acid (KMBA). The ACC pathway has first been characterized in higher plants and only two fungal species are known to produce ethylene via this pathway (Chagué, 2010). The KMBA pathway has been described in bacteria and many fungi. It involves deamination of L-methionine to KMBA by an unknown transaminase. KMBA is then further oxidized to ethylene in a non-enzymatic way in the presence of hydroxyl radicals (Chagué, 2010). KMBA can also passively convert to ethylene via photo-oxidation upon exposure to light, as was shown for *B. cinerea* (Cristescu *et al.*, 2002). In a third pathway ethylene is generated from the TCA cycle intermediate 2-oxoglutarate by an ethylene forming enzyme (EFE). EFE catalyses two simultaneous reactions, producing ethylene, succinate, guanidine and 1-pyroline-carboxylate from 2-oxoglutarate and L-arginine and needs Fe^{2+} as a cofactor (Figure 1.7). (Fukuda *et al.*, 1992; Chagué *et al.*, 2006; Eckert *et al.*, 2014). The EFE pathway has been described for several fungi and bacteria and the widespread presence of EFE homologs suggests that many more microorganisms have the capacity to produce ethylene via this route (Chagué, 2010; Eckert *et al.*, 2014).

The function of ethylene biosynthesis by microorganism is not always clear. Ethylene can promote spore germination in *B. cinerea* and *Colletotrichum gloeosporioides*, and several plant pathogens specifically produce ethylene as a virulence factor to promote host colonization (Chagué, 2010). Ethylene produced by *R. solanacearum* interferes with the signaling of Arabidopsis defense responses and *Efe* mutants of *Pseudomonas syringae* pv. *glycinea* were significantly reduced in their ability to grow *in planta* (Weingart *et al.*, 2001; Valls *et al.*, 2006). Moreover, some phytotoxin producers like *Verticillium dahliae* and *Fusarium oxysporum*, are able to produce microbial ethylene themselves, suggesting a role as toxin synergist (Chagué, 2010; Tzima *et al.*, 2010).

Ethylene can exert both positive and negative effects on plant ethylene biosynthesis. During normal growth and development, ethylene biosynthesis is typically regulated by a negative feedback loop to keep ethylene on a basal level. However, ethylene turns autocatalytic during fruit ripening and senescence, as well as during germination of pea seeds (Petruzzelli *et al.*, 2000; Alexander & Grierson, 2002; Wang *et al.*, 2002). Similarly, stress-induced ethylene is often autocatalytic and can be responsible for a large portion of damage observed upon pathogen attack (Stearns & Glick, 2003). It is not clear whether fungal ethylene could also trigger plant ethylene production. In the tomato-*B. cinerea* pathosystem, plant ethylene production precedes fungal ethylene, suggesting that plant ethylene production is a response to recognition of the fungus rather than to fungal ethylene (Cristescu *et al.*, 2002).

In the case of the necrotroph *C. miyabeanus*, ethylene is a clear susceptibility inducing factor, as application of ethylene signaling inhibitors strongly reduces symptoms, but externally applied ethylene exaggerated disease progression (De Vleesschauwer *et al.*, 2010). Microarray gene expression data and proteome analysis show a strong up-regulation of the ethylene biosynthesis pathway after *C. miyabeanus* inoculation (Kim *et al.*, 2014; Van Bockhaven *et al.*, 2015b). Moreover, the transcriptome of *C. miyabeanus*-infected plants at 12 hpi displayed strong similarities to that of rice seedlings treated with the ethylene precursor ACC (Garg *et al.*, 2012). Therefore, it seems feasible that *C. miyabeanus* could tap into the ethylene signaling pathway and enhances biosynthesis and signaling to induce susceptibility. In Chapters 2 and 4, we show that *C. miyabeanus* uses the phytotoxin ophiobolin A and tentoxin as virulence factors to promote disease. It is so far unclear if the production of these toxins is connected to the induction of ethylene biosynthesis and whether ethylene signaling could be involved in phytotoxin-induced effects. It was previously shown that *C. miyabeanus* can produce ethylene *in vitro* (Van Bockhaven, 2014). In this chapter we characterize two ethylene biosynthesis pathways and show that *C. miyabeanus* produces ethylene *in planta* during disease. Furthermore we analyse the effect of fungal ethylene production on disease progression and the involvement of toxins in ethylene enhanced susceptibility.

Results

C. miyabeanus infection triggers biphasic ethylene emission from rice leaves

Previously it was shown that ethylene signaling plays an important role in susceptibility of rice to *C. miyabeanus* and microarray gene expression analysis shows a strong induction of ethylene biosynthesis genes (De Vleesschauwer *et al.*, 2010; Van Bockhaven *et al.*, 2015b). To confirm the production of ethylene during infection and to get a better insight into the temporal dynamics, we monitored ethylene emission in real-time during infection of detached leaves using laser photo-acoustic detection (LPAD) (Figure 5.1). Similar patterns were obtained from an alternative setup, although there was some variation in the timing and levels of ethylene emission (Figure S5.1a).

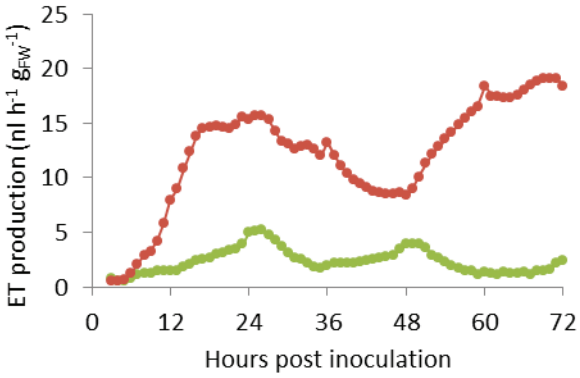


Figure 5.1. Temporal ethylene emission patterns after *Cochliobolus miyabeanus* strain Dm988 inoculation (red) of rice leaves compared with mock inoculation (green). Leaf pieces were inoculated with a spore suspension of Cm988 ($1x10^4$ spores ml^{-1}) or mock inoculated at T_0 = 11 am and incubated in 20-ml vials at room temperature under 12:12 light:dark. Ethylene was measured using laser photoacoustic detection (LPAD) during 72 hours post inoculation. Results of one representative experiment are presented.

Ethylene emissions from mock inoculated leaves followed a circadian rhythm in both setups, a pattern that was completely lost during infection. After inoculation of the leaf pieces, 2 distinct ethylene emission phases can be distinguished. A first peak emerged early during infection and coincides with pathogen penetration and recognition and ophiobolin A production by *C. miyabeanus* (Chapter 2). By the time the first peak decreases, small necrotic lesions are visible on the leaves. A second peak emerges from 36 hpi onwards, is characterized by the development of spreading chlorosis, and coincides with the production of tentoxin by the pathogen (Chapter 4). From these results it is clear that inoculation triggers a distinct ethylene emission pattern, confirming the up-regulation of ethylene biosynthesis genes seen in microarray data.

Previously it was shown that inhibition of plant ethylene signaling strongly enhances resistance (De Vleesschauwer *et al.*, 2010). When we blocked plant ethylene biosynthesis with aminooxyacetic acid (AOA, inhibitor of ACC synthase) and $CoCl_2$ (inhibitor of ACC oxidase), the plants became similarly resistant to brown spot disease (Figure 5.2a). This indicates that the

ability of *C. miyabeanus* to cause brown spot symptoms relies on plant ethylene metabolism. Successful infection is characterized by extensive cell death, associated with ophiobolin A production (Chapter 2). To test if ethylene signaling is needed for ophiobolin A induced cell death, we inhibited plant ethylene biosynthesis with AOA and $CoCl_2$ prior to treatment with 100 µM ophiobolin A. Ophiobolin A was unable to cause disease symptoms in the absence of ethylene (Figure 5.2b), suggesting that induction of ethylene is an integral part of the fungal infection strategy.

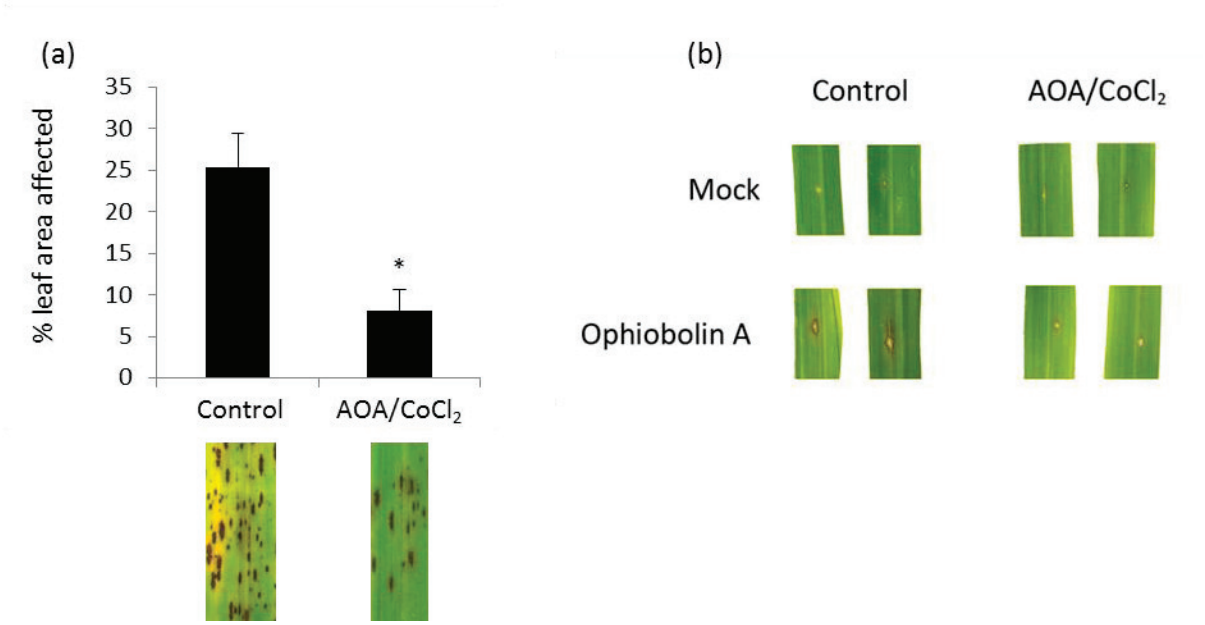


Figure 5.2. Effect of the plant ethylene biosynthesis inhibitors aminooxyacetic acid (AOA) (500 µM) and $CoCl_2$ (500 µM). (a) Rice leaf pieces were inoculated with a spore-solution ($1x10^4$ spores/ml) of *Cochliobolus miyabeanus* strain Cm988 24 hours after start of treatment with AOA and $CoCl_2$. Disease severity is measured as percentage affected leaf surface at three days post inoculation, using APS Assess software. Data presented are means ± SE of the pooled data of three independent repeats (n = 18) * indicate significant difference with WT by Wilcoxon Rank-Sum non-parametric test ($p < 0.05$). **(b)** Effect of ethylene biosynthesis inhibition on ophiobolin A-induced necrosis at 48 hpt. Leaf pieces were punctured and treated with a 10-µl drop of a mock solution, 100 µM ophiobolin A, 500 µM AOA and 500 µM $CoCl_2$ or a combination. Pictures feature representative symptoms caused by each treatment. Results are reproducible (n = 24).

C. miyabeanus produces ethylene *in vitro* via two distinct pathways

In fungi three ethylene biosynthesis pathways have been characterized (Figure 1.7). Experiments using the EFE inhibitor 2,2-bipyridyl strongly reduce disease symptoms, suggesting that *C. miyabeanus* uses the EFE pathway to produce ethylene (Van Bockhaven *et al.*, 2015a). However, because 2,2-bipyridyl also inhibits iron uptake, an essential cofactor for *C. miyabeanus*, effects could be secondary.

To test which ethylene pathways are operative in *C. miyabeanus*, we measured the ethylene emission of Cm988 spores germinating in liquid medium supplemented with a plant extract and appropriate precursors for each of the respective pathways (Figure 5.3). The fungus was not able

to produce ethylene from the plant ethylene precursor ACC, eliminating this pathway in *C. miyabeanus*. Interestingly, high levels of ethylene were produced from either methionine or KMBA. A small amount of ethylene was also emitted from mock inoculated medium supplemented with KMBA, indicating a passive transformation to ethylene, probably via a photo-oxidation reaction in the presence of light (Chagué *et al.*, 2002). Confirming previous results using 2,2-bipyridyl, *C. miyabeanus* is also able to produce ethylene from 2-oxoglutarate and arginine, although the levels of ethylene production were much lower compared with methionine or KMBA as precursor. When nitrate was added to the medium as a nitrogen source instead of the precursors, no ethylene was produced (Jonas Van Bockhaven, personal communication). This indicates that the fungus relies on external precursors for the production of ethylene.

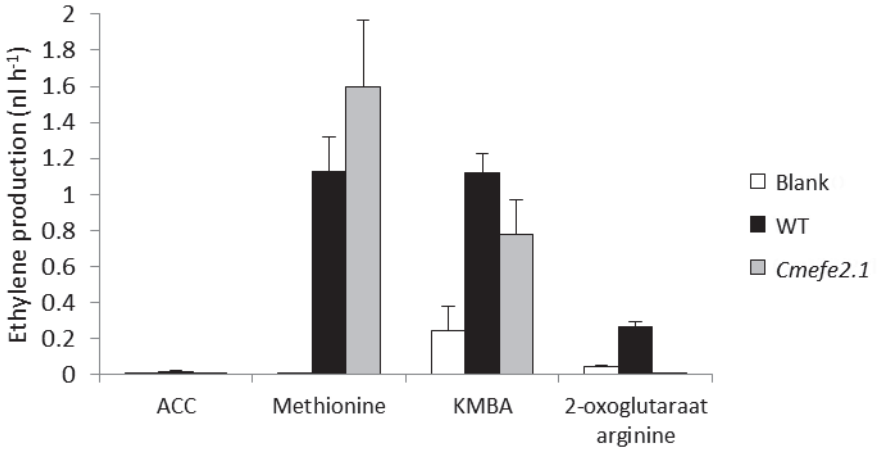


Figure 5.3. Ethylene emission from wild type *Cochliobolus miyabeanus* Cm988 and *Cmefe2.1* in liquid culture during 48 h. Fungal spores ($1x10^4$ spores) were inoculated in 5ml liquid Czapeck medium, supplemented with a sterile plant extract and ACC (10 mM), Methionine (5 mM), KMBA (10 mM) or 2-oxo-glutarate (5 mM) and arginine (1.25 mM) respectively. Ethylene was measured after 48 h of accumulation using laser photoacoustic detection (LPAD).

The ability to produce ethylene from 2-oxoglutarate suggests the existence of the EFE pathway in *C. miyabeanus*. A putative EFE homolog (CmEfe, JGI protein ID 109913) was identified in the genome sequence of *C. miyabeanus* strain WK1C based on a BlastP search with the EFE protein sequence from *Pseudomonas syringae* pv. *phaseolicola* as template (Uniprot accession P32021). Analysis of conserved domains using InterProScan 5 reveals that CmEfe features a non-haem dioxygenase N-terminal domain (DIOX_N, PF14226) and an oxoglutarate/iron-dependent dioxygenase domain (FE2OG_OXY, PF1471; 2OG-FeII_Oxy, PF03171) (Jones *et al.*, 2014). To confirm the function of *CmEFE* in ethylene production by *C. miyabeanus,* we deleted the gene via homologous recombination. The *Cmefe2*.1 mutant strain did no longer produce ethylene on a medium supplemented with 2-oxoglutarate and arginine. Ethylene production via methionine or KMBA was not affected (Figure 5.3). The production of ethylene via the EFE pathway probably does not play an important role in growth and development, as fungal growth rate and

conidiation were not different from the WT (Figure 5.4a and Figure 5.4b). Surprisingly, the *Cmefe2.1* mutant caused more disease symptoms than the WT strain (Figure 5.4c).

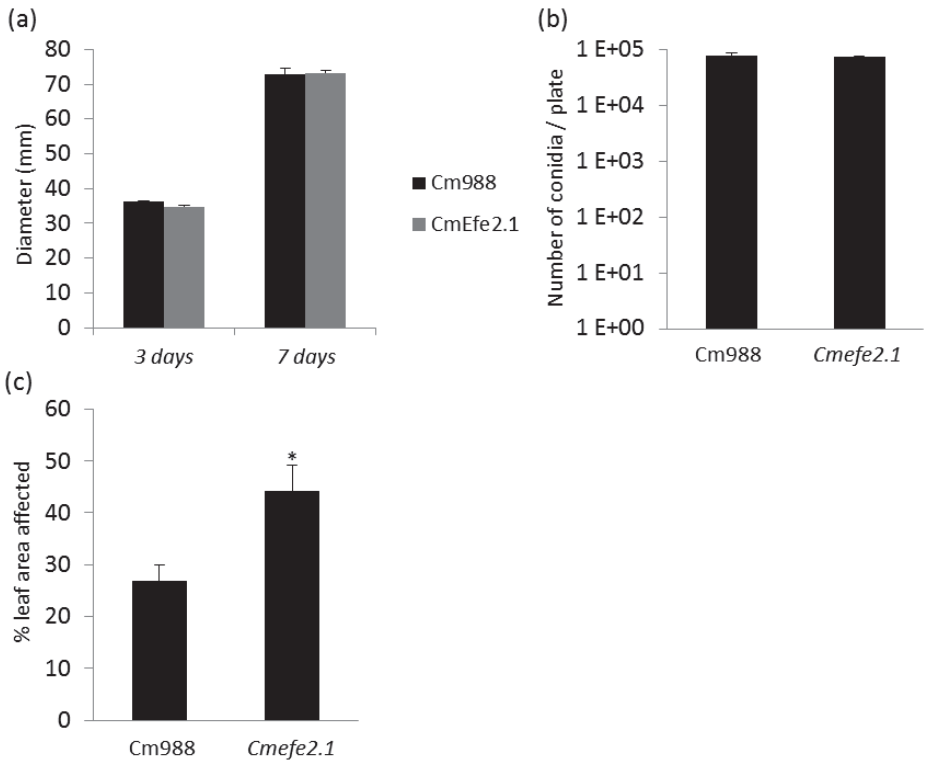


Figure 5.4. Effect of *CmEFE* deletion on fungal growth and development and brown spot disease severity. (a) Fungal growth rate was measured as the colony diameter of Cm988 WT and *Cmefe2.1* mutants after three or seven days of growth on PDA plates in the dark at 28°C. Results are means (± SE) of the pooled data of two independent experiments (n = 6). **(b)** Conidiation of Cm988 WT and the *Cmefe2.1* mutant was measured as the number of spores produced during 14 days of growth on PDA plates in the dark at 28 °C. Results are means (± SE) of the pooled data of two independent experiments (n = 6). **(c)** Difference in disease severity during infection with Cm988 WT and the *Cmefe2.1* mutant. Leaf pieces of five week old rice plants were spray inoculated with a spore suspension (1 x 10^4 conidia ml^{-1}) of the respective strain. The percentage affected leaf area was measured at 3 dpi using APS assess software. Data presented are means ± SE of pooled data of three independent experiments (n = 18). * indicate significant differences with WT by Wilcoxon Rank-Sum non-parametric test ($p < 0.05$).

Because the production levels of ophiobolin A and tentoxin are also linked to the severity of disease symptoms (see Chapters 2 and 4), we compared phytotoxin production patterns between WT and *Cmefe2.1* mutants (Figure 5.5a and Figure 5.5b). Toxin production displayed a high degree of variability, but no significant differences could be observed, indicating that there is no clear trade-off between EFE-steered ethylene production and toxin biosynthesis. Unfortunately, the enzymes and corresponding genes responsible for the transaminase reaction in the KMBA-based ethylene biosynthesis pathway are not known and no inhibitors are available to block fungal ethylene production from methionine.

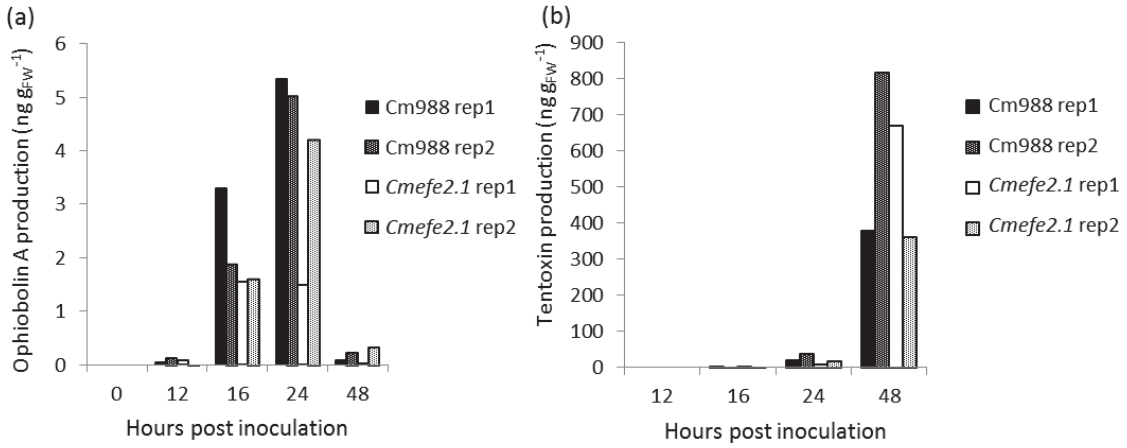


Figure 5.5. Temporal toxin production patterns during infection of rice (*Oryza sativa*) leaf pieces inoculated with *Cochliobolus miyabeanus* WT or *Cmefe2.1* mycelial suspension. Leaf pieces of five week old rice plants were spray inoculated with a spore suspension (1 x 10^4 conidia ml^{-1}) of the respective strain. Toxins were extracted from infected leaf material at the indicated time points post inoculation and quantified using UPLC-MS/MS. **(a)** Ophiobolin production. **(b)** Tentoxin production. Data of two biological replications are presented.

C. miyabeanus produces ethylene *in planta*

The above results show that *C. miyabeanus* possesses the capacity to produce ethylene *in vitro* via two distinct pathways. As ethylene can enhance plant susceptibility, we wondered whether fungal ethylene could contribute to rising ethylene emissions during infection and enhance disease development. To eliminate plant ethylene, plants were treated with a combination of 500 µM AOA and 500 µM $CoCl_2$, starting 24 hours before inoculation and administered throughout infection (Figure 5.6). Application of the inhibitors resulted in a complete abolishment of ethylene produced by mock-inoculated plants. Interestingly, small amounts of ethylene were measured from AOA/$CoCl_2$ treated leaf pieces after infection. AOA and $CoCl_2$ specifically block plant ethylene biosynthesis, and do not affect fungal ethylene production (Figure S5.2). Hence the observed ethylene most likely originates from the fungus. Fungal ethylene was detectable from 12 hpi onwards and rose to higher levels after 48 h.

Gene expression analysis of Cm988-inoculated plants revealed that *CmEFE* transcript levels respond to plant infection and peak at 48 hpi, concomitant with the second ethylene burst (Van Bockhaven, 2014). Pathogens rely strongly on the nutrients provided by the host to sustain their metabolism and the *in vitro* experiments showed the dependency on precursors for fungal ethylene production (Figure 5.3). Under normal conditions, leaves exhibit low 2-oxoglutarate levels, but microarray analysis revealed a significant up-regulation at 12 hpi of two glutamate dehydrogenases (OsGDH2 and OsGDH3) responsible for the conversion between glutamate and 2-oxoglutarate in rice (Yuan *et al.*, 2007; Van Bockhaven *et al.*, 2015a). At the same time, the oxaloacetate production and the plant ethylene biosynthesis pathway are also up-regulated, suggesting an increased production of the intermediate methionine (Kim *et al.*, 2014; Van

Bockhaven *et al.*, 2015b). However, the up-regulation of both fungal *CmEFE* and plant 2-oxoglutarate biosynthesis components suggests that the fungal ethylene observed during disease might originate from the EFE-pathway.

To test this hypothesis, we monitored the ethylene emission pattern of rice leaves infected with *Cmefe2.1* mutant spores, in the absence or presence of plant ethylene inhibitors (Figure 5.6). Strikingly, not only did the mutants still produce ethylene, the production levels were slightly enhanced compared with the WT strain when plant ethylene biosynthesis was blocked. According to this observation, the second ethylene peak in untreated rice leaves was higher than during infection with the parental strain (Figure 5.6). Similar patterns were obtained from the alternative setup (Figure S5.1b). This suggests that the KMBA and EFE pathways may be redundant for ethylene production during disease. Moreover, the increased level of disease caused by *Cmefe2.1* mutants might be directly related to higher ethylene production levels.

