MOLECULAR GENETICS OF HISTONE DEACETYLASE INHIBITORS FROM FILAMENTOUS FUNGI

By

Wanessa D. Wight

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Cell and Molecular Biology

2012

ABSTRACT

MOLECULAR GENETICS OF HISTONE DEACETYLASE INHIBITORS FROM FILAMENTOUS FUNGI

By

Wanessa D. Wight

Over the last century, thousands of secondary metabolites from filamentous fungi have been identified. Genes involved in secondary metabolite biosynthesis are often clustered and co