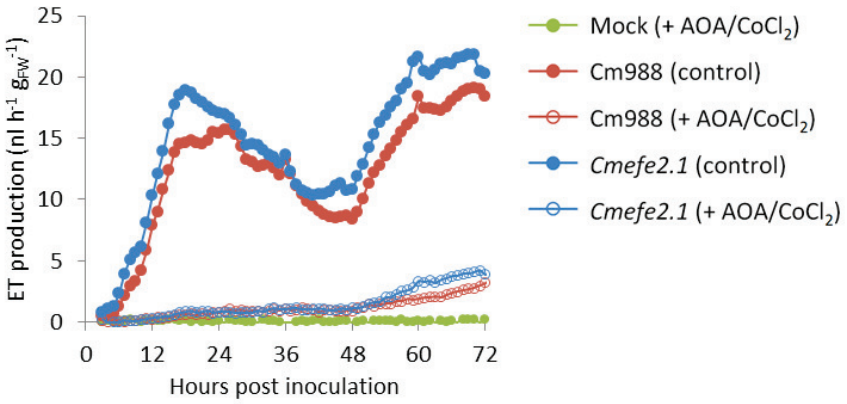


Figure 5.6. Temporal ethylene emission patterns during mock, WT or *Cmefe2.1 C. miyabeanus* infection of rice leaves, mock treatment (●) or treated with 500 µM AOA and 500 µM $CoCl_2$ (24 h before and continuously throughout infection) (○). Leaf pieces of five week old rice plants were spray inoculated with a spore suspension (1 x 10^4 conidia ml^{-1}) of the respective strain at T_0 = 10 am. Ethylene was measured in stop and flow mode at the indicated time points using laser photoacoustic detection (LPAD) from leaf pieces incubated in 20-ml vials at RT under 12:12 light:dark. Results of one representative experiment are presented.

To test if the fungal KMBA pathway could play a role during infection, we added the plant ethylene biosynthesis inhibitor aminoethoxyvinylglycine (AVG) (1 mM), methionine (5 mM), KMBA (10 mM) or a combination to the spore solution before inoculation (Figure 5.7). AVG specifically inhibits plant ethylene and has no effect on fungal ethylene biosynthesis (Figure S5.1). AVG strongly reduced disease symptoms, confirming the role of plant ethylene in susceptibility. When methionine or KMBA were added to the spore solution, symptoms were slightly enhanced, this suggests that methionine can be used by the fungus or the plant to enhance ethylene production although the difference was statistically not significant. To differentiate between methionine used by the plant or the fungus, we combined AVG with methionine. Surprisingly, a similar increase in leaf damage was measured when methionine was added in the absence (+ 12.9% leaf damage, $p > 0.05$) or presence (+ 10.6% leaf damage, $p < 0.05$)

of AVG. These data suggest that the fungus can produce ethylene via the KMBA pathway during disease and that fungal ethylene can enhance susceptibility.

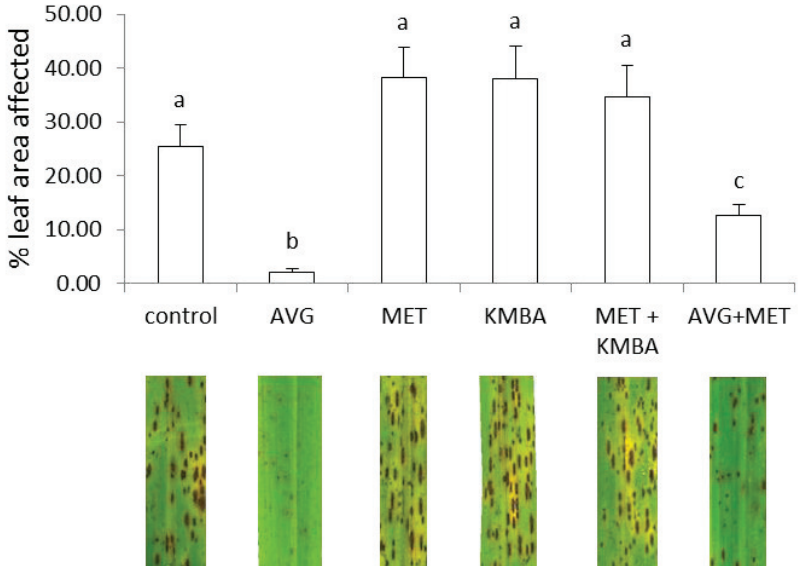


Figure 5.7. Effect of AVG (1 mM), MET (5 mM), KMBA (10 mM) or a combination, on brown spot disease severity. AVG: aminoethoxyvinylglycine, specific plant ethylene inhibitor; MET: Methionine, precursor of plant and fungal ethylene biosynthesis; KMBA: Precursor of plant and fungal ethylene biosynthesis. Chemicals or a solvent control were added to a *Cochliobolus miyabeanus* strain Cm988 spore solution ($1x10^4$ spores ml^{-1}) before spray-inoculation of rice (*Oryza sativa)* leaf pieces. The percentage affected leaf area at 3 days post inoculation was calculated using APS Assess software and used as a measure for disease severity. Results are means (± SE) of the pooled data of three independent biological repeats (n = 18). Statistical analysis by Kruskal-Wallis and Mann-Whitney U multiple comparison non-parametric tests after Bonferroni correction ($\alpha = 0.05$).

Connection between ethylene emissions and phytotoxin production

The first ethylene peak clearly coincides with fungal penetration and is induced by the presence of the pathogen or pathogen-derived compounds. Fungal ethylene could be a possible trigger for plant ethylene induction. However, the fungus clearly needs access to plant metabolites in order to induce its ethylene biosynthesis. Hence it is likely that other factors could trigger plant ethylene, after which fungal ethylene could enhance the ethylene responses.

Some fungal phytotoxins, like AAL-toxin, can trigger the plant ethylene pathway (Gechev *et al.*, 2004). Given that ophiobolin is produced in low amounts by germinating spores (Xiao *et al.*, 1991) and strong ophiobolin A production coincides with the first ethylene burst (Chapter 2), it is a valid candidate for an ethylene trigger. However, when rice suspension cells are treated with ophiobolin A, the ethylene responsive gene *EBP89* is not induced (Figure 5.8a). Furthermore, the first ethylene burst is very similar after inoculation with WT and ophiobolin-deficient *Cmops9.*1 mutant spores, indicating that ophiobolin production is not prerequisite for the first ethylene burst (Figure 5.8b).

The second ethylene peak coincides with tentoxin production and is characterized by spreading chlorosis (Chapter 4). As expected, the first ethylene burst was not different upon infection with tentoxin-deficient *Cmnps3* mutants compared with WT infection. However, ethylene emission patterns start to diverge from 24 hpi onwards, around the time of induction of tentoxin production *in planta*. Interestingly, *Cmops9.1* infection results in a similar reduced second ethylene peak (Figure 5.8b).

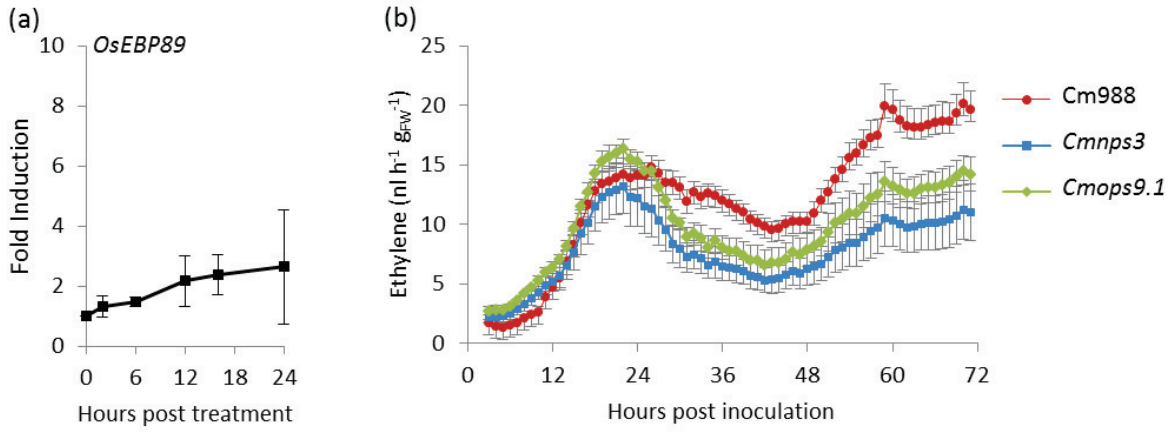


Figure 5.8. Effect of ophiobolin A and tentoxin on ethylene production. (a) Relative expression of the ethylene-responsive gene *OsEBP89* in rice suspension cells after treatment with 100 µM ophiobolin A. Gene expression levels were normalized using *OseIF1a* as an internal reference and expressed relative to control gene expression at each time point. Results represent means (± SE) of the pooled data of three independent experiments (3 biological x 2 technical repeats). **(b)** Temporal ethylene emission patterns from rice leaves after inoculation with a spore suspension (1x10^4 spores ml^{-1}) of wild type Cm988, *Cm988ΔCmops9.1* (ophiobolin-deficient mutant) or *Cm988ΔCmnps3.1* (tentoxin-deficient mutant) at T_0 = 10 am. Ethylene was measured using laser photo-acoustic detection (LPAD) from infected leaf pieces, incubated in 20 ml vials at RT under 12:12 light:dark. Data presented are means ± SE of 3 biological repeats.

Discussion

The gaseous plant hormone ethylene regulates many growth and developmental processes, and is involved in several responses to abiotic stresses and pathogen attack. Whereas ethylene effects in plant development are well described, its influence on plant resistance is more complex. As is the case for most plant hormones involved in plant-microbe interactions, the specific effect of ethylene on disease resistance depends strongly on the pathogen encountered.

Interestingly, ethylene has been associated with many processes leading to cell death. Studies with ethylene-signaling mutants show a reduced cell death phenotype and blocked reactive oxygen accumulation (Moeder *et al.*, 2002). Furthermore, ethylene plays a crucial role in the H_2O_2 burst that accompanies camptothecin-triggered PCD in tomato suspension cells (De Jong *et al.*, 2002). It seems plausible that ethylene is required, though not sufficient, for regular PCD and that its function is linked to controlling the extent of cell death, possibly via an enhanced production of ROS (Hoeberichts & Woltering, 2003). Furthermore, ethylene is involved in leaf senescence, a type of PCD that is not localized but occurs at organ-level and typically progresses slower than other types of PCD (Trobacher, 2009). Senescence is characterized by a remobilization of nutrients from the leaf to other organs, preceded by a switch from photosynthetic processes to catabolic break-down (Lim *et al.*, 2007). Although it was previously believed that ethylene generally confers resistance to necrotrophs, it is now clear that this view is overly simplistic (Adie *et al.*, 2007; De Vleesschauwer *et al.*, 2013). Given the close relationship between ethylene and cell death, it is actually not surprising to find that ethylene can enhance susceptibility towards several necrotrophs, and that some necrotrophs even produce phytotoxins that rely on the ethylene pathway (Asai *et al.*, 2000; Hoeberichts & Woltering, 2003; Gechev *et al.*, 2004; Mase *et al.*, 2012).

Ethylene production is often one of the early responses upon specific recognition of different wound-derived and pathogen elicitor molecules during pathogen attack and is part of a stereotypic defense response (Boller & Felix, 2009). However, temporal dynamics of ethylene during disease progression have been poorly studied. With the availability of sensitive photo-acoustic laser-based ethylene detection, a few ethylene production patterns have been described upon pathogen attack. Ethylene production during the *B. cinerea*–tomato interaction is characterized by a single ethylene peak induced after 24 h (Cristescu *et al.*, 2002). On the other hand, ethylene production in tobacco upon infection with avirulent *P. syringae* pv *tabaccci* is biphasic and reminiscent of a biphasic oxidative burst (Mur *et al.*, 2009). Biphasic stress-induced ethylene emission may be more general, whereby the first burst would initiate a defense response, such as induction of PR-genes, and the second peak is the effect of a positive feedback loop and is characterized by senescence and chlorosis (Stearns & Glick, 2003).

Our study shows that the ethylene production during *C. miyabeanus* infection of rice leaves is similarly biphasic. The two peaks differ from each other in several ways. The first peak is induced upon penetration of the pathogen and lasts for a relatively short period. Despite the fact that this peak coincides with ophiobolin A production and is characterized by the development of necrotic lesions, it seems to be independent of toxin production or disease-inducing ability of the pathogen. Although fungal ethylene could be involved in the induction of the peak, it could also represent a response triggered by PAMPs or other pathogen derived signals. The second ethylene burst, on the other hand, is induced two days later and lasts much longer. It is hallmarked by developing chlorosis, reminiscent of senescing tissue and the extent correlates with symptom development. This second peak coincides with tentoxin production. As we show in Chapter 4, tentoxin production is correlated with chlorosis and may accelerate senescence. Therefore tentoxin could be directly connected with the higher ethylene emissions during the second peak. Adding to this hypothesis is the observation that tentoxin deficient mutants trigger a much lower second ethylene burst. Interestingly, the inability to produce ophiobolin also results in lower ethylene production during the second phase. This may reflect the smaller leaf areas that are damaged by the mutants. On the other hand, preliminary results show that *CmOps9.1* also produces lower amount of tentoxin (Figure S5.3), therefore it is possible that the reduced levels of ethylene during *Cmops9.1* infection are due to lower tentoxin production. Although it is also possible that tentoxin has a direct ethylene-promoting effect, it is similarly plausible that the second ethylene peak is illustrative of the overall level of leaf senescence.

Given the clear susceptibility enhancing functions of ethylene during brown spot disease, it seems likely that *C. miyabeanus* can somehow hijack plant ethylene to enhance disease. Although ophiobolin A needs an active ethylene signaling pathway in order to cause symptoms in a pathogen-free system, ophiobolin A by itself is probably not responsible for the induction of ethylene. Like many plant pathogens, *C. miyabeanus* is able to produce ethylene by itself. We show here that *C. miyabeanus* possesses no fewer than two different ethylene-biosynthesis routes, reflecting an evolutionary advantage of the ability to produce ethylene. Although there are clear indications that the fungus produces ethylene during disease, it is not sure whether the presence of fungal ethylene is the actual trigger for plant ethylene production. Fungal ethylene may also have an additive effect and enhance the plant ethylene-response. Unfortunately, no inhibitors are known to repress the KMBA-pathway and genes involved in the pathway are not yet elucidated, therefore we could not completely inhibit fungal ethylene. Disconnecting the EFE-pathway does not abolish the first ethylene peak and eventually results in higher fungal ethylene emissions during the second phase. This seems ambiguous. If the pathogen can induce higher ethylene emission via the KMBA-pathway, why would the fungus rely on the EFE-pathway under normal circumstances? A trade-off would be expected, but neither ophiobolin nor tentoxin production was affected in *Cmefe2.1* mutants. Furthermore, growth and development *in vitro* were not different either. It is possible that CmEfe has additional yet to be resolved effects, or may affect fungal

development *in planta*. Furthermore, ethylene production from KMBA involves a two-step reaction. First, L-methionine is deaminated to KMBA by a specific unidentified transaminase (Primrose, 1977). Subsequently KMBA is non-specifically oxidized to ethylene in the presence of hydroxyl radicals, produced in a spontaneous reaction following reduction of Fe(III)EDTA by NADH:Fe(III)EDTA oxidoreductase (Ogawa *et al.*, 1990). On the contrary, the EFE-pathway involves a more efficient single-step reaction and may therefore be the preferred pathway (Weingart *et al.*, 2001).

It remains unclear what triggers the first ethylene burst. Recently, 31 secreted fungal proteins were identified from *C. miyabeanus* infected leaf tissue using a proteomics approach (Kim *et al.*, 2014) and comparative genomics predicted the existence of 143 small secreted proteins that could act as effector proteins in addition to several uncharacterized NRPS/PKS genes (Condon *et al.*, 2013). This enormous pool of candidate effectors that are yet to be characterized could provide the key to the induction of plant ethylene biosynthesis. A first preliminary screening of *in vitro* produced compounds, divided in fractions by UPLC, suggests the existence of ethylene-inducing factors (Figure S5.4). Future research could focus on these compounds.

Several roles for ethylene in defense responses have been reported. As mentioned earlier, ethylene can enhance susceptibility by supporting pathogen-triggered PCD or senescence. However, ethylene can also contribute to plant resistance by the induction of cell wall strengthening processes and induction of pathogenesis related proteins (Adie *et al.*, 2007). Our results show that the first ethylene-peak is prerequisite for the ophiobolin A triggered cell death during brown spot disease. Furthermore, ethylene contributes to spreading senescence during the second ethylene peak. The metabolic changes associated with senescence, especially those in amino acid metabolism, have previously been shown to be profitable to *C. miyabeanus* proliferation (Chattopadhyay & Bera, 1978). Furthermore, although ethylene can enhance phenylpropanoid metabolism in many plant species, an opposite effect seems at stake in rice (Hyodo & Yang, 1971; Chappell *et al.*, 1984; Tomas-Barberán *et al.*, 1997; Ballester *et al.*, 2011; Van Bockhaven *et al.*, 2015a). Treatment with the plant ethylene signaling inhibitor silver thiosulfate (STS) resulted in significantly higher accumulation of phenolic compounds at the infection site. Furthermore, previous experiments at our lab show that ethylene antagonizes phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (PPO) activity, resulting in reduced amounts of soluble phenolics produced during infection (Van Bockhaven, 2014). Given that phenolics have a clear beneficial effect on brown spot disease resistance, the antagonistic effect of ethylene can be another important role in pathogen-triggered susceptibility (Shabana *et al.*, 2008). It is obvious that induction of the ethylene biosynthesis pathway is highly desirable for *C. miyabeanus*. Future research should focus on the role of fungal ethylene and the mechanism of ethylene-induction during infection.

Materials and methods

Biological material and general growth conditions

C. miyabeanus strain Cm988 (De Vleesschauwer et al, 2010) and Cm988 mutants *CmOps9.1* (Chapter 2), *CmNps3.1* (Chapter 4) and *Cmefe2.1* were used. The strains were grown on potato dextrose agar (Difco, BD, Belgium) under dark conditions at 28°C.

The *Oryza sativa* japonica cultivar Nipponbare was grown in commercial potting soil (Universal; Snebbout, Belgium) in a growth room under 12 hours light/12 hours dark at 28°C as described previously (De Vleesschauwer et al, 2010).

Fungal transformation and PCR confirmation of *CmEFE* gene deletion

The *CmEFE* gene was deleted using homologous recombination as described by (Bi *et al.*, 2013). The entire selection marker fragment "hygB", including the promoter, was amplified from plasmid pUCATPH using M13R and M13F primers, then fused to the 5' and 3' flanking fragments of the target *CmEFE* gene. PCR amplification of transformation constructs was carried out with phusion high-fidelity DNA polymerase (Thermo Scientific) following the manufacturer's instructions. Primer sequences used for gene deletion can be found in the Primer List at the end of this thesis.

Transformation of *C. miyabeanus* was carried out as described in Chapter 2. Four confirmed transformants were selected and scanned for ethylene production on czapeck agar supplemented with a sterile rice extract, 2-oxoglutarate and arginine using GC-MS as described by (Van Bockhaven *et al.*, 2015a). *Cmefe2.1* was selected for further analyses.

Fungal growth rate and conidiation

A 6 mm diameter plug from a 7-day old colony of the respective strain was placed in the center of a PDA plate and incubated at 28°C in the dark. Fungal growth was measured as the average colony diameter after three or seven days of growth. After 14 days of growth, the conidia from each plate were suspended in 15ml if double distilled water and counted using a Bürker counting chamber. The experiment was repeated once, using 3 PDA plates per strain.

Quantification of ophiobolin A and tentoxin

Ophiobolin A was quantified as described in Chapter 2. Tentoxin was quantified as described in Chapter 4. Leaf pieces were inoculated with fungal mycelium as described in Chapter 2.

Infection assays

For inoculation of leaf pieces, the youngest fully developed leaves of 5-week old rice plants were cut in 7 cm pieces and kept in square petri dishes (9 x 9 cm, greiner bio one), lined with moistened tissue. *C. miyabeanus* conidia were harvested as described by (Thuan *et al.*, 2006) and resuspended in 0.02% Tween20 to a final density of $1x10^4$ conidia /ml. Leaf pieces were spray-inoculated with 0.5 ml conidial suspension/petri dish. The leaf pieces were kept in the dark at 28°C and moved to growth chamber conditions after 16h. Disease symptoms were quantified as percentage affected leaf surface at 3 dpi using APS Assess Software. Statistical analysis was performed using non-parametric Kruskal-Wallis Rank test ($\alpha = 0.05$).

Pharmacological Experiments

The ethylene biosynthesis inhibitors AOA (aminooxyacetic acid) and $CoCl_2$ were dissolved in water at the indicated concentrations and were applied by cutting the two youngest fully developed leaves in 7 cm segments and putting them in the chemical solutions 24 h prior to inoculation. Afterwards, the leaf segments were placed in square Petri dishes (Greiner Bio-One, 688102) lined with paper towels moistened with the inhibitor solution at the same concentration and inoculated as described for the Infection assays. Methionine, KMBA and the plant ethylene inhibitor AVG respectively were added to the spore solution before inoculation at the indicated concentration.

Quantification of ethylene accumulation

The production of ethylene by C. miyabeanus *in vitro* was measured from mycelium grown in liquid Czapek medium (Kubo et al., 1989) containing 40 mM sucrose, 5.6 mM KH2PO4, 0.14 mM KH2PO4, 2 mM MgSO4, 0.04 mM FeSO4, 6.7 mM KCl, 5 mM nitrogen and a sterile rice extract (pH 5.6). Nitrogen was supplied in the form of nitrate, 5 mM DL-methionine, 10 mM KMBA, 10 mM ACC or a combination of 5 mM oxoglutarate and 1.25 mM arginine respectively. A total amount of 5ml medium was inoculated with a conidial suspension ($1x10^4$ conidia/ml) and cultured in airtight vials at 28°C for 1 or 2 days. Total accumulated ethylene in the head space was detected and quantified using a sensitive photo-acoustic laser-based ethylene detector (Sensor Sense, ETD-300) in stop and flow modus and calculated as nL/h.

To study ethylene emission rates in infected leaf pieces, 2 different setups were used. The two youngest fully developed leaves of five-week old plants were detached and cut into pieces 1 day beforehand (to avoid interference of wound ethylene) and incubated in H_2O until spray inoculation. In the first setup, 7-cm pieces were spray-inoculated with 4 ml conidial suspension containing $1x10^4$ conidia/ml in 0.02% Tween20 and incubated in square cuvettes (9 x 9 cm) under 12:12 light:dark at 28°C. In the second setup, 4-cm pieces were spray-inoculated with 4 ml conidial suspension containing $1x10^4$ conidia/ml in 0.02% Tween20 and incubated vertically in 5ml cuvettes lined with moistened tissue paper under 12:12 light:dark at RT. In both setups the cuvettes were connected to a sensitive laser-based ethylene detector (Sensor Sense, ETD-300) in

combination with a real time gas flow through system (LPAD) as described by Cristescu et al. (2013) and real-time ethylene emissions were monitored during 72 hpi. Ethylene production was calculated as nl h^{-1} g^{-1} fresh leaf weight.

RNA extraction and quantitative RT-PCR

cDNA was prepared from treated rice suspension cells as described in Chapter 2. The amount of rice mRNA was normalized using elongation factor eIF1α (LOC Os03g08020) as internal control and mock treated samples were used as calibrator at each time point. The data were analysed using Stratagene's Mx3005P software. Nucleotide sequences of used primers can be found in the Primer List at the end of this thesis.

Supplementary figures

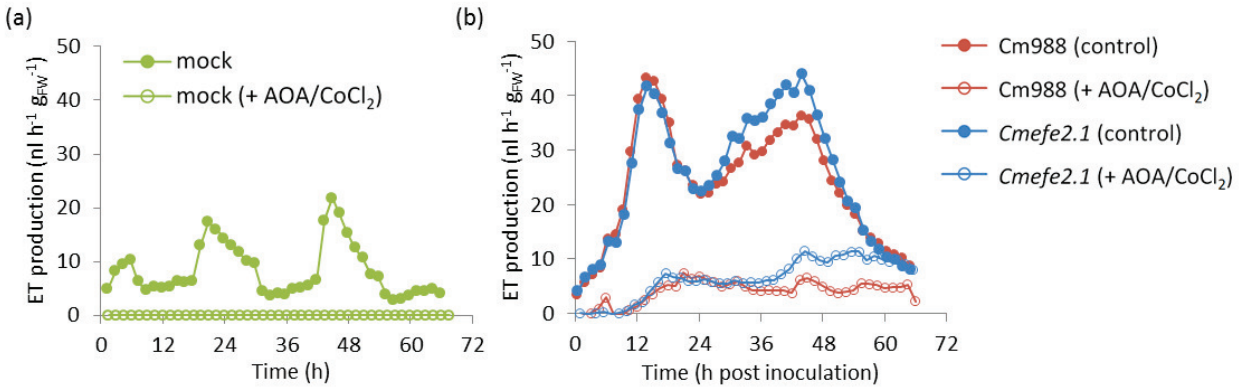


Figure S5.1. Temporal ethylene (ET) emission patterns from leaf pieces during mock treatment (●) or treatment with 500 µM AOA and 500 µM $CoCl_2$ (24 h before and continuously throughout infection) (○). (a) ET emissions from mock inoculated leaf pieces. **(b)** ET emissions after inoculation of leaf pieces with *Cochliobolus miyabeanus* WT or *Cmefe2.1 mutant* spores. Leaf pieces of five week old rice plants were spray inoculated with a spore suspension (1 x 10^4 conidia ml^{-1}) of the respective strain. Ethylene was measured using laser photoacoustic detection (LPAD). Leaf pieces were incubated in square petri dishes (9 x 9 cm) at 28°C under 12:12 light:dark. T_0 corresponds to 4 pm. Results of one representative experiment are presented. .

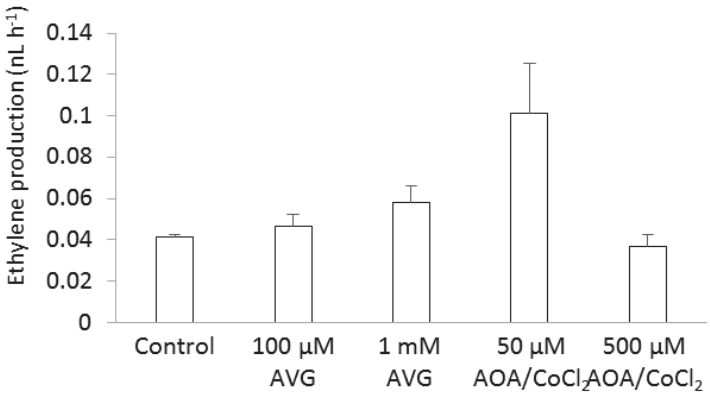


Figure S5.2. Effect of different plant ethylene biosynthesis inhibitors on the production of fungal ethylene via the KMBA pathway by *Cochliobolus miyabeanus* strain Cm988. Liquid Czapeck medium, supplemented with a sterile plant extract and methionine (5 mM) was inoculated with 1 x 10^4 conidia. Total ethylene was measured using LPAD after 24 h of accumulation in the head space. Data presented are means ± SD (n = 4).

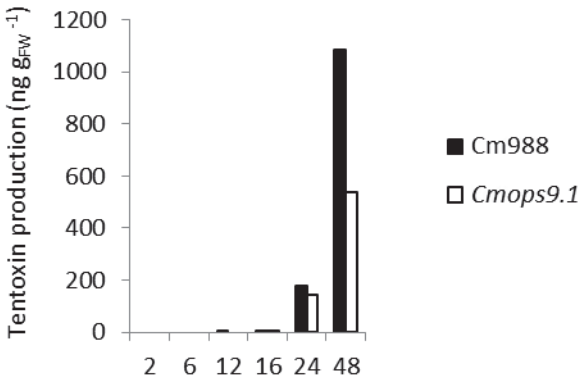


Figure S5.3. Temporal tentoxin production during infection of rice (*Oryza sativa*) leaf pieces by *Cochliobolus miyabeanus* wild type Cm988 and *Cmops9.1*. Leaf pieces of five week old rice plants were spray inoculated with a spore suspension (1 x 10^4 conidia ml^{-1}) of the respective strain. Tentoxin was extracted from infected leaf material at the indicated time points post inoculation and quantified using UPLC-MS/MS. Preliminary data (n = 1).

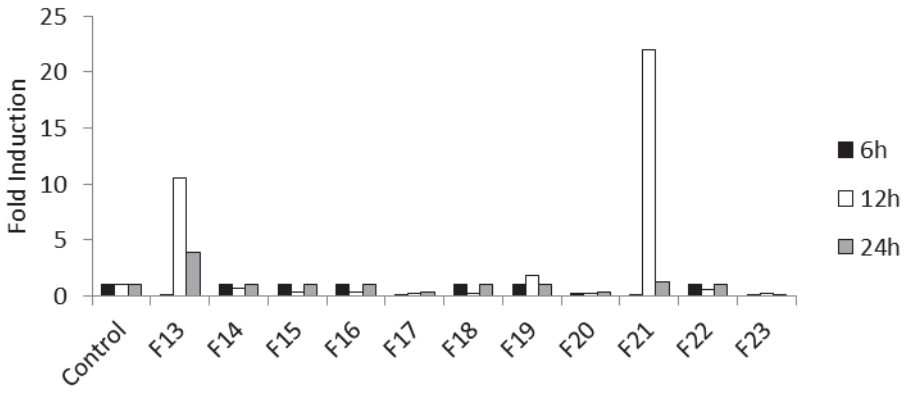


Figure S5.4. Fold induction of the ethylene-responsive gene *OsEBP89* in rice (*Oryza sativa*) suspension cells treated with different UPLC-seperated fraction containing compounds produced by *Cochliobolus miyabeanus* during growth in a liquid Fries medium. Compounds were extracted from sterile liquid culture supernatant using ethylacetate:acetonitrile and separated in different fractions using UHPLC. Fractions were collected each minute from T = 13 min until T = 23 min. Gene expression of *OsEBP89* was analysed at 6, 12 and 24 hours post treatment with the fractions. Expression levels were normalized using *OseIF1a* as an internal reference and expressed relative to gene expression upon mock treatment. Preliminary data (n = 1 x 2 technical repeats).

Chapter 6

General discussion and future perspectives

General discussion

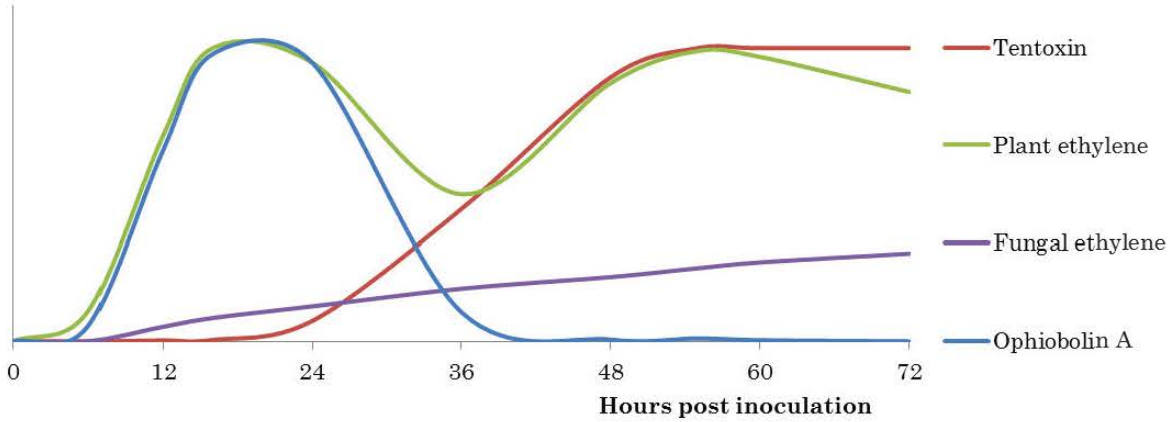
C. miyabeanus infection is characterized by induction of cell death during two distinct phases

Interaction between pathogens and plants causes radical changes in the physiology of both organisms. Ultimately, the pathogen relies on nutrients from the host plant for proliferation. Hence, pathogens not only depend on the ability to penetrate and colonize a host, but also need mechanisms to induce and utilize nutrient supplies (Solomon *et al.*, 2003). To safeguard and optimize nutrient availability, pathogens tamper with host metabolism by rerouting plant responses (Divon & Fluhr, 2007; Seifi *et al.*, 2014). The changes in host physiology brought about by the pathogen thus comprise an evasion of effective host defense responses as well as a switch in primary metabolism. At the same time, the physiology of the pathogen is influenced by a complex signaling network that enhances or suppresses metabolic pathways depending on nutrient availability. These regulatory networks can moderate both primary and secondary fungal metabolism (Divon & Fluhr, 2007; Yin & Keller, 2011).

The *C. miyabeanus* infection process involves a pre-penetration phase, followed by penetration and colonization and finally sporulation (Laluk & Mengiste, 2010). Investigation of the temporal dynamics of plant and fungal metabolism during disease clearly differentiates between two main phases in disease development after fungal penetration and before sporulation. These phases are characterized by two distinct ethylene emission peaks, synchronized with phytotoxin production (Figure 6.1). The results from our own study, combined with differential gene expression data and proteome analysis during infection, sheds new light on the previously poorly characterized molecular dynamics of rice brown spot disease (Kim *et al.*, 2014; Van Bockhaven *et al.*, 2015b).

Infection phase 1: induction of localized cell death

The first phase is characterized by the initial intimate contact between the host and its pathogen. Upon penetration, the starving fungus needs rapid access to plant nutrients in order to survive and evade plant defenses. Significant changes in plant metabolism, reminiscent of programmed cell death, are detected early on during infection. Microarray analysis of gene expression at 12 hpi shows a strong down regulation of photosynthesis and photorespiration and a switch from nitrogen assimilation to nitrogen remobilization, suggested by a down-regulation of genes involved in nitrate reduction and an up-regulation of glutamine synthetases (Van Bockhaven *et al.*, 2015b) (Figure 6.1). Interestingly, glutamine is not only used by the plant as a transportable intermediate to remobilize nitrogen to adjacent cells, but also represents a major nitrogen source for fungi. Furthermore, the first phase features a peak in ophiobolin A production, coinciding with a strong ethylene burst. Eventually, small necrotic spots develop towards the end of this phase, indicating localized cell death.



	12 hpi	36 hpi	Source
C. miyabeanus	Secreted in apoplast: α-amylase α-galactosidase	Secreted in apoplast: α-amylase Endo-1,4-ß-xylanase Exo-1,3-ß-glucanase α-N-arbinofuranosidase Cutinase precursor Endopeptidase (Alp1 homolog) Glyceraldehyde-3-P dehydrogenase Peroxidase/catalase	Proteomics study, Kim et al. (2014)
Rice	H_2O_2 Secreted in apoplast: Chitinase Glucan endo-1,3-ß-glucosidase LysM-containing protein ABC-transporter Major Facilitator Superfamily	H_2O_2 Metabolic changes: Decreased Calvin cycle Glycolysis (except for GAPDH) Increased OAA biosynthesis via TCA cycle Methionine and ET biosynthesis Cell redox homeostasis-involved proteins Ascorbate peroxidase Superoxide desmutase	Proteomics study, Kim et al. (2014)
	Metabolic changes: Down-regulation Photosynthesis (light-dependent reactions and Calvin cycle) Photorespiration Nitrate reduction MEP pathway Up-regulation Amino acid biosynthesis Phenylpropanoid pathway Cytosolic glutamine synthetases Mevalonate pathway		Microarray analysis, Van Bockhaven et al. (2015)

Figure 6.1. Overview of temporal dynamics during rice brown spot disease. The top chart represents interrelationships between phytotoxin production and ethylene emission as discussed throughout this thesis and is not to scale. The table summarizes major metabolic changes in *Cochliobolus miyabeanus* and rice during disease (*Oryza sativa)* as uncovered by proteome and microarray analysis (Kim *et al.*, 2014; Van Bockhaven *et al.*, 2015b)

Penetration of *C. miyabeanus* is likely assisted by cell wall degrading enzymes (CWDE), which is supported by the detection of several fungal CWDEs in the apoplast at 12 hpi (Kim *et al.*, 2014). These fungal CWDEs, together with additional plant secreted CWDEs, chitinases and a putative LysM-containing protein, likely release PAMPs and DAMPs that trigger a defense response. This defense response is characterized by induction of the phenylpropanoid pathway, H_2O_2 production and enhanced ethylene metabolism (Kim *et al.*, 2014; Van Bockhaven *et al.*, 2015b). The production of the antioxidant enzymes ascorbate peroxidase and superoxide dismutase is induced by the plant in an attempt to restore cell redox homeostasis (Kim *et al.*, 2002). At the same time, the fungus produces high amounts of ophiobolin A (Figure 6.1).

As we discussed in Chapter 2, ophiobolin A most probably targets the cell membranes, resulting in ion leakage and extracellular alkalinisation. Like many phytotoxins, they eventually trigger a form of programmed cell death (Bury *et al.*, 2013). Interestingly, ophiobolin A-treatment and *C. miyabeanus* infection both trigger *OsNAC4* gene expression. OsNac4 is a phosphorylation dependent transcription factor that acts as an important inducer of HR-like cell death during incompatible interaction of the biotrophic pathogen *Acidovorax avenae* and rice (Kaneda *et al.*, 2009). This observation adds to the contention that necrotrophs can induce similar responses as their biotrophic counterparts. Interestingly, ophiobolin A treatment also induces *OsMPK5* gene expression, a MAPK able to phosphorylate OsNac4 (Kishi-Kaboshi et al. 2010). However, evidence for a direct link between OsMPK5 and OsNac4 during *C. miyabeanus* infection is lacking. Moreover, the difference in *OsMPK5* gene expression between wild type and *Cmops1* mutants is small, and abscisic acid-induced OsMPK5 suppresses ethylene-mediated rice susceptibility and increases resistance to brown spot (De Vleesschauwer et al. 2010). Therefore it seems likely that other processes are involved in the induction of OsNac4 gene expression.

Furthermore, ophiobolin A-treatment induces *OsPAL1* gene expression. OsPal1 catalyzes the first step in phenylpropanoid biosynthesis and is typically induced by the plant upon the detection of danger signals. Induction of the phenylpropanoid pathway results in the production of antifungal phenolic compounds, which are effective against *C. miyabeanus* (Vidhyasekaran *et al.*, 1992; Shabana *et al.*, 2008). It is not clear whether ophiobolin A itself is perceived as a danger signal or whether the plant senses membrane damage. Regardless, *OsPAL1* gene expression in ophiobolin deficient mutants is strongly reduced, indicating that ophiobolin A could be a key factor in the induction of the phenylpropanoid pathway upon pathogen attack. Although high amounts of ophiobolin A are only detected after penetration, germinating spores produce small amounts of ophiobolin that could be detected early by the plant in addition to PAMPs and DAMPs (Xiao *et al.*, 1991).

Plants also respond to *C. miyabeanus* penetration with an early and strong ethylene burst, reaching a maximum at 12hpi (see Chapter 5). The production of ethylene is reflected in an induction of ethylene biosynthesis genes (Van Bockhaven *et al.*, 2015b). Although ethylene on its

own does not induce programmed cell death in healthy plants, as part of a stress response it can be involved in signaling pathways leading to cell death (Mase *et al.*, 2012). Ethylene could be part of an archetypal defense response, induced by the detection of PAMPs or DAMPs and similar biphasic ethylene responses have been observed in other-plant pathogen interactions (Stearns & Glick, 2003; Boller & Felix, 2009). However, the induction of ethylene enhances susceptibility to *C. miyabeanus* and ethylene has an inhibiting effect on the phenylpropanoid pathway in rice (Van Bockhaven, 2014). This is contrasting to most plant species, where ethylene typically enhances biosynthesis of phenolic compounds (Hyodo & Yang, 1971; Chappell *et al.*, 1984; Tomas-Barberán *et al.*, 1997). It is tempting to speculate that without ethylene, phenylpropanoid biosynthesis may be more enhanced. Hence, it is likely that *C. miyabeanus* is somehow able to steer the early defense response towards ethylene-mediated signaling pathways, assisting the fungal infection process in multiple ways.

The first ethylene emission peak coincides with ophiobolin A production by the fungus. We showed in Chapter 2 that ophiobolin A is a major virulence factor during disease and in Chapter 5 that ethylene is involved in ophiobolin A-induced cell death. Although phytotoxins that rely on ethylene signaling for the induction of cell death can often trigger ethylene themselves, ophiobolin A is probably not the trigger for the strong first ethylene response. This is substantiated by the fact that ophiobolin-deficient mutants still cause a similar plant ethylene burst. Therefore, another factor must be responsible. In Chapter 5 we show that *C. miyabeanus* can produce ethylene via two distinct pathways from 2-oxoglutarate via EFE and from methionine via KMBA respectively. Fungal ethylene could act autocatalytically to induce ethylene biosynthesis via a positive feedback loop. The induction of *CmEFE* gene expression, combined with the induced expression of two glutamate dehydrogenases suggests that the EFE pathway is active during disease (Chapter 5). However, deletion of *CmEFE* does not affect the initial plant ethylene burst. One explanation could be that the second ethylene biosynthesis pathway via KMBA has taken over the role of CmEFE. Elimination of plant ethylene biosynthesis through the use of inhibitors revealed that small amounts of ethylene are produced by the fungus starting at 12 hpi, and slightly increase overtime. However, because inhibition of plant ethylene strongly inhibits symptom development, this fungal production pattern may not reflect the true fungal ethylene dynamic during disease. It is also possible that the first ethylene emission peak is not triggered by fungal ethylene, but requires another elicitor. Induction of *CmEFE* during normal infection is strongest at 48 hpi, and fungal ethylene emission in the plant ethylene-deficient background started around 12 hpi for both WT and *Cmefe2.1*, suggesting that the plant ethylene burst may precede the fungal ethylene production. Furthermore, *C. miyabeanus* needs an external source of precursors to produce ethylene *in vitro* and *in planta* (Chapter 5), therefore it seems likely that the fungus needs access to plant nutrients before it can induce ethylene biosynthesis. However, fungal ethylene could also function to enhance the plant ethylene response after the onset of plant ethylene biosynthesis by increasing the total ethylene emissions.

Alternatively, fungal ethylene could act as a toxin synergist during the first phase to promote ophiobolin A induced cell death. This is supported by failure of ophiobolin A to induce cell death when plant ethylene biosynthesis is inhibited (Chapter 5). In the case of AAL from *Alternaria alternata* and Fumonisin B1 from *Fusarium moniliforme*, ethylene is also necessary for cell death induction (Moore *et al.*, 1999; Asai *et al.*, 2000; Gechev *et al.*, 2004). Furthermore, ethylene acts as a synergist of phytotoxic fractions during *Verticillium* wilt of tomato (Cronshaw & Pegg, 1976). Hence, the simultaneous production of fungal ethylene and phytotoxins may enhance disease symptoms.

Although ophiobolin A signaling needs ethylene for cell death induction, it is not clear whether ethylene is prerequisite for ophiobolin production. Other plant specific triggers may be involved in the up-regulation of *CmOPS1*. Typically, secondary metabolite biosynthesis in filamentous fungi is regulated by a complex signaling network that integrates environmental signals. It is likely that a similar system steers ophiobolin biosynthesis (Keller *et al.*, 2005; Wu *et al.*, 2012). As discussed in Chapter 3, the ophiobolin biosynthesis gene cluster comprises a putative redox-regulated bZIP transcription factor. Therefore, presence of ROS may be a possible trigger for ophiobolin production *in planta*. Interesting in this regard is the fact that on top of the already mentioned plant metabolic changes, terpene metabolism is also perturbated upon infection. This is characterized by an up-regulation of the mevalonate pathway and a down regulation of the MEP pathway respectively (Van Bockhaven *et al.*, 2015b). Ophiobolins are produced from the end products of the mevalonate pathway and feeding experiments showed that ophiobolins can be produced from exogenous mevalonate pathway intermediates (Nozoe *et al.*, 1968). It is thus tempting to speculate that the fungus causes a change in terpene metabolism in order to secure its supply of ophiobolin precursors, allowing for much higher production levels than when it must rely on its own metabolism. Preliminary experiments monitoring gene expression levels of key genes from the plant MEP and mevalonate pathway during infection, indicate that the terpenoid metabolism is similarly affected by WT and ophiobolin-deficient mutants (data not shown).

The coevolution of plants and pathogens has resulted in sophisticated strategies to survive. Whereas fungi develop mechanisms to evade plant defense, plants need to invest in ways to withstand phytotoxic effects. In this regard it is interesting to find that plant-originating transporter proteins of the ABC-transporter family and the Major Facilitator Superfamily (MFS) are detected in the apoplast at 12 hpi (Kim *et al.*, 2014). Although these transporters could be used for the detoxification of endogenous compounds like ROS and phenolics, it is tempting to speculate that these transporters could also detoxify ophiobolin A or other fungal factors (Tommasini *et al.*, 1997; Rea *et al.*, 1998). A comparison of ophiobolin-induced cell death in animal and plant suspension cells shows that much higher concentrations of ophiobolin A are needed to kill plant cells. This suggests that plants may have developed ways to detoxify the compound (Bury *et al.*, 2013).

The biosynthesis of ophiobolin A stops abruptly after 48 hours and together with a drop in ethylene production concludes the first phase. We hypothesise that strong virulence factors like ophiobolins are needed for rapid induction of cell death and to counteract the plant defense responses. By the time the disease enters its second phase, the plant has been considerably debilitated and the pathogen has regained strength. During the second phase the fungus promotes senescence in order to release more nutrients.

Infection phase 2: progressing senescence

Whereas the first phase mainly involved an on-going warfare characterized by the induction of defense responses by the plant and the production of CWDEs and ophiobolins by the fungus, the second phase is hallmarked by the induction of senescence and spreading chlorosis and eventually wilting of the leaf in severe cases. Most of the physiological changes observed during the first phase, such as reduction in photosynthesis and photorespiration, and an induction of methionine and ethylene biosynthesis are prolonged during the second phase (Kim *et al.*, 2014). Furthermore, proteins involved in maintaining the cell redox homeostasis are still highly present at 36 hpi. The lack of newly produced carbohydrates from the calvin cycle eventually results in a down regulation of glycolysis (Figure 6.1).

By the end of the first phase, the pathogen no longer produces ophiobolins, but switches to the production of tentoxin (Chapter 4). Strikingly, the second wave of phytotoxin production coincides with a second ethylene peak. It seems likely that during this phase, senescence, ethylene biosynthesis and tentoxin production are interconnected and reflect the same phenomenon.

Although rice seedlings were previously described to be insensitive to tentoxin action, our results indicate that tentoxin plays a role in the induction of chlorosis (Chapter 4). Tentoxin is a methylated tetrapeptide, comprised of glycine, alanine, leucine and phenylalanine. Because fungi rely on plant nitrogen sources during infection, it can be expected that the amino acids used for tentoxin production originate from the plant metabolism. These amino acids can be produced via *de novo* biosynthesis, however it is more likely that they are released from degraded plant proteins as glycolysis is down-regulated during the second phase (Kim *et al.*, 2014). The plant breaks down its own proteins to preserve nitrogen sources during senescence (Araújo *et al.*, 2011). However, some of these amino acids are also useful in defense related pathways. Phenylalanine is a precursor for phenolic compounds that could protect the plant against *C. miyabeanus*, and glycine is decarboxylated to serine during photorespiration (Figure 6.2). Victorin can induce senescence in oats by targeting glycine decarboxylase in the photorespiration, therefore it seems plausible that inhibiting photorespiration through drainage of glycine could have a similar effect (Navarre & Wolpert, 1999). This would support the hypothesis that tentoxin should be made *in planta* in order to have an effect and would also explain the insensitivity of rice seedlings to tentoxin treatment. However, it is also possible that tentoxin only causes symptoms under certain circumstances, e.g. when the plant is already stressed or relies strongly on photorespiration.

Although tentoxin could cause small necrosis like symptoms on punctured leaves 33% of the times, this does not resemble the spreading chlorosis seen during disease and may reflect an additional activity. Furthermore, the observed variability in tentoxin production might be related to variations in the availability of free amino acids. The observation that ophiobolin-deficient mutants also produce less tentoxin may be reflective of less damage caused during the first phase, possibly resulting in lower amino acid availability. The ethylene emissions may be directly correlated to the amount of senescence, which is likely enhanced by tentoxin production.

Senescence is a form of programmed cell death that is typically induced depending on plant developmental age, determined by several endogenous and external environmental factors (Lim *et al.*, 2007). However, it is not always easy to distinguish between cause and effect. Senescence is associated with a rapid reduction of photosynthesis, but a decline of photosynthesis below a certain threshold level could also act as a senescence-inducing signal (Quirino *et al.*, 2000). Similarly, ethylene is an important positive regulator and acts autocatalytically in a positive biosynthesis feedback loop during senescence (Chang *et al.*, 2008; Trobacher, 2009). Tentoxin production induces chlorosis, which typically leads to lower photosynthetic capacity. This could enhance senescence, associated with higher ethylene emissions. However, tentoxin-production may rely on free amino acids released during programmed cell death in the first phase of infection. Therefore, it seems that pathogen attack predisposes the plant for premature senescence, and that tentoxin production during the second phase can enhance the process. Furthermore, it is expected that fungal ethylene may contribute to the ethylene emissions during the second phase, thereby accelerating on-going senescence. This is supported by the observation that fungal ethylene production gradually increases and is maximal during the second phase in leaves treated with plant ethylene biosynthesis inhibitors (Chapter 5). Furthermore, *CmEFE* reaches maximal expression levels at 48 hours post inoculation with wild type spores (Van Bockhaven, 2014). *C. miyabeanus* relies on external precursors to produce ethylene. 2-oxoglutarate is needed for ethylene production via the EFE pathway. During infection glutamate-dehydrogenases are upregulated (Van Bockhaven *et al.*, 2015b). Although these enzymes could work in both directions, conversion of glutamate to 2-oxoglutarate would drain the glutamate source for N-remobilization via glutamine. Alternatively, the fungus could biosynthesize ethylene from methionine via the KMBA pathway. This way, the fungus could take over the plant-ethylene production from methionine when the plant is no longer able to induce ethylene biosynthesis itself.

Also during this second phase, *C. miyabeanus* releases a higher diversity of CWDEs in the apoplast, probably as a means to provide the fungus with the needed carbon sources (Kim *et al.*, 2014). Eventually the leaf wilts and the fungus will have drained all useful nutrients from the macerated tissue. To safeguard its perseverance, a new round of sporulation is induced announcing another disease cycle. As discussed above, throughout the disease process the fungus may rely directly on building blocks released by the plant for the production of phytotoxic

compounds, thereby disturbing plant physiology. An overview of the different metabolic interfaces and the corresponding affected plant processes is given in Figure 6.2.

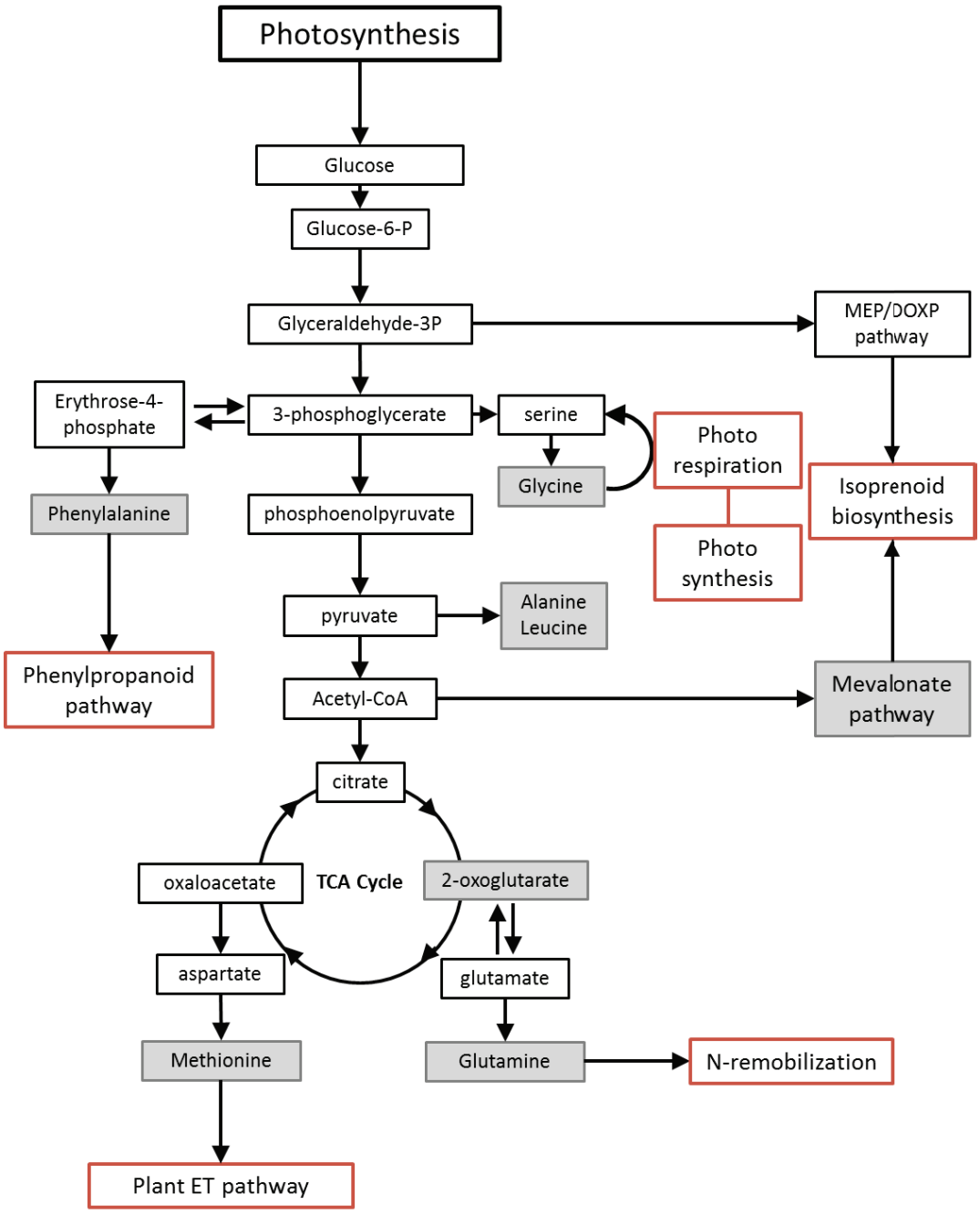


Figure 6.2. Schematic overview of possible plant-pathogen metabolic interfaces during brown spot disease. Grey squares: Plant intermediates that could be used by *C. miyabeanus* either for production of phytotoxins or as a nutritional source. **Red squares**: plant metabolic processes that may be impacted. *C. miyabeanus* uses mevalonate pathway intermediates to produce ophiobolins; this may eventually impact plant isoprenoid biosynthesis. Biosynthesis of tentoxin consumes phenylalanine, glycine, alanine, leucine and possibly SAM for the methylation reactions. Drainage of phenylalanine by the fungus could reduce phenylpropanoid biosynthesis resulting in a reduction of phenolic compounds, whereas consumption of glycine may impact photorespiration and trigger senescence. During ethylene biosynthesis via the EFE pathway, *C. miyabeanus* could dissipate 2-oxoglutarate, which is then lost to either the TCA-cycle or nitrogen remobilization via glutamate and glutamine. Furthermore, glutamine is a major nitrogen source for fungi and consumption prevents further nitrogen remobilization.

Predisposition to brown spot disease: a pivotal role for ethylene

As discussed profoundly above, *C. miyabeanus* causes clear metabolic changes in the plant during successful brown spot infection, including a general reduction of photosynthetic processes and photorespiration and a shift in amino acid metabolism, (Kim *et al.*, 2014; Van Bockhaven *et al.*, 2015b). These physiological changes illustrate a switch from energy storage to catabolic processes, generating a ready supply of metabolites for the fungus. We have shown that ethylene plays a central role in these physiological changes and is associated with both phases of symptom development.

Typically, hormonal crosstalk regulates plant adaptation to abiotic stresses, including nutrient deficiencies, via a central stress response system. It has become increasingly clear that in addition to abscisic acid and cytokinins, ethylene plays a major role in this regard (Lynch & Brown, 1997; Rubio *et al.*, 2009; Peleg & Blumwald, 2011). The same hormonal pathways are also involved in defense responses to particular plant pathogens (Pieterse *et al.*, 2012a). Several abiotic and biotic stresses as well as growth and developmental demands constantly feed into this hormonal web and trade-offs shape the ultimate plant physiology. These trade-offs not only exist between pathogens with opposite lifestyles, but also between different (a)biotic stresses. Generally, plants suffering from nutrient deficiencies or drought stress are hyper-susceptible to brown spot disease (Ou, 1985; Chakrabarti, 2001; Carvalho *et al.*, 2010; Barnwal *et al.*, 2013). Especially deficiency in iron, magnesium, manganese, calcium, potassium and nitrogen seems to make plants vulnerable. Interestingly, these stresses have all been associated with induction of ethylene biosynthesis and/or physiological changes reminiscent of the perturbations caused by successful *C. miyabeanus* infection.

Nutrient stress is initially sensed by the roots, and triggers rapid changes in gene expression, even before nutrient concentrations decrease in plant tissues (Schachtman & Shin, 2007). Activation of specific signal transduction pathways in response to nutrient deficiencies has mainly been studied in regard to changes in root development. For instance, induction of ethylene biosynthesis is essential in the development of root hairs upon iron deficiency (Lynch & Brown, 1997; Schmidt, 1999; Pierik *et al.*, 2007; Iqbal *et al.*, 2013). Most evidence indicates that ethylene production is enhanced under iron shortage. However, iron is also an essential cofactor of ACC synthase, hence an inhibition of ethylene biosynthesis could be expected in case of extreme iron deficiency (Lynch & Brown, 1997). Interestingly, too much iron promotes disease. It is not known whether this is caused by stimulation of ACC synthase. *C. miyabeanus* also has high requirements for iron, thus excess iron could be advantageous for infection. This is reflected in the reduction of virulence when the fungal siderophore *NPS6* is deleted, although this could also be due to enhanced sensitivity to H_2O_2 (Oide *et al.*, 2006). Moreover, high iron concentrations can become toxic and cause oxidative stress and weaken plant tissue (Connolly & Guerinot, 2002). Furthermore, iron and magnesium are vital cofactors of normal photosynthesis and shortage is

reflected in a decrease of photosynthetic processes (Jiang *et al.*, 2007; Taiz & Zeiger, 2010a). Magnesium is the central atom of chlorophyll and is essential to harvest solar energy and drive photochemistry. Magnesium starvation results in chlorophyll decline and triggers ethylene biosynthesis genes (Hermans *et al.*, 2010; Iqbal *et al.*, 2013). Manganese can activate several enzymes in the TCA cycle and other metabolic pathways, but is also an essential element during photosynthesis. Low manganese availability resulted in higher ethylene emissions and a reduction in photosynthetic capacity of rice leaves (Lidon *et al.*, 2004). Furthermore, deficiency can result in chlorosis and has been shown to enhance ozon-induced ethylene production (Taiz & Zeiger, 2010b; Iqbal *et al.*, 2013). Limited calcium supplies results in weakened cell walls and plasma membranes, rendering the cells more prone to pathogen attack (Iqbal *et al.*, 2013). On top of that, low calcium may result in accelerated senescence. However, calcium is also involved in ethylene production and severe calcium deficiencies result in impeded ethylene responses to pathogens (Lynch & Brown, 1997). In this context it is interesting to note that increasing brown spot severity has been observed upon calcium excess (Datnoff, 1994).

The macronutrients nitrogen, phosphorus and potassium have been associated with changes in primary metabolism and ethylene signaling as well. Phosphorus deficiency typically invokes ethylene production in roots, associated with primary root elongation (Lynch & Brown, 1997; Borch *et al.*, 1999; Schmidt, 1999; Rubio *et al.*, 2009; Iqbal *et al.*, 2013). Being essential during photosynthesis, a down-regulation of photosystem subunits and small subunits of RuBisCO has been reported in phosphorus deficient plants. Furthermore, plants seem to change their primary metabolism in order to bypass ATP-dependent enzymes (Hammond *et al.*, 2004; Ticconi & Abel, 2004; Rubio *et al.*, 2009; Tripathi *et al.*, 2014). Changes in Arabidopsis in response to phosphorus deprivation are detectable within 24 hours and are overall characterized by a down regulation of genes involved in photosynthesis and nitrogen assimilation. Furthermore catabolic genes involved in degradation of proteins, fatty acids, lipids and isoprenoids are induced (Ticconi & Abel, 2004). One experiment showed that during a dry season phosphorus nutrition could reduce disease incidence (Singh *et al.*,2005). However, low phosphorus has also been associated with brown spot resistance, whereas high phosphorus status can enhance susceptibility under certain conditions (Datnoff, 1994; Alam *et al.*, 2010; Sunder *et al.*, 2014). Given the many functions phosphorus fulfils in the plant, it seems likely that phosphorus plays an ambiguous role and acts at the cross road of different signaling pathways (Rubio *et al.*, 2009).

A reduced content of all photosynthetic components is usually observed in nitrogen-deficient rice leaves. Furthermore, nitrogen starvation of rice seedlings induced expression of S-adenosyl methionine synthetase and an ethylene responsive gene, indicating an up-regulation of ethylene biosynthesis (Chen *et al.*, 2003a,b). Besides, GAPDH was up-regulated, an effect that is also observed during *C. miyabeanus* infection and associated with several other stress responses (Chen *et al.*, 2003b; Kim *et al.*, 2014). Although some reports mention NH^{4+} as a superior nitrogen source compared to NO^{3-} in terms of disease resistance, excess NH^{4+} also induces susceptibility

(Chattopadhyay & Dickson, 1960; Sunder *et al.*, 2014). Finally, potassium-deficiency closes the list of nutrient stresses predisposing plants to brown spot susceptibility (Chakrabarti, 2001). Like many of the elements mentioned above, potassium is a cofactor for enzymes involved in photosynthesis and respiration. Low potassium conditions are generally associated with increased ethylene biosynthesis and ROS production (Ashley *et al.*, 2006; Schachtman & Shin, 2007; Iqbal *et al.*, 2013). It is clear that nutrient deficiencies typically enhance disease, however for some elements, excess is similarly detrimental, probably for different reasons. Furthermore, nutrients interact with each other, and deprivation of one nutrient can affect the accumulation of another (Schachtman & Shin, 2007). Therefore it is essential to maintain a good balance between different nutrients.

In seasons with irregular and low rainfall, brown spot disease incidence is appreciably higher and plants in drought-prone fields are more susceptible to the disease (Chakrabarti, 2001; Pannu *et al.*, 2005). This explains the high incidence of brown spot in rainfed upland rice in Asia. Microarray analyses shows that the metabolic profile under drought conditions is very similar to metabolic changes during successful infection. In general, photosynthetic light reactions and photorespiration are strongly reduced, along with a metabolic switch to degradation pathways, including protein and lipid degradation and an induction of amino acid biosynthesis (Degenkolbe *et al.*, 2009). Drought stressed rice plants not only suffer from water deficiency, but are more prone to nutrient deficiencies as water shortage reduces the availability of nutrients for the plants (Fukai *et al.*, 1999). Rice seems to be especially vulnerable to decreased nutrient uptake under dry conditions, compared to maize and soybean (Tanguilig *et al.*, 1987). On the other hand, drought tolerant plants often exhibit enhanced nutrient uptake. For instance, drought tolerant wheat showed an increased potassium uptake and drought resistant chickpea cultivars accumulated more nitrogen, phosphorus, potassium, calcium, zinc, manganese and boron under drought stress conditions (Sinha, 1978; Gunes *et al.*, 2006). Evidently, nutrient deficiencies could underlie the enhanced susceptibility in drought stressed plants. Surprisingly, more drought resistant rice cultivars resisted the switch to degradation pathways, although photosynthesis was similarly inhibited (Degenkolbe *et al.*, 2009). In this regard it seems a worthwhile approach to test nutrient efficiencies of drought resistant rice varieties and their resistance towards brown spot.

The production of endogenous phenolics or the application of exogenous phenolic compounds can promote resistance to brown spot (Shabana *et al.*, 2008; Van Bockhaven *et al.*, 2015a). Interestingly, many abiotic stresses increase the phenolic content in the leaves. For example, nitrogen deficiency in wheat, tomato and Arabidopsis enhances the polyphenol content in the leaves (Dixon & Paiva, 1995; Solecka, 1997; Cartelat *et al.*, 2005). A similar relationship between nitrogen deficiency and flavonoid content of leaves was observed in paddy rice fields (Li *et al.*, 2013b). Furthermore, in drought resistant maize and triticale genotypes, increased levels of phenolics and antioxidants are present in the epidermis where they are used for photoprotection

(Hura *et al.*, 2008, 2009). Moreover, stress-induced ethylene production has been directly linked to increasing phenylpropanoid biosynthesis in some plant species (Hyodo & Yang, 1971; Chappell *et al.*, 1984; Tomas-Barberán *et al.*, 1997; Ballester *et al.*, 2011). In rice however, ethylene has been shown to have an antagonistic effect on disease-triggered phenol accumulation. It is unclear to which extent ethylene production may counteract stress-induced phenolic biosynthesis in rice. Additionally, manganese is also involved in the shikimate pathway as a cofactor of PAL, therefore manganese-deficiency could also reduce production of phenolics, as is the case for wheat (Engelsma, 1972; Brown *et al.*, 1984).

To conclude, most of the nutrient deficiencies result in an induction of ethylene biosynthesis or sensitivity. Stress-related ethylene production has been associated with reduction of photosynthesis and the induction of senescence, processes reminiscent of the physiological changes observed during successful disease. Hence a picture emerges in which nutrient deficiency and/or drought pave the way for successful infection by changing the plant physiology towards a favourable environment for *C. miyabeanus* and by reducing effective plant defenses. Whereas highly virulent strains may be able to infect even healthy plants due to the production of high amounts of phytotoxins that push plants towards cell death, less virulent genotypes may not need huge amount of phytotoxins to cause disease on predisposed plants.

In this regards it is interesting to mention that factors that steer plant physiology in the opposite direction can enhance resistance. It has been shown convincingly that silicon application is particularly successful in making plants more resistant to brown spot (Dallagnol *et al.*, 2009; Prabhu *et al.*, 2012; Van Bockhaven *et al.*, 2015a,b). Interestingly, silicon can alleviate multiple other abiotic stresses as well. Proteome and microarray analyses have shown that silicon application shifts the plant metabolism in the opposite direction of the physiological changes caused by brown spot disease or nutrient-deficiency. In general, photosynthesis is enhanced by silicon, whereas amino acid metabolism and ethylene metabolism are inhibited (Van Bockhaven *et al.*, 2015b). In conclusion we can state that any stress that triggers ethylene biosynthesis, impacts photosynthesis or steers plants towards premature senescence seems to predispose rice plants to brown spot susceptibility. Similarly, factors that alleviate the stress or shift plant metabolism in the opposite direction, can enhance plant resistance.

Additional research is needed to verify the central role of ethylene in disease predisposition. First of all infection tests should be performed on plants that are subjected to controlled nutrient deficiencies and water shortage to confirm enhanced disease severity. Furthermore ethylene emissions should be monitored from plants subjected to the same stresses. Finally, to analyse the role of ethylene in the predisposition of abiotically stressed plants, we could compare disease severity in mock treated plants and plants treated with ethylene biosynthesis inhibitors.

Future perspectives

The impact of ophiobolin A, tentoxin and ethylene production on fungal colonization and crop yield loss

Although it has been clearly shown throughout this thesis that production of ophiobolin A, tentoxin and ethylene is correlated with symptom development during brown spot disease, we did not investigate the effect on fungal colonization. Many phytotoxins promote fungal host colonization by enhancing access to host nutrients (Pedras *et al.*, 2001; Divon & Fluhr, 2007; Zhou *et al.*, 2012). It is hence likely that mutants which are no longer able to produce ophiobolin A may colonize the host slower than the wild type strain. Similarly, the lack of tentoxin production could influence the colonization rate, independent of the fact whether tentoxin has a direct phytotoxic effect or an indirect effect on the plant physiology. The phytohormone ethylene has been shown before to play an ambiguous role during disease. On one hand, ethylene can contribute to development of disease symptoms, such as chlorosis and senescence, without affecting pathogen proliferation (Bent *et al.*; Lund *et al.*, 1998; van Loon *et al.*, 2006). On the other hand, ethylene may influence plant defense in a positive or negative way depending on the interaction and eventually impact pathogen colonization (Knoester *et al.*, 1998; Hoffman *et al.*, 1999; Thomma *et al.*, 1999; Geraats *et al.*, 2003; van Loon *et al.*, 2006). Hence, in order to draw conclusion on the effect of the different discussed factors on overall fungal virulence, the fungal biomass in the host tissue should be monitored during infection using real-time qPCR-analysis.

Furthermore, the eventual impact of the discussed virulence factors on total yield of the rice crop should be investigated. Therefore, field experiments should be conducted to compare the impact of wild-type and knock-out mutants on commercial rice varieties. These experiments would allow investigating the importance of certain virulence factors on fungal dispersion over the rice field by secondary infection rounds throughout the season. In addition it would allow measuring the impact of differential disease severity on the eventual yield, both in terms of quantity and quality of the produce. Additionally, it would be interesting to analyse the effect of the gene deletions on the survival rate of the mutant strains in between the cropping seasons. Hence ideally, the field experiments would stretch over multiple growing seasons.

The role of tentoxin during brown spot disease

In Chapter 4 we show that the ability to produce tentoxin is correlated with disease inducing ability and that tentoxin production *in planta* seems to be linked with chlorosis development. However, it is not clear how exactly tentoxin affects rice plants. Surprisingly, treatment with pure tentoxin did not cause chlorosis symptoms on healthy plants and damage to the plant was recorded as a small necrotic ring around the puncture wound in only one third of the cases (Chapter 4). It is possible that tentoxin is only active under certain circumstances. For example

physiological changes induced by a stress response could be prerequisite for tentoxin effects. This would also explain the production of tentoxin during the second phase of infection. Furthermore, the target of tentoxin in rice is not known. Unlike rice, seedlings of sensitive species suffer from severe chlorosis upon tentoxin treatment. These species possess chloroplast ATP-synthases of which the F1-subunit is sensitive to inhibition by tentoxin (Meiss *et al.*, 2008). The inhibition of ATP-synthase results in a depletion of ATP and subsequent reduction of PPO incorporation in the thylakoids (Sommer *et al.*, 1994; Holland *et al.*, 1997). The inactivation of PPO can decrease the biosynthesis of phenolic phytoalexins and at the same time cause overenergization of the thylakoids. Interestingly, tentoxin could also inhibit photophosphorylation in insensitive species *in vitro*, but needed higher concentrations (Steele *et al.*, 1976). A second proposed target is the cell membrane, although there is evidence that tentoxin membrane-effects may be indirect and caused by interruption of energy supply trough photophosphorylation (Böcher & Novacky, 1981). Tentoxin can also act as a K^+ ionophore and alter K^+ efflux in artificial lipid bilayer membranes (Klotz, 1988). Finally, tentoxin can enhance long-distance K^+-transport from roots to shoot in insensitive species if administered for prolonged periods (Erdei & Klotz, 1988; Klotz & Erdei, 1988), although the involvement of these effects may be minimal during brown spot disease, which results in extended cell death within three to four days. Given the likely implication of chlorosis, future investigations in the role of tentoxin during brown spot disease should reassess the involvement of photophosphorylation. Furthermore, it is tempting to speculate that the mere production of tentoxin, based on plant-derived building-blocks, could contribute to disease severity as drainage of amino acids results in their loss to nitrogen remobilization. Overall this would result in a reduction of nutrients available for the plant. Furthermore, the consumption of phenylalanine and glycine could inhibit the phenylpropanoid pathway and photorespiration respectively. Future investigations should comprise the monitoring of amino acid levels during disease.

Signaling pathways associated with phytotoxin action

In Chapter 5 we show that in order to trigger cell death, ophiobolin A needs active plant ethylene biosynthesis. Furthermore, although ophiobolin A seems to be able to perturb cell membranes and cause ion leakage independent of the presence of Ca^{2+}, a Ca^{2+} signature is needed for the induction of severe cell death (Chapter 2). Moreover, cell death induced by ophiobolin A is characterized by induction of *OsNAC4*, a transcription factor also involved in HR-like cell death. Although these results show that ophiobolin A triggers a plant response and relies on plant signaling pathways to induce cell death, additional pathway components have not been characterized. Furthermore it is not known what happens downstream of ethylene signaling. Future investigation could focus on the involvement of ethylene biosynthesis and signaling components, by using mutant or transgenic rice lines. Furthermore, the implication of ethylene responsive transcription factors would be expected, therefore it would be a good idea to analyse the gene expression of known ethylene responsive genes upon ophiobolin A treatment. In the case

of fumonisin B1 and AAL-toxin induced cell death, two homologous ethylene responsive transcription factors are involved, MACD1 in *N. benthamiana* and its ortholog ERF102 in Arabidopsis, respectively (Mase *et al.*, 2013). As these phytotoxins also affect membrane functions, a rice MACD1 ortholog may be a good starting point to find additional ophiobolin cell death associated factors. Besides, two rice ERF-TF could induce HR-cell death when transiently overexpressed in tobacco (Ogata *et al.*, 2012).

The observed changes in terpenoid biosynthesis upon infection by *C. miyabeanus* trigger the question whether this could be related to ophiobolin production. Intriguingly, the plant-specific MEP pathway is down-regulated upon infection, whereas the MVA pathway is up-regulated. *C. miyabeanus* can use MVA intermediates and end products to synthesise ophiobolins (Nozoe *et al.*, 1968). As a way to preserve its own reserves, the fungus may tap into the plant biosynthesis pathway and drain MVA pathway intermediates. Experiments using isotope labelling could enhance our understanding of the building blocks used by *C. miyabeanus*.

As mentioned above, almost nothing is known about the effects of tentoxin on rice plants and no reports are available on plant signaling pathways that may be involved. Most research on tentoxin and ophiobolin action *in planta* is more than 30 years old and has mainly focussed on primary plant targets (Arntzen, 1972; Bennett, 1976; Chattopadhyay & Samaddar, 1976, 1980a; Vaughn & Duke, 1984; Au *et al.*, 2000). The availability of high throughput techniques like microarray analysis could shed a new light on the downstream actions of these phytotoxins. Furthermore it could also provide better insight into mechanisms of pathogen-triggered physiological changes, such as the perturbation of terpenoid biosynthesis.

Regulation of fungal phytotoxin production

From the fungal side, little is known about biosynthesis pathways and regulatory networks that mediate phytotoxin production in *C. miyabeanus*. In Chapter 2 and 4 we identify the key biosynthesis genes responsible for ophiobolin and tentoxin production respectively. Furthermore in Chapter 3 we characterized a putative ophiobolin biosynthesis gene cluster *in silico*. Future investigations could focus on functional analysis of the different cluster components. To this purpose, site-directed mutations of the different genes as well as co-expression analysis should shed more light on their involvement in ophiobolin production. Furthermore, deletion of P450 monooxygenases or redox-associated enzymes would likely imply a change in chemical structure of ophiobolin biosynthesis end products (Črešnar & Petrič, 2011). Therefore, chemical characterization of newly produced compounds could clarify the function of each modifying enzyme.

With the boost in fungal genome sequences that have become available during recent years, the complexity of fungal secondary metabolite regulatory networks has become clear. Whereas specific transcription factors located in the gene clusters can fine-tune phytotoxin production,

global transcription factors regulate entire clusters on a higher level, steered by environmental and developmental conditions (Yu & Keller, 2005). Many fungi sense their environment using histidine protein kinases, which transduce the signal to response regulators in a two-component system, who in turn activate a MAPK or directly influence gene transcription (Wolanin *et al.*, 2002; Oide *et al.*, 2010). Several two-component systems have been reported in *C. heterostrophus*, regulating reproductive development, virulence, stress responses, osmotic adaptation and dicarboximide resistance (Yoshimi *et al.*, 2004; Oide *et al.*, 2010). Moreover, *C. heterostrophus* virulence and sporulation as well as melanin biosynthesis are regulated by a MAPK pathway (Lev & Horwitz, 2003; Eliahu *et al.*, 2007). Furthermore, many global regulators of secondary metabolism, such as LaeA and VeA, are conserved and widespread among filamentous fungi. In *C. heterostrophus* sexual and asexual development and secondary metabolism are regulated by interplay between LaeA, Llm1 and velvet proteins, similar to regulation in *A. nidulans* (Bayram & Braus, 2012; Bi *et al.*, 2013). Although extensive studies on regulatory networks in *C. miyabeanus* are lacking, homologs have been found for some of these regulators. Ophiobolin and tentoxin biosynthesis show a distinct production pattern during infection in rice, but the environmental triggers and signal transduction pathways are not known. Investigation in the regulatory components involved in the induction of phytotoxin biosynthesis may provide new insights in the complex molecular interaction between rice and *C. miyabeanus*.

The role of fungal ethylene during disease

The importance of ethylene during the infection process has been discussed throughout this thesis and it is clear that ethylene is essential for full-scale disease development. Given that ethylene enhances susceptibility, it is not very surprising to find that *C. miyabeanus* can produce ethylene by itself. However, the role of fungal ethylene during disease remains elusive. Depending on the timing of fungal ethylene production, different hypotheses could be proposed.

If fungal ethylene production precedes plant ethylene biosynthesis, it may act as an autocatalyser of plant ethylene biosynthesis to trigger a plant ethylene response via a positive feedback loop. At the same time it could act as a toxin synergist along with ophiobolin, allowing for a strong cell death-induction (Chapter 5). *C. miyabeanus* needs plant precursors to produce ethylene. In this regard it is interesting to mention that rice leaf exudates contain small amounts of methionine (Purkayastha & Mukhopadhyay, 1974). Therefore it is not unlikely that the fungus can produce small amounts of ethylene before penetration. However, our results suggest that fungal ethylene starts to accumulate later than the first ethylene emission peak. In a plant ethylene-deficient background, fungal ethylene is detectable after 12 hours and increases more strongly during the second phase, while in the non-inhibited leaves, total ethylene starts to rise much earlier. Furthermore, *CmEFE* gene expression is most strongly induced at 48 hpi. Therefore, fungal ethylene may play a more important role during the second phase instead of the

first phase of infection. The second phase is characterized by progressing senescence and additional fungal ethylene could enhance this process.

To clarify the true role of fungal ethylene, future investigations should focus on the timing of fungal ethylene production in relation to plant ethylene induction. Therefore, a method should be developed to distinguish between fungal and plant ethylene. However, given that the fungus relies on plant nutrients, epitope tagging probably won't provide conclusive evidence. Ideally, the fungal KMBA pathway should be elucidated. So far, no biosynthesis genes have been characterized in any fungal species. In the KMBA pathway, methionine is converted to KMBA in a specific transaminase reaction, followed by a non-specific oxidation to ethylene in the presence of hydroxyl-radicals (Primrose, 1977; Ogawa *et al.*, 1990). A plant methionine transaminase has been identified in Arabidopsis, BRANCHED-CHAIN AMINOTRANSFERASE4 (BCAT4) (Schuster *et al.*, 2006). Screening of the *C. miyabeanus* strain WK1C genome sequence for BCAT4 orthologs reveals the presence of four putative methionine transaminases (Table 6.1). Similar to BCAT4, they are all predicted by InterProScan5 to be branched-chain amino acid aminotransferases II (IPR005786), belonging to class IV aminotransferases (IPR001544) (Jones *et al.*, 2014).

JGI protein ID	Genome location	E-value	%identity	% coverage
104809	scaffold_161:34133-35542	7.04E-037	36.9	70.5
32524	scaffold_7:187703-189133	2.11E-030	34.1	52.6
35557	scaffold_42:44411-45754	5.22E-023	34.6	46.5
9393	scaffold_222:7874-9127	9.58E-020	43.4	32.6

Table 6.1. Putative methionine transaminases in the genome of *C. miyabeanus* strain WK1C, retrieved by BlastP using BCAT4 as a template.

Site-directed mutation of each of these corresponding genes could reveal their putative function in the KMBA pathway. In addition, screening of *C. miyabeanus* restriction enzyme-mediated integration (REMI)-mutants may also result in the identification of the methionine transaminase responsible for conversion of methionine to KMBA. REMI is a useful technique to randomly disrupt fungal genes by plasmid insertion and allows subsequent identification of the disrupted genes (Turgeon *et al.*, 2010). The technique has been successfully applied in several fungal species, including *Cochliobolus* spp. (Lu *et al.*, 1994; Bölker *et al.*, 1995; Sánchez *et al.*, 1998). Eventually, the development of a double mutant in both EFE and KMBA ethylene-biosynthesis would allow us to determine whether fungal ethylene is indispensable for plant ethylene biosynthesis and/or infection.

Identification of additional virulence factors

As plant-pathogenic fungi typically produce an arsenal of virulence factors, including phytotoxins, effectors, cell wall degrading enzymes, siderophores and so on, it is expected that many more compounds will be identified from *C. miyabeanus* in the future. The development of a

double mutant in *CmOPS1* and *CmNPS3* could provide a convenient tool to find additional virulence factors that may be masked in the presence of these pertinent phytotoxins. A preliminary screening of compounds secreted by the wild type Cm988 strain in liquid culture and separated using UPLC, shows the presence of several ethylene-inducing fractions (Chapter 5). Further analysis of these fractions could provide more insight in the strong ethylene response of rice plants upon penetration of *C. miyabeanus*.

During fungal infection, chitin oligosaccharides are released from fungal cell walls by chitinase activity. Some plants possess LysM containing receptors that induce a plant defense response upon perception of chitin oligomers that act as PAMPs (Miya *et al.*, 2007). Recently it was shown that *Cladosporium fulvum* produces a LysM-containing protein that acts as a virulence factor, Ecp6, and subsequent screening of fungal genome sequences revealed that many filamentous fungi carry putative LysM-containing proteins (de Jonge & Thomma, 2009). Production of LysM-proteins by fungi can have several functions. First of all it could avoid recognition of PAMPs by sequestering the chitin oligomers. Furthermore it could shield the fungal hyphae from chitinases. Finally, LysM proteins could prevent conformational changes of plant receptors by interacting with the substrate (de Jonge & Thomma, 2009; de Jonge *et al.*, 2010; Kombrink & Thomma, 2013). A quick screening of predicted proteins from *C. miyabeanus* strain WK1C for proteins with a Pfam LysM motif (PF01467), revealed the presence of 13 putative LysM-proteins. It would be interesting to investigate a possible role for these LysM proteins in *C. miyabeanus* virulence. Chitinases can be induced upon ethylene signaling (Kasprzewska, 2003). Whereas the induction of ethylene during brown spot disease overall promotes susceptibility, induction of chitinases could be detrimental to the pathogen. Rice indeed secretes chitinases and a LysM-containing protein in the protoplast (Kim *et al.*, 2014). It is not unthinkable that *C. miyabeanus* would try to protect its hyphae by covering them in LysM-proteins (Kombrink & Thomma, 2013).

Plants try to protect their cells from extensive cell death by inducing antiapoptotic genes. A well-known cell death inhibitor is Bax-Inhibitor (BI) and many BI-homologs have been shown to be involved in plant resistance (Matsumura *et al.*, 2003; Hückelhoven *et al.*, 2003; Watanabe & Lam, 2006; Kawai-Yamada *et al.*, 2009). Whereas rice suspension cells were able to strongly induce *OsBI-1* expression upon treatment with pure ophiobolin A, *OsBI-1* induction was no longer observed during infection (Chapter 2). It is possible that *C. miyabeanus* produces additional factors that can repress *OsBI-1*, in an attempt to enhance cell death. Similar effects have been observed for *M. oryzae* cell wall derived sphingolipid cerebrosides that can down-regulate *OsBI-1* expression (Koga *et al.*, 1999; Matsumura *et al.*, 2003). Given that a similar cerebroside elicitor has been found in *C. miyabeanus*, it may be worth analysing its effect on *OsBI-1* expression during infection (Umemura *et al.*, 2000).

Breeding towards brown spot disease resistance

Resistance breeding is a critical principle of sustainable pest management. Given that rice is the most important staple crop worldwide, improvement in rice production and reduction of diseases can have immediate and strong socio-economic implications. This is especially true for rice brown spot disease, as it affects mainly resource-poor farmers which still make up the majority of rice farmers in South-East Asia (Savary *et al.*, 2012b; Barnwal *et al.*, 2013). Past breeding efforts towards brown spot disease resistance have not achieved very good results. Overall, the search for resistant germplasm has been unsystematic, ignoring variability in strain virulence and soil and weather conditions (Hossain *et al.*, 2004; Arshad *et al.*, 2013). Fortunately, efforts are finally increasing and several available rice genotypes are being tested under different field conditions and new promising quantitative trait loci (QTL) have been reported (Hossain *et al.*, 2004; Sato *et al.*, 2008; Katara *et al.*, 2010; Yaqoob *et al.*, 2011; Kumar *et al.*, 2013).

Qualitative resistance genes, or susceptibility genes in the case of necrotrophs, can provide strong sources of resistance, or susceptibility, to host-specific pathogens. However, brown spot disease relies on a multitude of non-selective virulence factors and no qualitative resistance genes are expected to be found. Hence, efforts should focus on quantitative disease resistance, which is actually superior to qualitative resistance in regards of durability. Nowadays, breeding programs make extensive use of effectors to find corresponding interaction partners in plants. The era of effectoromics has resulted in the discovery of many previously unidentified R-genes that are complementary to existing resistance sources to mainly biotrophic pathogens. However, necrotrophic effectors can also be used to screen for quantitatively more resistant germplasm (Dangl *et al.*, 2013; Vleeshouwers & Oliver, 2014). Although the widespread use of effectors may be recent, resistance breeding using selective agents, such as phytotoxins, has existed for a long time (Ling *et al.*, 1985; Buitatti & Ingram, 1991; van den Bulk, 1991; Švábová & Lebeda, 2005). The identification of new virulence factors could complement breeding efforts.

Given the essential role of phytotoxins in necrotroph disease, it is an interesting thought that broad spectrum toxin efflux transporters might improve resistance. For example, pleiotropic drug-resistant (PDR)-like ABCG transporters are potentially involved in wheat resistance to deoxynivalenol, produced by *F. graminearum* during Fusarium head blight (Muhovski *et al.*, 2014). So far, 23 PDR transporters have been identified in rice, of which only one has been characterized. Apart from exporting phytotoxins, they could also contribute to resistance through secretion of antimicrobial compounds (Moons, 2008). Furthermore, transient expression of yeast *PDR5* in tobacco resulted in export of toxins (Muhitch *et al.*, 2000). In Chapter 3, we identified a putative toxin transporter in close vicinity to the ophiobolin biosynthesis gene cluster, Orf1. If the functionality of Orf1 in ophiobolin export would be confirmed, it would be interesting to test whether expression of the gene in rice could provide protection.

Furthermore, given the importance of plant cell death throughout the infection process, breeding towards preventing cell death responses may be successful in enhancing resistance to necrotrophs. However, localized cell death responses are prerequisite to ward off biotrophs. Better insight in the differences between HR and necrotroph-induced cell death could therefore provide targets that avoid trade-offs in disease resistance (van Kan, 2006). In this regard, we should be careful when focussing on the impact of ethylene on susceptibility. Whereas reducing ethylene responses upon infection could confer resistance to brown spot, it may at the same time enhance susceptibility to blast (Iwai *et al.*, 2006). As mentioned earlier, nutritional stress and drought predispose plant to brown spot disease by changing plant physiology and inducing ethylene. However, these physiological changes are less pronounced in drought resistant cultivars, suggesting that they may also exhibit improved resistance to brown spot. Hence it seems opportune to test this correlation, which could provide a major advantage in the challenges created by climate change, such as increasing water shortages and extended periods of drought.

Primer list

Primer name	Sequence (5' – 3') *	Purpose	Chapter
NLC38	CGTTGCAAGACCTGCCTGAA	Forward primer for HygB (Catlett *et al.*, 2003)	2, 4, 5
NLC37	GGATGCCTCCGCTCGAAGTA	Reverse primer for HygB (Catlett *et al.*, 2003)	2, 4, 5
M13F	CGCCAGGGTTTTCCCAGTCACGAC	Reverse primer to amplify HygB (Catlett *et al.*, 2003)	2, 4, 5
M13R	AGCGGATAACAATTTCACACAGGA	Forward primer to amplify HygB (Catlett *et al.*, 2003)	2, 4, 5
DW729	ATCTTGGGACCTGGGAACTT	Forward primer of 5' flank for *CmOPS1* deletion	2
DW730	*TCCTGTGTGAAATTGTTATCCGCT* GTGGCTTATGAACGGTGGTT	Reverse primer of 5'flank for *CmOPS1* deletion with added HygB region (complementary to M13R)	2
DW731	*GTCGTGACTGGGAAAACCCTGGCG* CGTTTGGACGTGTGTTTGTC	Forward primer of 3' flank for *CmOPS1* deletion with added HygB region (complementary to M13F)	2
DW734	ATCATTTGGCTATGGCGGTA	Reverse primer of 3' flank for *CmOPS1* deletion	2
DW735	TCTTAGCACCGACCTCCCTA	Internal forward primer for *CmOPS1*; deletion verification	2
DW736	TGAGCCACTTGTTGATGGAG	Internal reverse primer for *CmOPS1*; deletion verification	2
DW733	GTGGGGGTTGATCCTTACAA	Forward primer outside of 5' flank for *CmOPS1* deletion; deletion verification	2
DW737	AGCAATGGGTCAAGAGTCGT	Reverse primer outside of 3' flank for *CmOPS1* deletion; deletion verification	2
CmAct1.2_F	AAGTCGCAGCTCTCGTCATC	Forward primer for *CmActin*; normalizer for qRT-PCR fungal gene expression analysis	2
CmAct1.2_R	TGAGTCCTTCTGGCCCATAC	Reverse primer for *CmActin*; normalizer for qRT-PCR fungal gene expression analysis	2
CmOphio2_F	CACCGACCTCCCTAACTTGA	Forward primer for *CmOPS1*; quantitative RT-PCR gene expression analysis	2
CmOphio2_R	TTGAGCCACTTGTTGATGGA	Reverse primer for *CmOPS1*; quantitative RT-PCR gene expression analysis	2
eIF1a_for	TTTCACTCTTGGTGTGAAGCAGAT	Forward primer for *OseIF1a*; normalizer for qRT-PCR	2
eIF1a_rev	GACTTCCTTCACGATTTCATCGTAA	Reverse primer for *OseIF1a*; normalizer for qRT-PCR	2
OsNAC4_F1	GGTGAAGGAGGACAACGACT	Forward primer for *OsNAC4*; qRT-PCR	2
OsNAC4_R1	TCAGAATGGTGGCAGGATTGT	Reverse primer for *OsNAC4*; qRT-PCR	2
OsBI1-F	CGGAGGAGAAGAAGAGGAAG	Forward primer for *OsBI-1*; qRT-PCR	2

OsBI1-R	ACGACGACAAACCTGAGAAG	Reverse primer for *OsBI-1*; qRT-PCR	2
Jamyb-F	CCCTCGTCAATTACATCTCTGA	Forward primer for *OsJAMyb*; qRT-PCR	2
Jamyb-R	GAGTGGAGGTCGAGGATGAG	Reverse primer for *OsJAMyb*; qRT-PCR	2
MPK5-F	ACGAGGACCAAATGAAGCAGC	Forward primer for *OsMPK5*; qRT-PCR	2
MPK5-R	AGCAGCCACAACTTGCAGAGA	Reverse primer for *OsMPK5*; qRT-PCR	2
OsPAL1-F	AGCACATCTTGGAGGGAAGCT	Forward primer for *OsPAL1*; qRT-PCR	2
OsPAL1-R	GCGCGGATAACCTCAATTTG	Reverse primer for *OsPAL1*; qRT-PCR	2
CmNODE8391_UF1	GCCGTTTTGCTGCTATCTT	Forward primer of 5' flank for *n8391* deletion	4
CmNODE8391_UR1	*TCCTGTGTGAAATTGTTATCCGCT* AAGGTGTCAGAACGCTACG	Reverse primer of 5'flank for *n8391* deletion with added *HygB* region (complementary to M13R)	4
CmNODE8391_DF1	*GTCGTGACTGGGAAAACCCTGGCG* GGATGAGGACAAGGAAAGG	Forward primer of 3' flank for *n8391* deletion with added *HygB* region (complementary to M13F)	4
CmNODE8391_DR1	GCGGGTGTGTGAATAAAGA	Reverse primer of 3' flank for *n8391* deletion	4
CmNODE8391_ amp1F1	AGAAGTCATCAACAGCAGCA	Internal forward primer for *n8391* ; deletion verification	4
CmNODE8391_ amp1R1	CCTGACTGAAACCCCTGAC	Internal reverse primer for *n8391* ; deletion verification	4
8391_UpVER1	TGGTTAGACGCACCCATACA	Forward primer outside of 5' flank for n8391 deletion; deletion verification	4
8391_DownVER1	AGTTGTGCACTCCTCTCTCC	Reverse primer outside of 3' flank for n8391 deletion; deletion verification	4
CmEFE_FP1	GCTAATACAACTGCCGACGA	Forward primer of 5' flank for CmEFE deletion	5
CmEFE_RP1	*TCCTGTGTGAAATTGTTATCCGCT* CGCTATAGTCAGCGATGTTATCC	Reverse primer of 5'flank for CmEFE deletion with added HygB region (complementary to M13R)	5
CmEFE_FP2	*GTCGTGACTGGGAAAACCCTGGCG* GGCAATGCCCTTACAAGGTA	Forward primer of 3' flank for CmEFE deletion with added HygB region (complementary to M13F)	5
CmEFE_RP2	TGCCATTCTCTCCTCAGCTC	Reverse primer of 3' flank for CmEFE deletion	5
CmEFE_qF1	TCACTAAAGATCTTGCATTGTCTGA	Internal forward primer for CmEFE; deletion verification	5
CmEFE_qR1	GCTTGCATGACAGTCTTCATCT	Internal reverse primer for CmEFE; deletion verification	5
CmEFE_UpVER1	CCGCTTTTGGGCAGTTAAT	Forward primer outside of 5' flank for CmEFE deletion; deletion verification	5
CmEFE_DownVER1	CGAAGACTGGGCGATTCTAA	Reverse primer outside of 3' flank for CmEFE deletion; deletion verification	5

* Sequences in *italics* are complementary to M13F and M13R sequences respectively.

Addendum

Connecting growth and defense: the emerging roles of brassinosteroids and gibberellins in plant innate immunity

Authors

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Abstract

Brassinosteroids (BRs) and gibberellins (GAs) are two groups of phytohormones that regulate many common developmental processes throughout the plant life cycle. Fueled by large-scale 'omics' technologies and the burgeoning field of plant computational biology, the past few years have witnessed paradigm-shifting advances in our understanding of how BRs and GA are perceived and their signals transduced. Accumulating evidence also implicates BR and GA in the coordination and integration of plant immune responses. Similar to other growth regulators, BR and GA play ambiguous roles in molding pathological outcomes, the effects of which may depend not only on the pathogen's lifestyle and infection strategy, but also on specialized features of each interaction. Analysis of the underpinning molecular mechanisms points to a crucial role of GA-inhibiting DELLA proteins and the BR-regulated transcription factor BZR1. Acting at the interface of developmental and defense signaling, these proteins likely serve as central hubs for pathway crosstalk and signal integration, allowing appropriate modulation of plant growth and defense in response to various stimuli. In this review, we outline the latest discoveries dealing with BR and GA modulation of plant innate immunity and highlight interactions between BR and GA signaling, plant defense and microbial virulence.

Introduction

Plant hormones are small signaling molecules that play diverse roles throughout the lifespan of plants. They not only orchestrate intrinsic developmental programs, but also convey environmental inputs and drive adaptive responses to abiotic and biotic stresses. Historically, plant immunity research has focused on the role of three hormones: salicylic acid (SA), jasmonic acid (JA), and ethylene (ET). Upon infection, plants produce a highly specific blend of these 'defense hormones', with the exact combination seemingly depending on the pathogen's lifestyle. In the dicot model plant *Arabidopsis thaliana*, resistance to biotrophic pathogens is usually dependent on SA, whereas necrotrophic pathogens tend to be resisted through a combination of JA and ET signaling (Pieterse *et al.*, 2012). Moreover, interaction between these two pathways is most often antagonistic, which has led many authors to suggest that plant immunity follows a binary model with SA and JA/ET having opposing influences (Navarro *et al.*, 2008). Although valid for many plant-pathogen interactions, this traditional view is overly generalized, and accumulating findings in both dicot and monocot systems suggest a more complex reality (Van der Ent & Pieterse, 2012; Pieterse *et al.*, 2012a). For instance, in rice (*Oryza sativa*), one of the most important food crops worldwide and a model for molecular genetic studies in cereals, disease resistance seems to be controlled by a highly complex signaling network that does not support a dichotomy between the effectiveness of the SA, JA and ET pathways and the lifestyle of a given pathogen (De Vleesschauwer et al., 2010; Riemann et al., 2013; De Vleesschauwer et al., 2013).

Moreover, new developments indicate that plant defense networking is more than just SA and JA/ET, with more integrative models implicating a coordinated range of hormones in configuring the plant's response to pathogen attack. These hormones include abscisic acid (ABA), which is best known for its role in abiotic stress adaptation. Depending on the pathogen type, plant species, and even timing of infection, ABA can act as both a positive and negative regulator of disease resistance, at least in part by feeding into the SA-JA-ET backbone of the plant immune system (Asselbergh et al., 2008; Cao et al., 2011). Other recently identified defense regulators include cytokinins (CKs), auxin, brassinosteroids (BRs) and gibberellins (GAs), all of which have been intensively studied in the context of plant growth and development. Although much remains to be discovered about their precise role and function in plant-microbe interactions, recent data are now beginning to unveil how these 'developmental hormones' modulate host immunity, and how microbe-induced perturbations of these classic growth regulators contribute to virulence (Robert-Seilaniantz et al., 2011; Pieterse et al., 2012). In case of auxin, for instance, it is now well established that the auxin and SA pathways act in a mutually antagonistic manner during plant defense, whereas auxin and JA signaling share many commonalities. Moreover, a growing body of evidence indicates that some pathogens either produce auxin themselves or increase plant auxin biosynthesis upon infection tot manipulate the plant's defensive and developmental machinery (Valls et al., 2006; Kazan and Manners, 2009).

In this review, we survey recent progress in deciphering the immune-regulatory role of brassinosteroids (BRs) and gibberellins (GAs), with a special focus on the cellular components and hormone signals involved in regulating crosstalk between BR/GA signaling, plant defense and microbial virulence. For more detailed information on auxin- and cytokinin-modulated immunity, we refer the reader to a number of excellent recent review papers dealing with these topics (Choi et al., 2011; Naseem et al., 2012; Denance et al., 2013).

Brassinosteroids: multi-taskers in plant-microbe interactions

Role of brassinosteroids in plant physiology

Discovered nearly 40 years ago, brassinosteroids are a unique class of polyhydroxylated steroidal phytohormones with important roles in regulating myriad physiological and developmental processes, including pollen tube elongation, seed germination, photomorphogenesis, flowering and senescence. To date, more than 50 brassinosteroids have been described, of which brassinolide (BL) and castasterone are the most active forms (Bajguz, 2007). Typically, BR-deficient and BR-insensitive mutants display dwarfism. Underlying this phenotype is the involvement of BRs in controlling cell division, expansion, and even differentiation (For review see Müssig, 2005; Fridman and Clouse, 2011; Savaldi-Goldstein, 2013). In regard to these physiological effects, BRs often have been shown to interact with other growth regulators, such as GA and auxins (Fridman & Savaldi-Goldstein, 2013). Additionally, a clear role in reaction to abiotic stresses was demonstrated (Bajguz & Hayat, 2009). Recent advances, however, indicate that brassinosteroids are also involved in a complex molecular interaction network that steers host defense responses upon pathogen attack (Choudhary *et al.*, 2012).

BR signaling in plants

Over the past decade, molecular genetic studies using Arabidopsis (*Arabidopsis thaliana*) and rice (*Oryza sativa*) as model plants have identified numerous genes involved in BR biosynthesis and gene regulation. Together with more recent biochemical approaches, these studies have provided fascinating insights into the various aspects of BR signaling, establishing the BR pathway as one of the best-understood signal transduction pathways in plants (for review see Li, 2010; Ye et al., 2011; Tong and Chu, 2012; Zhu et al., 2013). According to current concepts, active brassinosteroids such as BL and castasterone bind directly to the extracellular domain of the leucine-rich repeat receptor-like kinase (LRR-RLK) BRI1, thereby inducing a series of biochemical responses, including BRI1 dissociation from the negative regulator BKI1, subsequent heterodimerization of BRI1 with, and activation of, the co-receptor BAK1, phosphorylation of the BRI1-interacting signaling kinase (BSK1), and activation of the protein phosphatase BSU1. These events eventually culminate in inhibition of the shaggy-like kinase BIN2 and resultant

activation of the homologous transcription factors (TFs) BZR1 and BES1/BZR2. Finally, activated BZR1 and BES1 migrate to the nucleus where they bind BR-responsive promoters, causing transcriptional changes that ultimately shape BR signaling outputs (Tang *et al.*, 2011).

Implications of BR in basal defense and disease susceptibility

Besides their critical role in orchestrating growth and developmental processes, BRs are also increasingly implicated in plant responses to pathogen attack. However, compared to the well-characterized signaling pathway, the role of BRs in plant defense is less well understood, and even controversial. Although BRs have long been tagged as positive regulators in plant disease resistance, recent findings have revealed many previously unexpected roles of BR in shaping pathological outcomes (Jaillais *et al.*, 2011; Albrecht *et al.*, 2012; Belkhadir *et al.*, 2012; De Vleesschauwer *et al.*, 2012; Nahar *et al.*, 2013; Shi *et al.*, 2013).

Originally, field trials with exogenously administered BRs suggested a positive effect on the resistance of diverse crops to a broad array of pathogens (Khripach, 2000). This was supported by a report by Nakashita et al. (2003) who studied the impact of exogenously applied BRs on innate immunity. The authors reported a positive but variable effect of BL on disease resistance of tobacco and rice to distinct leaf pathogens in small-scale disease trials. This effect was found to be not only local, but also systemic (Nakashita *et al.*, 2003). More recently, BR application was also shown to protect barley from several *Fusarium* diseases (Ali *et al.*, 2013).

However, the impact of BR on disease resistance is not always positive as demonstrated by the inability of exogenous BL to alter resistance of Arabidopsis to *Pseudomonas syringae* pv. *tomato* (*Pto*) and *Alternaria brassicicola* (Albrecht *et al.*, 2012). Moreover, two recent studies revealed that BL pretreatment renders rice hyper-susceptible to the root pathogens *Pythium graminicola* and *Meloidogyne graminicola*, unmasking BRs as potential negative regulators of plant immunity (De Vleesschauwer *et al.*, 2012; Nahar *et al.*, 2013). In case of *P. graminicola*, it was even suggested that the pathogen hijacks the rice BR machinery to promote infection, thus exploiting BRs as virulence factors (De Vleesschauwer *et al.*, 2012). More examples of BR-effects on distinct plant-pathogen interactions are reviewed by Bajguz and Hayat (2009).

Together these apparently conflicting findings clearly illustrate the importance of BR homeostasis in the establishment of plant immunity. Given the central importance of BRs in regulating plant size, these observations are compatible with the idea that BRs merge immune system function with normal growth and developmental programs, thus serving as important regulators of the innate trade-off between disease resistance and plant growth. Although initially seen as mainly positive players in disease resistance, the situation now seems more complicated with positive, negative as well as neutral BR effects being reported. Other plant hormones, such as SA, JA and ET can also have diverse effects on disease resistance and for many pathosystems the effectiveness of the SA/JA/ET-pathways can be linked to the lifestyle of the invading pathogen

(i.e. biotrophy, hemibiotrophy and necrotrophy). In contrast, the effects of BR are seemingly independent of either the plant species or type of pathogen involved, suggesting that BRs function as complex multifaceted regulators of plant immunity, with apparently divergent outcomes.

Molecular mechanisms of BR-mediated immunity

Although much remains to be revealed about the precise mechanisms via which BRs dictate the output of plant-pathogen interactions, exciting new developments connect BRs to a wide variety of defense-related pathways involved in innate immunity triggered by conserved microbial signatures, microbial-induced cell death, hormone signaling, oxidative metabolism and secondary metabolite production (Figure A1).

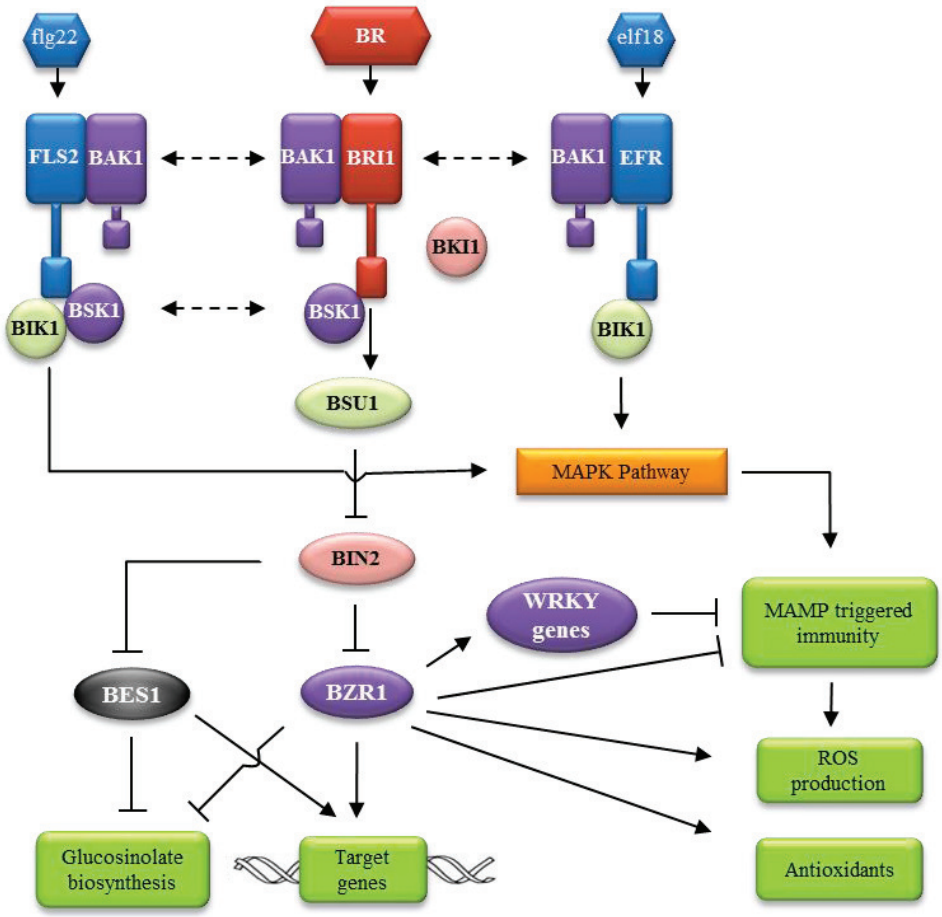

Figure A1.Model illustrating molecular mechanisms by which BR could influence disease resistance. The BR signaling pathway is linked to the PTI signaling pathway on 3 levels by sharing the components BAK1 and BSK1 and via BZR1-steered negative regulation of MAMP-triggered immune responses. Downstream in the BR signaling pathway, core TFs BES1 and BZR1 transduce the BR signal to the nucleus where they alter gene expression, regulate ROS and antioxidant production, and suppress glucosinolate biosynthesis.

BRs modulate the efficiency of PAMP-triggered immunity

To date, most studies aimed at understanding how BR molds pathological outcomes have focused on the role of the LRR-RLK BAK1. Originally identified as a co-receptor for BRI1 and,

hence, a positive regulator of BR responses, BAK1 appears to be positioned at the intersection of multiple signaling routes. Indeed, besides its role in BR signaling, BAK1 is also involved in the regulation of microbe-induced cell death (Kemmerling et al., 2007), and interacts physically with various pattern recognition receptors (PRRs), including the flagellin receptor FLS2, to drive so-called PAMP-triggered immunity (PTI) (Fradin *et al.*, 2009; Bar *et al.*, 2010; Chaparro-Garcia *et al.*, 2011).

In view of this, it was long speculated that BR would either increase plant immunity through BAK1 activation or, alternatively, antagonize plant defenses through competition for BAK1. However, recent work by Albrecht et al. (2012) revealed that exogenously administered BR does not reinforce PTI responses in *Arabidopsis*, but instead unidirectionally antagonizes FLS2-mediated immune signaling (Albrecht *et al.*, 2012; Belkhadir *et al.*, 2012). Moreover, it was shown that BAK1 is not rate-limiting and that BR-controlled inhibition takes place downstream and independent of BAK1 by a so far unknown mechanism (Albrecht *et al.*, 2012). Besides, null *bak1* mutants expressing the hypermorphic *bak1*^{elg} allele showed enhanced BR signaling but failed to mount resistance to *Pto* DC3000 upon treatment with the flagellin epitope flg22 (Jaillais *et al.*, 2011). Another mutation in BAK1 (*bak1-5*) did not impair BR signaling, but compromised PTI signaling, resulting in hypersusceptiblity to a weakly virulent *Pto* DC3000 mutant deficient in production of the phytotoxin coronatine (Schwessinger *et al.*, 2011). This adds to the hypothesis that BAK1 pathway specificity is regulated by differential phosphorylation and extracellular domain interactions between receptor and coreceptor (Kemmerling *et al.*, 2007; Oh *et al.*, 2010; Schwessinger *et al.*, 2011; Jaillais *et al.*, 2011). Together with previous studies (Chinchilla *et al.*, 2009), these data also suggest that BAK1 function in cell death control and innate immunity is independent of its function in BR signaling.

At the same time, recent findings by Belkhadir et al. (2012) point to a more complex scenario. By tuning the BR pathway response in *Arabidopsis*, these authors elegantly demonstrated that BR can act both antagonistically and synergistically with PTI responses. Conditions of excess and depleted endogenous BR content as well as increased BR signaling triggered by BRI1 overexpression all attenuated BAK1-mediated PTI, suggesting the existence of a narrow range of BR concentrations that prime innate immunity. In contrast, in plants expressing the hypermorphic *BRI1*^{sud1} allele, flg22-triggered PTI signaling was enhanced in a BAK1-dependent manner. Despite these enhanced PTI responses, *BRI1*^{sud1} plants displayed enhanced susceptibility to the obligate biotrophic pathogen *Hyaloperonospora arabidopsidis*, whereas the same slight increase in BR signaling enhanced resistance in *bak1* mutant plants. Overall these data not only support a scenario wherein BAK1 acts as a mediator for the synergistic activities of BRs on PTI responses, but also indicate that BR can act on plant defenses independently of BAK1.

Interestingly, two recent papers have shed more light on the molecular mechanisms via which BR suppresses PTI responses in a BAK1-independent manner. In the first study, Lozano-Duràn et al. (2013) propose that BR antagonism of PTI happens downstream of BIN2 and that the key BR transcription factor BZR1 is required and sufficient for the suppression of PAMP-induced immune signaling. BZR1 activates several WRKY genes, which subsequently act as co- and secondary negative regulators of PAMP-triggered ROS production and gene expression, independent of MAPK signaling. The authors propose a model in which BZR1 and WRKY40 cooperate to down-regulate the expression of defense-related gene expression. The second study by Shi et al. (2013) focuses on the role of the BRI1-associated signaling kinase BSK1. Acting as a crucial BRI1 substrate in the BR signaling pathway, BSK1 also serves as a positive regulator of flg22-induced ROS production and SA accumulation by physically interacting with FLS2. Moreover, inhibition of BSK1 increased susceptibility to both virulent and avirulent pathogens (Shi *et al.*, 2013). Although this would imply that negative BR-PTI crosstalk may also occur upstream of BIN2, these findings suggest that competition for BSK1 between the BRI1 and FLS2 signaling pathways could add to the BAK1-independent negative effect of BR on the flg22-response.

Recently, the receptor-like cytoplasmic kinase Botrytis-Induced Kinase 1 (BIK1) was added to the list of signaling components shared by the BR and PTI pathways (Lin et al., 2013). Although BIK1 negatively regulates the BR-signaling pathway and positively regulates the FLS2-PTI signaling pathway, its functions in both processes are mechanistically uncoupled. Consistent with FLS2-BIK1 complex dynamics in flagellin signaling, BIK1 associates with BRI1, and is released from the BRI1 receptor upon BR treatment. However, in contrast to BAK1-dependent FLS2-BIK1 dissociation, BAK1 is dispensable for BRI1-BIK1 dissociation. Moreover, unlike FLS2 signaling which depends on BAK1 to phosphorylate BIK1, BRI1 directly phosphorylates BIK1 to transduce BR signaling. Thus, BIK1 relays the signaling in plant immunity and BR-mediated growth via distinct phosphorylation by BAK1 and BRI1, respectively.

BRs orchestrate crosstalk among defense-signaling pathways

Associations between BR signaling and innate immunity have also been found in hormonal crosstalk, as reviewed by Choudhary et al. (2012). Following stress perception, BRs interact with a range of hormones, such as SA, JA, abscisic acid (ABA), auxins and GA. In many cases, the nature and direction of these hormone signal interactions can be rapidly adjusted depending on the prevailing conditions, equipping the plant with a powerful regulatory mechanism to fine-tune its defense response to the type of stress encountered. This is nicely exemplified in rice where BRs enhance resistance to foliar pathogens in an SA-independent manner; yet, antagonize SA-mediated defenses to the root pathogen *P. graminicola* (Nakashita *et al.*, 2003; De Vleesschauwer *et al.*, 2012). Furthermore, several SA-marker genes were found to be induced upon BL treatment in Arabidopsis, raising the prospect of synergistic BR-SA crosstalk as well (Divi *et al.*, 2010). In

addition, BRs negatively interact with JA in the regulation of growth processes in Arabidopsis (Choudhary *et al.*, 2012) and, accordingly, disable JA-induced resistance to the rice root knot nematode *M. graminicola* (Nahar et al., 2013). Interestingly, BRs can also cross-communicate with auxins. As auxins are well known modulators of plant immunity, either directly or via crosstalk with the SA/JA signaling network, bidirectional BR-auxin interplay may also contribute to the ambivalent effects of BRs in disease and resistance (Choudhary *et al.*, 2012; Pieterse *et al.*, 2012). Finally, BRs also interact with GA. In the rice-*P. graminicola* interaction, for instance, BRs were shown to dampen effective immune responses by interfering at multiple levels with GA metabolism (De Vleesschauwer et al., 2012). Operating at both the level of biosynthesis regulation and signal transduction with BR suppressing GA biosynthesis and transcriptionally activating GA repressor genes, this BR-GA antagonism results in indirect stabilization of the rice DELLA protein and GA signaling inhibitor, SLR1 (De Vleesschauwer *et al.*, 2012). More recent findings, however, indicate that crosstalk between BR and GA is also mediated by direct physical interactions between the BZR1 transcription factor and GA-inhibiting DELLA proteins (see below).

BRs fine-tune oxidative and secondary metabolism

Evidence for yet another mode of BR action is provided by the ability of BRs to influence the production of reactive oxygen species (ROS) (Foreman *et al.*, 2003; Bajguz & Hayat, 2009; Almagro *et al.*, 2009; Xia *et al.*, 2009; Swanson & Gilroy, 2010; Suzuki *et al.*, 2012; Baxter *et al.*, 2013). ROS are secondary messenger molecules generated during development and in response to stress that serve myriad functions, including the activation of phytoalexin biosynthesis and regulation of cell death. The involvement of BR in ROS and antioxidant production and the interplay between BR signaling and ROS signaling is ambiguous. A narrow range of BR concentrations stimulate ROS production by membrane-bound NADPH oxidases, and BR-induced ROS production has been shown to be central in the establishment of stress tolerance (Xia *et al.*, 2010). However, depending on hormone titers, plant age, and the type of tissue, BRs can also stimulate antioxidant production and scavenge ROS (Bajguz & Hayat, 2009; Fariduddin *et al.*, 2013). Finally, BRs may also influence disease resistance by modulating secondary metabolite production, as evidenced by the repressive effect of BZR1 and BES1 on glucosinolate production in Arabidopsis (Guo *et al.*, 2013).

In summary, consistent with the apparent multifaceted role of BR in determining the outcome of plant-pathogen interactions, abovementioned studies have revealed a wide variety of underpinning mechanisms, ranging from modulation of PAMP perception to downstream signaling and orchestration of oxidative metabolism and production of secondary metabolites. Although the importance of BR in the regulation of plant immunity is evident, the precise mechanism(s) involved and its role in either stimulating disease resistance or susceptibility appears to depend greatly on the plant-attacker combination, indicating that the function of plant

growth hormones in pathogen defense cannot easily be generalized. In the upcoming sections, we attempt to distill current literature on the immune-regulatory role of another class of phytohormones, GAs, and highlight how these master growth regulators participate in influencing disease outcomes.

GA and DELLA proteins: novel master regulators of plant immunity

GAs: pivotal growth regulators and architects of the first green revolution

Gibberellins (GAs) are tetracyclic 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 (Hauvermale et al., 2012). The origin of research into GAs can be traced back to Japanese plant pathologists in the late 1800s that were studying a devastating rice disease referred to as 'bakanae' (foolish seedling). Symptoms of the disease included excessive seedling elongation, slender leaves, and stunted roots. In 1938, Yabuta and his associate Yusuke Sumiki tied the elongation of bakanae rice to a substance derived from the fungal pathogen *Gibberella fujikuroi* (teleomorph: *Fusarium moniliforme*) and named the compound after the pathogen it was isolated from: gibberellin.

Although it was not until the mid 1950s that researchers became aware of GAs as naturally occurring substances in higher plants, the involvement of GAs in determining plant stature has since revolutionized modern agriculture. During the first Green Revolution, breeding programs capitalized on increasing crop productivity by selecting semidwarf varieties that responded well to nitrogen fertilization without lodging and displayed increased partitioning of assimilates into grains (Khush, 2001). It was subsequently discovered that most Green Revolution varieties harbored mutations affecting GA synthesis. These mutants include the 'miracle' rice line IR8, which produced record yields throughout Asia and was found to contain a defective GA20 oxidase gene (Monna *et al.*, 2002; Sasaki *et al.*, 2002; Spielmeyer *et al.*, 2002). Through extensive physiological and molecular genetic studies of mutant plants impaired in GA perception, biosynthesis or signaling, it is now clear that GAs are master regulators of plant growth, particularly in response to environmental conditions such as light, flooding, temperature and salt, and biotic stress (Claeys *et al.*, 2013).

GA signaling in plants: of molecular glue and DELLA repression

As with BRs, research over the past few years has uncovered the principal steps associated with GA perception and signal transduction in Arabidopsis and rice. According to the canonical GA signaling model, GA promotes plant growth and development by inducing the degradation of

DELLAs, a class of nuclear growth-repressing proteins. In rice, binding of GA to the soluble GA receptor GID1 causes the GID1 N-terminal lid domain to act as molecular glue and interact with the single DELLA protein, SLENDER RICE 1 (SLR1) (Ueguchi-Tanaka *et al.*, 2007; Murase *et al.*, 2008). Subsequently, the stabilized trimeric complex consisting of GA, GID1 and SLR1 is targeted for polyubiquitination by the F-box protein GID2, which leads to rapid degradation of SLR1 by the 26S proteasome and resultant relief of the SLR1-imposed growth restraint (Gao *et al.*, 2011). A similar yet more complicated pathway is operative in *Arabidopsis* with three GA receptors (GID1a, GID1b and GID1c), five DELLA proteins (RGA, GAI, RGL1, RGL2 and RGL3), and the F-box protein SLY1 (Hauvermale *et al.*, 2012).

Implications of GA and DELLAs in basal defense and disease susceptibility

Despite being first discovered in a fungal plant pathogen, GAs and their signaling components have only recently been implicated in plant responses to pathogen attack. Among the first to suggest a role for GA in plant immunity were Zhu et al. (2005), who reported that the outer capsid protein P2 of the rice dwarf virus interacts with plant ent-kaurene oxidases, leading to decreased GA levels in the infected plants.

However, GA-related immunity research failed to gain momentum until it was shown that Arabidopsis mutants lacking four of the five DELLAs exhibited high levels of SA-dependent resistance when challenged with the hemibiotrophic pathogen *P. syringae* (Navarro *et al.*, 2008). In contrast, quadruple DELLA mutants showed attenuated induction of the JA-reporter gene *PDF1.2*, which correlated with enhanced susceptibility against the necrotrophic fungus *Alternaria brassicola* (Navarro et al., 2008). On the basis of these and other findings, it has been contemplated that DELLAs modulate the balance of SA/JA signaling during plant immunity, promoting JA perception and/or signaling, and repressing SA biosynthesis and signaling (Navarro et al., 2008). This ambivalent role of DELLAs also seems well conserved in monocot species as experiments with defined wheat and barley lines differing in DELLA status revealed a similar resistance trade-off with increased susceptibility to biotrophs and enhanced resistance to necrotrophs (Saville *et al.*, 2012).

In rice, strikingly different results have been obtained in that exogenously administered GA was found to lower resistance to the hemibiotrophic rice pathogens *Magnaporthe oryzae* (*Mo*) and *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (Yang *et al.*, 2008; Qin *et al.*, 2013). Moreover, transgenic rice overexpressing Elongated Uppermost Internode (EUI), a GA deactivating enzyme, accumulated low levels of GA and SA and displayed enhanced resistance to *Mo* and *Xoo*, whereas loss-of-function mutations in EUI were more vulnerable to these pathogens (Yang et al., 2008). These phenotypes and additional analyses of rice mutants compromised in GA perception or synthesis suggest that GA impairs (hemi)biotroph resistance in rice (Tanaka *et al.*, 2006; Qin *et al.*, 2013). However, GA can also act positively on rice immunity as shown for the necrotrophic

rice root rot pathogen *P. graminicola* (De Vleesschauwer *et al.*, 2012). Therefore, opposite to the situation in Arabidopsis, barley and wheat, rice GA signaling appears to promote resistance to necrotrophs and susceptibility to (hemi)biotrophs.

Emerging from these studies is the view that GAs play ambiguous roles in the plant innate immune signaling network. In some cases, they contribute to the development of disease symptoms, whereas in other cases they are required for defense responses and induction of plant resistance. Much like BRs and the classic defense hormones SA, JA and ET, GAs thus seem to behave as multifaceted regulators of plant immunity, the effect of which may differ not only on the plant species and type of pathogen involved, but also on specialized features of each interaction.

Mechanisms of GA- and DELLA-mediated plant immunity

Whilst offering a first insight into the immune-regulatory role of GA and DELLAs, most of abovementioned reports have only scratched the surface and much remains to be learned about the precise mechanisms via which GA and DELLAs modulate plant immunity. However, fueled by large-scale proteomic analyses and recent advances in computational biology, exciting new progress is now beginning to unveil the molecular secrets of GA and DELLA protein action.

GA and DELLAs contribute to fine-tuning of ROS production

Although earlier studies provided circumstantial evidence connecting GA to the regulation of phytoalexin biosynthesis and the expression of pathogenesis-related proteins (Zhu *et al.*, 2005; Tanaka *et al.*, 2006), Achard et al. (2008) made a first inroad into the molecular underpinnings of GA-mediated immunity by showing that DELLAs modulate ROS production during periods of adversity. Opposite to GA-induced oxidative stress (Schopfer, 2001; Fath, 2001), DELLAs elevate the induction of genes involved in ROS detoxification, thus reducing ROS levels and restraining plant cell death. Upon attack by a necrotrophic pathogen, such DELLA-mediated cell death restriction contributes to enhanced disease resistance, as was shown for the grey mould pathogen *Botrytis cinerea* and the mutualist fungus *Piriformospora indica* (Achard *et al.*, 2008; Jacobs *et al.*, 2011). Implicit here is the view that necrotrophic pathogens such as *G. fujikuroi* exploit GAs as virulence factors to degrade DELLAs and establish host susceptibility, not only by interfering with SA/JA-dependent defenses as described above, but also by tilting the ROS-controlled life-or-death balance towards death. Moreover, considering the intricate interaction of ROS and SA in a self-amplifying system (Shirasu *et al.*, 1997), it has been hypothesized that attenuation of SA signaling by DELLAs results, at least in part, from diminishing ROS levels (Grant & Jones, 2009).

GA signaling orchestrates cell wall development and modifies carbon and energy metabolism

Plant defense is a highly energy-intensive process (Bolton, 2009). Although pathogen recognition and downstream immune signaling have been intensively studied, the question of how plants are able to recruit energy to fuel defense responses has received only scant attention. Mounting evidence, however, indicates that large carbon and nitrogen fluxes into secondary metabolism during the immune response cannot occur without influencing reactions in primary metabolism (Bolton, 2009; Seifi *et al.*, 2013a,b).

Noteworthy in this respect is a recent study that investigated the effect of GA on carbon and energy metabolism during rosette growth in Arabidopsis (Ribeiro *et al.*, 2012). Combined metabolite profiling and translational analyses revealed that low GA regimes modulate global changes in primary metabolism, thereby uncoupling growth from carbon availability. Likewise, overexpressing gain-of-function versions of the Arabidopsis DELLA proteins GAI and RGL1 in transgenic poplar trees had profound consequences on cellular metabolism, suggesting increased respiration in roots and reduced carbox flux through the lignin biosynthetic pathway in shoots as well as a shift towards defense compounds associated mainly with the phenylpropanoid pathway (Busov *et al.*, 2006). By analogy with auxin-mediated disease susceptibility (Zhang *et al.*, 2007), GA may thus modify plant metabolism profiles, resulting in decreased production of antimicrobial compounds or increased nutrient efflux favoring microbes.

Interestingly, GA-induced metabolism changes may also result in cell wall modification, the role of which in plant immunity is well documented (Nishimura *et al.*, 2003; Hernandez-Blanco *et al.*, 2007; Curvers *et al.*, 2010). GA and DELLA have long been known to respectively induce and repress cell wall relaxation by altering the expression of xyloglucan endotransglucosylase/endohydrolases (XTHs) and expansins (Ogawa, 2003; Gallego-Bartolomé *et al.*, 2011; Park *et al.*, 2013). Although loosening the cell wall is a vital process during growth and development, it may also render the plant more vulnerable to biotic intruders by facilitating pathogen entry or allowing enhanced nutrient leakage. Indeed, recent evidence showed that rice plants overexpressing expansin genes become more vulnerable to *Xoo* and *Mo*, two pathogens that exploit GAs as virulence factors and are sensitive to DELLA-mediated defenses (Tanaka *et al.*, 2006; Ding *et al.*, 2008; Yang *et al.*, 2008; Fu *et al.*, 2011). These findings raise the possibility that DELLAs may induce disease resistance, at least in part, by counteracting pathogen-induced cell wall disturbance.

DELLAs mediate stress-induced cell differentiation and direct immunity-associated changes in plant cytoskeleton architecture

Cell divisions, including meiosis, mitosis, and endoreduplication, are essential processes for both vegetative and reproductive development in plants. The anaphase-promoting complex/cyclosome (APC/ C) is an evolutionarily conserved E3 ubiquitin ligase critical for cell cycle progression by degrading cell cycle proteins (Inzé and De Veylder, 2006). A recent study by Bao et al. (2013) revealed an unexpected effect of APC/C activity on plant immunity by showing that deregulation of two negative APC/C regulators, *OSD1* and its homolog *UVI4*, is associated with activation of disease resistance genes and enhanced immunity to *Pto* (Bao et al., 2013). Interestingly, *UVI4* was also found to be down-regulated following DELLA stabilization, resulting in mitotic exit and onset of endoreduplication (Claeys et al., 2013). While the causality of DELLA stabilization on plant disease resistance is by no means clear, these findings suggest a potential role of DELLAs in plant immunity by interfering with cell cycle-dependent expression of immunity genes.

In a similar vein, DELLAs have recently also been found to control plant cytoskeleton dynamics by physically interacting with the prefoldin complex, a cochaperone required for tubulin folding (Locascio et al., 2013). In the absence of GA, the prefoldin complex interacts with DELLAs in the nucleus, which severely compromises alpha/beta-tubulin heterodimer availability, affecting microtubule organization. In the presence of GA, however, DELLAs are degraded, which keeps the complex in an active state in the cytosol. Particularly interesting in this regard is a series of recent studies unmasking the cytoskeleton as a dynamic platform for sensing and responding to a diverse array of fungal, bacterial and oomyceteous pathogens (Tian et al., 2009; Lee et al., 2012; Porter et al., 2012; Henty-Ridilla et al., 2013). With transient increases in actin filament density being unveiled as an important contributing factor during PTI (Henty-Ridilla et al., 2013), elucidating whether DELLA-mediated plant immunity involves perturbation of cell cycle regulation is an important challenge ahead.

Straddling the line: DELLA-JAZ interactions balance plant growth and defense

Although abovementioned studies also implicate a non-transcriptional mode of action, DELLAs are generally thought of as transcriptional regulators. Yet, the lack of known DNA-binding domains and the moderate enrichment of promoter targets determined by chromatin immunoprecipitation (Zentella *et al.*, 2007; Hauvermale *et al.*, 2012) suggest that DELLAs require additional factors to exert transcriptional activity. In compliance with this concept, DELLAs were recently shown to physically interact with Switch (SWI)/Sucrose Nonfermenting (SNF)-type chromatin-remodeling complexes responsible for modulation of chromatin structure (Sarnowska et al., 2013). Moreover, it is becoming increasingly clear that DELLAs orchestrate growth and developmental processes through direct protein interaction with multiple TF targets. For

example, DELLAs mediate hypocotyl elongation by interacting with Phytochrome Interacting Factors (PIFs) (de Lucas *et al.*, 2008; Feng *et al.*, 2008; Gallego-Bartolomé *et al.*, 2010; Hauvermale *et al.*, 2012), and they control floral transition and fruit patterning by respectively binding to Squamosa Promoter Binding-Like (SPL) and Alcatraz (ALC) factors (Arnaud *et al.*, 2010; Yu *et al.*, 2012).

In a similar vein, several lines of evidence suggest that DELLAs modulate plant immunity via competitive binding to JA ZIM-domain (JAZ) proteins, a family of JA signaling repressors (Hou *et al.*, 2010; Wild *et al.*, 2012; Yang *et al.*, 2012). JAZ proteins bind and inhibit the activity of a wide array of TFs, including the key JA transcriptional activator MYC2 (Kazan & Manners, 2012, 2013). Recently, three groups have shown that DELLAs compete with MYC2 for binding to JAZs, thereby releasing free MYC2 to activate JA-responsive gene expression and enhance resistance to necrotrophic pathogens (Navarro et al., 2008; Hou et al., 2010; Yang et al., 2012; Wild et al., 2012). In the presence of GA, however, DELLAs are rapidly degraded, leading to inhibitory JAZ-MYC2 interactions and disruption of JA signaling (Figure A2). This so-called 'relief of repression' model not only elegantly explains how plants mediate the balance between growth and defense, but also offers novel insights into how GA may disable JA-mediated pathogen resistance by degrading DELLAs and releasing JAZs to bind and inhibit MYC2 (Navarro et al., 2008; Hou et al., 2010).

Interestingly, MYC2 can also modulate the level of the DELLA protein RGL3. Unlike other DELLAs, *RGL3* is induced by JA in a MYC2-dependent fashion. In turn, JA-induced RGL3 interacts with JAZs and releases MYC2 to enhance JA-responsive gene expression (Wild et al., 2012). Therefore, DELLAs seem to positively regulate JA responses not only through blocking JAZ repressor action, but also through acting directly as JA-responsive regulators. Importantly, phenotypic analyses revealed that the *rgl3-5* mutant exhibits enhanced susceptibility to the necrotroph *Botrytis cinerea* and increased susceptibility to the hemibiotroph *Pto*, demonstrating the biological significance of this mechanism. Moreover, the finding that *RGL3* expression is strongly induced by *Pto* but not by a mutant strain defective in production of coronatine, a JA-mimicking phytotoxin, raises the possibility that pathogens co-opt the JA pathway to modulate DELLA expression *in planta* (Wild et al., 2012).

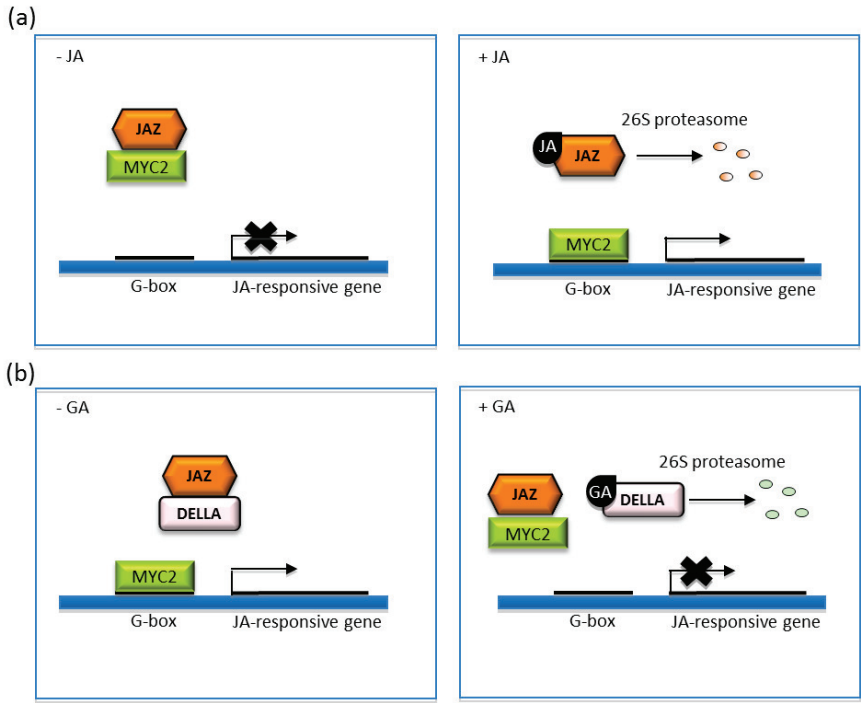

Figure A2. 'Relief of repression' model illustrating how DELLA-JAZ interactions orchestrate JA signaling. (a) In the absence of JA, stabilized JAZs sequester and inhibit the JA transcriptional activator MYC2, thereby preventing JA-responsive gene expression. JA promotes degradation of JAZs by the 26S proteasome, thus releasing free MYC2 to trigger expression of JA-responsive genes via binding to G-box motifs. **(b)** Without GA, stabilized DELLAs compete with MYC2 for binding to JAZs, which in turn liberates MYC2 to activate JA-responsive genes. In the presence of GA, DELLAs are rapidly degraded, enabling JAZs to bind MYC2 and block JA signaling output.

The spider in the web: do DELLAs act as a nexus for pathway crosstalk and signal integration?

Together the abovementioned studies clearly establish the importance of DELLA in the regulation of JA-GA crosstalk and the conflicting association between plant growth and defense. However, this may not be the end of story as rapidly accumulating evidence suggests that DELLAs serve as main hubs within an intricate signaling network, integrating, transmitting and processing multiple environmental signals and developmental cues (Hauvermale et al., 2012; Hou et al., 2013; Kazan and Manners, 2012).

Because of their well-studied growth-repressing properties and their accumulation in response to biotic and abiotic stresses (Achard *et al.*, 2006, 2008; Navarro *et al.*, 2008), DELLA proteins have long been hypothesized to act at the interface of developmental, physiological, and stress signaling. In support of this assumption, a large body of data indicates that DELLAs lie at the node of multiple hormone signaling pathways. The effect of GA on DELLA is probably the most important and the most direct; however, several other hormones have been shown to regulate plant growth by affecting DELLA levels. Auxin, for instance, is well known to promote root growth by triggering GA-induced DELLA proteolysis, whereas JA, ABA, cytokinin and ET all

enhance DELLA stabilization, and delay its degradation by GA (Fu & Harberd, 2003; Vriezen *et al.*, 2004; Brenner *et al.*, 2005; Achard *et al.*, 2007; Wild *et al.*, 2012; Yang *et al.*, 2012). Moreover, DELLA may also serve as a point of integration of hormone and metabolite signaling based on recent findings that sucrose, but not glucose, stabilizes DELLA to repress growth and induce anthocyanin biosynthesis in Arabidopis (Li *et al.*, 2013a).

Other than harmonizing signals from multiple upstream effectors, DELLAs also interact with a variety of regulatory TFs, thus controlling the expression of a multitude of genes functioning in myriad cellular activities and biological processes (de Lucas *et al.*, 2008; Feng *et al.*, 2008). Thus far, most attention has been paid to abovementioned DELLA-JAZ interaction and its role in orchestrating GA-JA signal interactions. However, with an increasing number of TFs and other proteins being identified as binding targets of JAZs, JAZ proteins appear to be main hubs for orchestrating not only multiple JA downstream processes, but also the interplay between JA and many other phytohormones, including SA, ET, ABA and GA (Song *et al.*, 2011; Qi *et al.*, 2011; Kazan & Manners, 2012). Therefore, interactions with single JAZ proteins may enable DELLA to tap into multiple hormone signaling pathways and control the outcome of multi-dimensional signal interactions.

Interestingly, three recent studies have revealed similar interactions with the key BR-regulatory TF BZR1, further diversifying DELLA's functional portfolio. Analogues to the mechanism described for JAZs and PIFs, DELLAs sequester BZR1 into inactive protein complexes, thereby inhibiting its DNA binding activity and ultimately blocking BR signal transduction (Bai *et al.*, 2012; Gallego-Bartolomé *et al.*, 2012; Li *et al.*, 2012). In addition, large-scale chip-chip experiments have identified a few thousand BZR1 targets, including numerous defense-related proteins such as the flg22 receptor *FLS2*, as well as PIF4, a dark- and heat-activated TF which together with BZR1 controls a central transcription network involved in cell elongation and seedling photomorphogenesis (Sun *et al.*, 2010; Yu *et al.*, 2011; Oh *et al.*, 2012). Through its ability to inhibit BZR1 and PIF4 individually and target the BZR1-PIF4 heterodimer, DELLA also impinges on this core transcription module, thereby enabling coordinated regulation of growth and development by GA, BR, temperature and light signals (Bai et al., 2012).

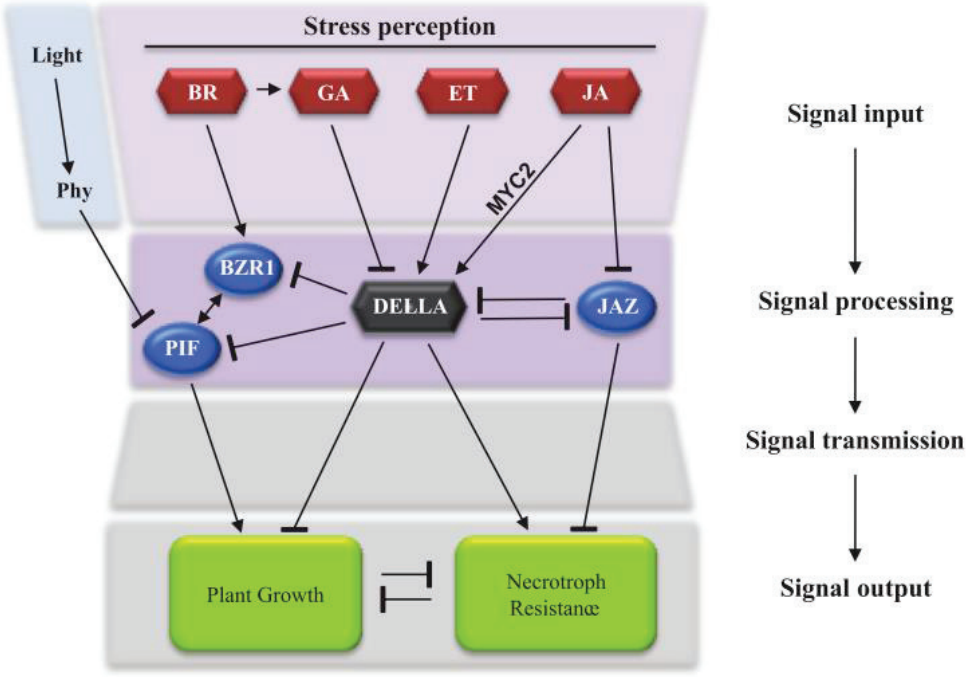

Figure A3. Model describing how the PIF-BZR1-DELLA-JAZ decision module integrates and processes various environmental and developmental cues to balance plant growth and defense to necrotrophic pathogens in *Arabidopsis thaliana*. Perception of environmental stress signals triggers asymmetric activation of phytochrome, BR, GA, ET, and JA signaling to modulate the levels of PIFs, BZR1, DELLAs and JAZs, respectively. BZR1 and PIFs form a functional complex that regulates large suites of genes contributing to cell growth, whereas JAZ proteins bind and inhibit numerous TF targets to suppress JA-induced processes, including resistance to necrotrophic pathogens. DELLAs on the other hand, physically interact with BZR1, PIFs and JAZs to inhibit their DNA-binding ability and, hence, block their transcription activity. We propose that the dynamic nature and balance of DELLA-TF interactions act as a central rheostat, enabling plants to flexibly tailor the allocation of limited resources into growth and/or defense responses.

In summary, the emerging roles of BZR1, PIFs and JAZs as coordinators of a wide network of (hormone) signaling crosstalk illustrate the functional versatility of these proteins. Considering the intricate interconnection of DELLAs with each of these regulators, it is tempting to speculate that the balance of DELLA-BZR1/PIF/MYC2 interaction act as a central command system that integrates responses from independent hormones and signals of adverse conditions, permitting flexible and appropriate modulation of plant growth and defense in response to diverse stimuli. Moreover, considering the combinatorial diversity in DELLA-MYC2/PIF/BZR1 interactions and the ability of each protein to control a vast array of both common and unique target genes, this decision module may not only accept different inputs, but also send out branched outputs. In Figures A3 and A4, we propose a model for how dynamic DELLA-TF interactions may fine-tune the interplay and balance of plant defense and growth responses mounted through multiple signaling pathways in the model plants Arabidopsis and rice.

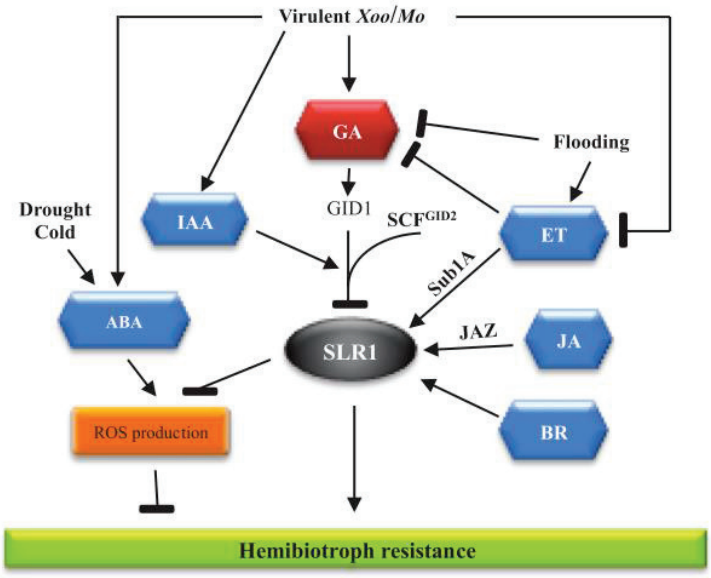

Figure A4. The DELLA protein SLR1 coordinates and adjusts interplay between environmental and phytohormonal signals to shape the immune response of rice (*Oryza sativa*). Opposite to DELLA-mediated necrotroph resistance in Arabidopsis, SLR1 mounts resistance to (hemi)biotrophic rice pathogens such as the blast fungus *Magnaporthe oryzae* (*Mo*) and the bacterial leaf blight pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). The model provides a mechanistic framework for abiotic stress (ABA)-induced susceptibility (Jiang et al., 2010; Koga et al., 2004) to *Xoo* and *Mo*, as well as for immunity triggered by JA (Mei et al., 2006; Riemann et al., 2013), BR (Nakashita et al., 2003) and/or flooding-induced ET (Singh et al., 2004), but also may explain how virulent strains of *Mo* and *Xoo* can attenuate SLR1-orchestrated immunity by hijacking the GA and/or IAA machinery in order to promote GID2-mediated degradation of SLR1 by the 26S proteasome (Ding et al., 2008; Fu et al. 2011; Domingo et al., 2009). Manipulation of ABA signaling by *Xoo* and *Mo* was shown by Xu et al. (2013) and Jiang et al. (2010), respectively. Blunt lines indicate antagonistic interactions; arrowheads depict positive interactions. Abbreviations: GA, gibberellic acid; IAA, indole acetic acid; ABA, abscisic acid; ET, ethylene; JA, jasmonic acid; BR, brassinosteroids; ROS, reactive oxygen species.

Conclusions and further perspectives

Contrary to the relative wealth of information on how BR and GA are perceived and their signals transduced, their role and significance in the regulation and integration of plant immune responses has long been ignored. Emerging from the studies reviewed here is a complex picture whereby GA and BR play a widespread and ambivalent role in plant defense, acting as either positive or negative regulators of disease resistance by interfering at multiple levels with biotic stress signaling cascades. Moreover, exciting new developments suggest that a number of GA/BR regulatory elements such as DELLAs and BZR1 constitute central nodes within the hard wiring of the plant's signaling network. Situated at the interface of developmental and defense signaling, these proteins likely serve as main hubs for pathway crosstalk and signal integration, allowing flexible and appropriate modulation of plant growth and defense in response to various endogenous and exogenous cues.

Despite these paradigm-shifting advances, our understanding of precisely how BR and GA impact plant innate immunity is still lagging far behind that of the classic defense hormones SA, JA and ET. For instance, there is little if no information available about the spatiotemporal dynamics of BR and GA during a given plant-microbe interaction, the dynamics and stability of DELLA and BZR1 protein complexes in response to pathogen attack remain elusive, and there is still much to be learned about the function and action of many of the downstream DELLA/BZR1 target genes. In addition, there is a paucity of knowledge on the causality of DELLA stabilization on disease resistance and it remains to be tested whether the temporal oscillation of DELLA accumulation and BR synthesis may contribute to the circadian rhythm of plant immunity (Wang et al., 2011; Arana et al., 2011; Belkhadir et al., 2012).

Moreover, it will be very interesting to assess whether GA and BR signaling are sites of manipulation by pathogens during infection. Although unequivocal evidence is still lacking, several authors have suggested that pathogens may deploy specific effectors to modify the host's GA and BR homeostasis for their own advantage (Shan *et al.*, 2008; De Vleesschauwer *et al.*, 2012). In addition, plant pathogens may produce GA or BR themselves to trick the plant into activating inappropriate responses. Genome sequencing of the obligate biotroph *Albugo laibachii* revealed the presence of a near-complete BR biosynthesis pathway (Kemen *et al.*, 2011), while several *Fusarium* species are known to produce toxins that resemble brassinosteroids (Robert-Seilaniantz *et al.*, 2007). Furthermore, several fungal and bacterial species synthesize GA or GA-like compounds that are chemically similar to plant GAs and have similar effects on plant physiology (Bottini *et al.*, 2004; Kawaide, 2006). Since pathogens that are deficient in hormone production do not display a strong growth phenotype, it is tempting to speculate that pathogens produce BR and GA or mimics thereof to tap into the plant's defense signaling network and subdue host immunity.

In conclusion, despite significant progress made over the recent years, we are only just beginning to understand the immune-regulatory roles of BR and GA. Given their apparent pluriform function in plant disease and resistance, unraveling the complexity of BR- and GA-modulated disease resistance will prove a hard nut to crack. However, elucidating the molecular mechanisms via which GA and BR interact with and feed into the plant immune network will not only advance our fundamental understanding of how plants integrate and balance immune system function and normal growth signals in response to the environment, but ultimately, also will be instrumental in developing novel strategies for biologically based, environmentally friendly and durable disease control in various agricultural settings.

Acknowledgements

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Summary

Plants are constantly threatened by pathogenic microorganisms trying to access plant nutrients for their own growth and reproduction. Especially pathogens with a necrotrophic lifestyle deploy aggressive virulence strategies to promote host cell death and acquire nutrients from dead plant tissue. Often they produce potent phytotoxins that interact with cellular targets and derange plant metabolism in order to induce cell death. The necrotrophic fungus *Cochliobolus miyabeanus* causes brown spot disease of rice (*Oryza sativa*). Brown spot disease is currently the most important yield reducer of rice and is prevalent in all rice-producing regions of the world. The disease is characterized by the development of necrotic lesions early during infection, followed by spreading chlorosis around the lesions and eventual withering of the leaf. Although recent proteome and differential gene expression analysis have revealed interesting facts about changes in plant metabolism during infection, less effort has been put in the investigation of the virulence mechanisms employed by *C. miyabeanus*. It is known that *C. miyabeanus* is capable of producing non-selective phytotoxic ophiobolins, of which ophiobolin A displays the highest biological activity. However, the involvement of ophiobolin A in disease is still uncertain. Moreover, no additional *C. miyabeanus* virulence strategies have been revealed. Furthermore it is clear that ethylene can enhance plant susceptibility to brown spot disease. As ethylene biosynthesis genes are up-regulated during infection, it seems plausible that the fungus can somehow hijack the plant ethylene metabolism, but the mechanism remains elusive. To extent our knowledge on how *C. miyabeanus* interacts with rice, we investigated different fungal virulence strategies and their dynamics during disease. The availability of the fungal genome sequence of *C. miyabeanus* and the ease with which *C. miyabeanus* genes can be site-directed mutated, provided us with invaluable tools to investigate the function of known and new virulence factors.

We first focused on ophiobolin A and investigated its role in disease development. Ophiobolins are cyclic sesterterpenes and their biosynthesis is mediated by a terpene cyclase. We identified the corresponding gene, *C. miyabeanus* ophiobolin synthase (*CmOPS1*), in the genome of *C. miyabeanus* strain WK1C. Subsequent phenotypic analysis of *Cmops1* deletion mutants confirmed the role of CmOps1 in ophiobolin biosynthesis. We show that ophiobolin A is an important virulence factor and is only produced during the first 24 hours of disease. Furthermore, the virulence abilities of different field strains are reflected in the production level of ophiobolin A during disease. Ophiobolin A likely targets the cell membranes, causing extracellular alkalinisation, and triggers a plant response that is reminiscent of defense responses to biotrophs. Ophiobolin A was shown to be a key factor in the induction of phenylalanine ammonia lyase

(*OsPAL1*) upon infection and induces the positive cell death regulator *OsNAC4*. Ophiobolin A eventually causes cell death, both in a Ca^{2+} dependent and independent way.

In silico genome analysis predicts that *CmOPS1* is part of a wider ophiobolin gene cluster, featuring two specific transcription factors, a P450 monooxygenase, two oxidoreductases and an ABC transporter. The involvement of each of these components is yet to be confirmed *in vivo*. Homologous ophiobolin gene clusters are conserved in a diverse group of filamentous fungi belonging to different Pezizomycotina classes. This suggests an evolutionary advantage for fungi with different life styles, apart from plant pathogenicity. Although vertical gene transfer provides a good explanation for acquisition of the cluster by most of the species, *Oidiodendron maius* may have acquired the cluster horizontally from an ancestor of *Coccidioides immitis*.

Ophiobolin-deficient mutants could still cause disease, although to a lesser extent. Therefore we aimed to identify new virulence factors in a second part of this work. We compared metabolites of virulent and less virulent *C. miyabeanus* strains using ultra-high performance liquid chromatography coupled to high resolution Orbitrap mass spectrometry (UHPLC-Orbitrap-HRMS). The production of high amounts of tentoxin by a highly virulent strain was revealed by principal component analysis of produced metabolites and confirmed by UHPLC coupled to tandem-quadrupole mass spectrometry (MS/MS). This is the first report of tentoxin production by a non-*Alternaria* plant pathogen. Furthermore, we identified for the first time a non-ribosomal peptide synthetase (NRPS) responsible for the production of tentoxin, CmNps3. The encoding gene is a homolog of the *Cochliobolus heterostrophus* ChNps3. Infection tests with wild type and *Cmnps3* knock-out mutants show that tentoxin acts as a virulence factor and is correlated with chlorosis development. Interestingly, tentoxin is only produced during the second phase of infection. Although rice has previously been classified as a tentoxin-insensitive species, our data shows that tentoxin-production by *C. miyabeanus* affects symptom development. The molecular mechanism by which tentoxin interacts with plant cells remains elusive. Besides, the mere drainage of plant amino acids to produce tentoxin may weaken the plant, as nitrogen is lost to surrounding healthy tissue.

Finally, because ethylene is a known susceptibility inducing factor, we investigated the interplay between ethylene and ophiobolin A and tentoxin. We show that *C. miyabeanus* can produce ethylene itself via two distinct pathways, the EFE (ethylene forming enzyme) pathway and the KMBA (α-keto-γ-methylthiobutyric acid) pathway. Real-time ethylene measurements during disease show a remarkable ethylene production pattern, featuring two distinct ethylene emission peaks, each coinciding with a wave of phytotoxin production. Our efforts to find a trigger for the first ethylene peak showed that ophiobolin A is not responsible for ethylene production, but needs ethylene to cause symptoms. The involvement of fungal ethylene in the induction of plant ethylene biosynthesis remains inconclusive. Fungal ethylene may also act as a toxin synergist or enhance the plant ethylene response. During the second ethylene burst, tentoxin production is

associated with ethylene emissions and the onset of senescence and chlorosis. During this phase it is plausible that fungal ethylene acts autocatalytically to enhance the already started senescence process. The exact function of fungal ethylene during disease warrants additional research.

To conclude, an image of the brown spot infection process emerges in which *C. miyabeanus* triggers a plant response early on during infection and deranges the plant metabolism in order to provide a nutrient flux and eventually kill the tissue. To this end, ophiobolin A is produced during the first phase of infection triggering rapid cell death and debilitating the plant. A coinciding first ethylene peak facilitates the cell death induction process and enhances susceptibility. In a second phase, the fungus no longer produces ophiobolins, but switches to the production of tentoxin and causes chlorosis. This is correlated with spreading senescence and a strong and prolonged ethylene burst. It is expected that fungal ethylene plays an important role in this phase as an autocatalytic enhancer of progressing senescence.

The results of this work broaden our knowledge on the virulence mechanisms deployed by *C. miyabeanus* and substantiate the central role of ethylene in disease development. Besides, the identification of a new virulence factor could complement future breeding efforts towards quantitatively more resistant genotypes. At the same time, this work generates many new questions regarding regulation of fungal phytotoxin production as well as the mechanisms by which tentoxin and fungal ethylene contribute to disease development.

Samenvatting

Planten worden voortdurend bedreigd door pathogene micro-organismen die uit zijn op de nutriënten van de plant om hun groei en voortplanting te waarborgen. Pathogenen met een necrotrofe levensstijl gebruiken in het bijzonder erg agressieve virulentiestrategieën om celdood in de gastheer te bewerkstelligen en nutriënten uit het dode plantenweefsel op te nemen. Vaak produceren deze necrotrofen fytotoxines die interageren met een specifieke component van de gastheercel en ontregelen zo het metabolisme van de plant. In de meeste gevallen leidt dit finaal tot celdood. *Cochliobolus miyabeanus* is een necrotrofe schimmel die brown spot ziekte veroorzaakt in rijstplanten (*Oryza sativa*). Brown spot komt voor in alle rijstproducerende gebieden ter wereld en veroorzaakt van alle rijstziekten momenteel de belangrijkste opbrengstverliezen. De ziekte manifesteert zich in een vroeg stadium als necrotische lesies op rijstbladeren en evolueert snel naar chlorose die zich uitbreidt rondom de lesies. In ernstige gevallen kan dit leiden tot het volledig verwelken van het geïnfecteerde blad.

Recent onderzoek naar veranderingen in het proteoom en de genexpressie in geïnfecteerde rijstbladeren heeft aangetoond dat er tijdens de infectie significante veranderingen optreden in het metabolisme van de plant. Er is echter nog amper onderzoek gevoerd naar de virulentiemechanismen van de schimmel. Het is wel geweten dat *C. miyabeanus* in staat is niet-specifieke fytotoxische ophiobolines te produceren, waarvan ophioboline A de meest bioactieve variant is. Het is niet duidelijk of ophioboline A een rol speelt tijdens de infectie en verder zijn er momenteel geen alternatieve virulentiestrategieën gekend. Het plantenhormoon ethyleen maakt rijstplanten extra gevoelig voor brown spot ziekte. Aangezien de ethyleen biosynthese-genen geactiveerd worden tijdens de infectie, lijkt het aannemelijk dat de schimmel het ethyleen-metabolisme van de plant kan kapen. Hoe dit in zijn werk gaat, is niet geweten. We hebben in deze studie verschillende virulentiestrategieën van *C. miyabeanus* en hun dynamiek tijdens de infectie onderzocht om zo onze kennis omtrent de interactie van de schimmel met de rijstplant uit te breiden. De beschikbaarheid van de volledige genoomsequentie en het gemak waarmee de genen van *C. miyabeanus* gericht kunnen uitgeschakeld worden, bleken van onschatbare waarde voor het bestuderen van de functie van zowel nieuwe als gekende virulentiefactoren.

In een eerste deel hebben we ons geconcentreerd op de rol van ophioboline A tijdens het verloop van de ziekte. Ophiobolines zijn cyclische sesterterpenen en hun biosynthese wordt geregeld door een terpeen-cyclase. We hebben het overeenkomstige gen, *C. miyabeanus* ophiobolin synthase (*CmOPS1*), geïdentificeerd in het genoom van de *C. miyabeanus* stam WK1C. Vervolgens hebben we aangetoond dat CmOps1 inderdaad onmisbaar is voor de biosynthese van ophiobolines door het gen uit te schakelen in *Cmops1* mutanten. We tonen aan dat ophioboline A

een belangrijke virulentiefactor is en enkel geproduceerd wordt tijdens de eerste 24 uur van de infectie. Verder tonen we ook dat het virulentieniveau van verschillende veldstammen overeenstemt met het productieniveau van ophioboline A tijdens de ziekte. Waarschijnlijk interageert ophioboline A met de celmembranen en veroorzaakt zo een stijging van de extracellulaire pH. Ophioboline A lokt een reactie uit in de plant die kenmerken deelt met de verdedigingsreactie tegen sommige biotrofen. We tonen ten slotte dat ophioboline A een sleutelrol speelt in de stimulatie van phenylalanine ammonia lyase (*OsPAL1*) en de positieve celdoodregulator *OsNAC4*. Ophioboline A veroorzaakt uiteindelijk celdood op een Ca^{2+}-afhankelijke en een Ca^{2+}onafhankelijke wijze.

Op basis van een *in silico* analyse van de genomische sequentie rondom *CmOPS1* voorspellen we dat *CmOPS1* behoort tot een vermeende ophioboline gencluster die verder nog bestaat uit twee specifieke transcriptiefactoren, een P450 monooxygenase, twee oxidoreductasen en een ABC-transporter. Het belang van elk van deze componenten in de biosynthese van ophioboline moet nog *in vivo* aangetoond worden. We vonden homologen van de ophioboline gen cluster terug in een diverse groep van filamenteuze schimmels die behoren tot verschillende klassen van de Pezizomycotina. Dit doet ons vermoeden dat het bezit van deze gencluster evolutionair niet enkel voordelig is voor plantpathogenen, maar ook voor schimmels met een andere levensstijl. De meeste van de schimmels hebben de cluster allicht verticaal overgeërfd van hun voorouders. Toch lijkt het erop dat een horizontale genoverdracht heeft plaats gevonden van een voorouder van *Coccidioides immitis* naar een voorouder van *Oidiodendron maius*.

Mutanten die niet langer ophiobolines kunnen maken, zijn toch nog in staat om in beperkte mate ziekte te veroorzaken. Daarom zijn we op zoek gegaan naar niet-gekende virulentiefactoren in een tweede deel van onze studie. We hebben het metaboloom van meer en minder virulente *C. miyabeanus* stammen tijdens infectie vergeleken aan de hand van ultra-hoge performantie vloeistofchromatografie (UHPLC), gekoppeld aan hoge resolutie Orbitrap massaspectrometrie. Uit een principale-componentenanalyse op 5000 geproduceerde metabolieten bleek dat de sterk-virulente stam waarschijnlijk tentoxine aanmaakt. Dit werd bevestigd aan de hand van UHPLC gekoppeld aan tandem-quadropool massaspectrometrie (MS/MS). Dit is de eerste keer dat tentoxine wordt vastgesteld in een plantpathogeen die niet behoort tot *Alternaria* spp. Vervolgens hebben we ook voor de eerste maal een niet-ribosomaal eiwitsynthetase geïdentificeerd dat verantwoordelijk is voor de biosynthese van tentoxine, CmNps3. Het coderende gen blijkt een homoloog van *C. heterostrophus* ChNps3. Infectietesten waarbij de wild type stam vergeleken wordt met *Cmnps3* deletie-mutanten toonden aan dat tentoxine een virulentiefactor is en gecorreleerd is met de ontwikkeling van chlorose. Interessant genoeg wordt tentoxine enkel aangemaakt gedurende de tweede fase van de infectie. Hoewel rijst eerder werd ondergebracht in de groep van planten die ongevoelig zijn voor tentoxine, blijkt uit onze data dat productie van tentoxine door *C. miyabeanus* tijdens infectie bijdraagt aan symptoomontwikkeling. Het

moleculaire mechanisme waarmee tentoxine interageert met de plantencel is voorlopig niet gekend. Het is mogelijk dat het aftappen van plantenaminozuren om tentoxine te maken op zich al de plant verzwakt, omdat zo stikstof verloren gaat dat anders geremobiliseerd kan worden naar omliggende gezonde weefsels.

Omdat ethyleen de gevoeligheid van rijst voor de ziekte bevordert, hebben we ten slotte ook de wisselwerking tussen ethyleen en de verschillende fytotoxines onderzocht. We tonen aan dat *C. miyabeanus* zelf ethyleen kan aanmaken via twee afzonderlijke routes, via de EFE (ethylene forming enzyme) en de KMBA (α-keto-γ-methylthioboterzuur) route. Realtime ethyleenmetingen gedurende het gehele infectieproces tonen een opmerkelijk ethyleenemissiepatroon, gekenmerkt door twee afzonderlijke ethyleenpieken. Elk van deze pieken valt samen met een piek in de productie van fytotoxines. Tijdens onze inspanningen om te achterhalen wat de eerste ethyleenpiek veroorzaakt, stelden we vast dat ophioboline A niet verantwoordelijk is voor de activatie van ethyleenbiosynthese, maar wel ethyleen nodig heeft om symptomen te kunnen veroorzaken. Het belang van schimmelethyleen in de activatie van plantethyleenbiosynthese is nog steeds onduidelijk. Het schimmelethyleen kan tevens dienst doen als een toxine-synergist of kan de ethyleenrespons in de plant versterken. De mate waarin tentoxine wordt geproduceerd komt overeen met de sterkte van de tweede ethyleenpiek en het moment van productie valt samen met het begin van senescentie en chlorose in het geïnfecteerde blad. Tijdens deze tweede fase is het erg waarschijnlijk dat het schimmelethyleen een autokatalytische functie vervult om zo de reeds gestarte senescentie te bevorderen. Aanvullen onderzoek zal nodig zijn om de exacte rol van het schimmelethyleen te achterhalen.

We kunnen concluderen dat *C. miyabeanus* vroeg tijdens de infectie een reactie uitlokt die het metabolisme van de plant verstoort om zo een aanvoer van nutriënten te verzekeren en het weefsel af te doden. Hiertoe maakt de schimmel tijdens de eerste fase van de infectie ophioboline A aan om snelle celdood te bevorderen en de plant te verzwakken. Dit wordt vergemakkelijkt door een samenvallende eerste ethyleenpiek die tevens de gevoeligheid van de plant vergroot. In een tweede fase produceert de schimmel niet langer ophiobolines, maar schakelt over op de aanmaak van tentoxine en veroorzaakt chlorose. Deze fase valt samen met een uitbreidende senescentie en een sterke en aanhoudende ethyleenpiek. Het lijkt er sterk op dat schimmelethyleen in deze fase een belangrijke rol speelt als autokatalysator van senescentie.

De resultaten van deze studie vergroten onze kennis omtrent de virulentiemechanismen die ingezet worden door *C. miyabeanus* en benadrukt een centrale rol voor ethyleen tijdens de ziekteontwikkeling. Daarnaast kan de identificatie van een nieuwe virulentiefactor bijdragen tot toekomstige veredelingsinspanningen om kwantitatief resistentere genotypen te vinden. Tegelijkertijd roept dit werk nieuwe vragen op betreffende de onderliggende mechanismen die de fytotoxineproductie in de schimmel regelen, alsook het mechanisme waarmee tentoxine en schimmelethyleen bijdragen tot de ziekteontwikkeling.

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Curriculum vitae

Personal information

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Education

2009: Master's degree in bioscience engineering: cell and gene biotechnology, Ghent University, Ghent (Belgium) (Great distinction)
Major: Molecular Plant Breeding

Master Dissertation: 'The importance of hormone signaling pathways in the response of rice to infection by *Meloidogyne graminicola*'

Promotors: Prof. L. Gheysen (dept. of molecular genetics) and Prof. M. Höfte (dept. of crop protection)

2007: Bachelor's degree in bioscience engineering: cell and gene biotechnology, Ghent University, Ghent (Belgium) (Great distinction)

2004: Secondary school graduation, Science and Mathematics, Sint-Vincentiusinstituut, Dendermonde (Belgium)

Additional training

Comparative genomics in eukaryotes (1 day), 2013

International workshop for young researchers: Bioinformatics tools for NRPS discovery (University of Lille, France) (3 days), 2013

Effective scientific communication (20 contact hours), 2014

Communication skills (2 days) and negotiation skills (1 day), 2012

Summer school : 'Let's talk science' (3 days), 2014

Experience abroad

August-September 2008: Internship for master dissertation at the International Rice Research Institute (IRRI) in Los Baños (the Philippines)

October-November 2013: Six weeks study leave abroad at the lab of Prof. B. Gillian Turgeon (Section of Plant Pathology and Plant-Microbe Biology), Cornell University, Ithaca (US)

Professional record

January 2011 – December 2014: IWT strategic basic research scholarship
+January 2015 – March 2015: PhD scholarship UGent
PhD candidate at the Department of Crop Protection, Faculty of Bioscience Engineering, Ghent University, Ghent (Belgium)

PhD thesis: 'Interplay between phytotoxins and ethylene mediates rice brown spot disease, caused by *Cochliobolus miyabeanus'*

Promotor: Prof. Monica Höfte

Co-promotor: Dr. David De Vleesschauwer

May 2010 – November 2010: Research associate at the Institute for Agricultural and Fisheries research (ILVO), Melle (Belgium)

Publications

Peer reviewed

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

Submitted

De Bruyne L, Van Poucke C, Di Mavungu JD, Mohd Zainudin NAI, Vanhaecke L, De Vleesschauwer D, Turgeon BG, De Saeger S and Höfte M. 2015. Comparative chemical screening and genetic analysis reveal tentoxin as a new virulence factor in Co*chliobolus miyabeanus*, the causal agent of brown spot disease on rice.
Submitted to Molecular Plant Pathology

Mohd Zainudin NAI, Condon B, De Bruyne L, Van Poucke C, Bi Q, Li W, Höfte M, and Turgeon BG. 2015. Virulence, host selective toxin production, and development of three *Cochliobolus* phytopathogens lacking the Sfp-type 4'-phosphopantetheinyl transferase Ppt1 .
Submitted to Molecular Plant Microbe Interactions

Participation at conferences and symposia

XVI IS-MPMI conference, 6-10 July 2014, Rhodes, Greece

Oral presentation: De Bruyne L, Van Poucke C, Wu D, Turgeon B G, De Saeger S & Höfte M. Ophiobolin A is involved, but is not essential in brown spot disease in rice

66th International Symposium on Crop Protection, 20 May 2014, Ghent, Belgium

Oral presentation: De Bruyne L, Van Poucke C, Wu D, Turgeon B G, De Saeger S & Höfte M. Phytotoxin production by the rice brown spot pathogen, *Cochliobolus miyabeanus*

35th Mycotoxin Workshop, 22-24 May 2013, Ghent, Belgium

Poster presentation: De Bruyne L, Van Poucke C, Diana Di Mavungu J, De Vleesschauwer D, De Saeger S & Höfte M. Phytotoxin production by *Cochliobolus miyabeanus,* the causal agent of brown spot disease on rice

65th International Symposium on Crop Protection, 21 May 2013, Ghent, Belgium

Poster presentation: De Bruyne L, Van Poucke C, Diana Di Mavungu J, De Vleesschauwer D, De Saeger S & Höfte M. Phytotoxin production by *Cochliobolus miyabeanus*, the causal agent of brown spot disease on rice

1st International Brassinosteroids Conference, 27-29 June 2012, Barcelona, Spain

Poster presentation: De Bruyne L, Höfte M & De Vleesschauwer D. The role of brassinosteroids in the interaction of rice with *Magnaporthe oryzae* and *Cochliobolus miyabeanus*

64th International Symposium on Crop Protection, 22 May 2012, Ghent, Belgium

Poster presentation: De Bruyne L, Höfte M & De Vleesschauwer D. Brassinosteroid-mediated disease resistance in rice

17th Symposium on Applied Biological Sciences, 10 February 2012, Leuven, Belgium

Poster presentation: De Bruyne L, De Vleesschauwer D & Höfte M. The role of brassinosteroids in the interaction of rice with *Magnaporthe oryzae* and *Cochliobolus miyabeanus*

Teaching (practical courses)

Molecular plant pathology

Plant biotechnology: case studies (praktijkstudie)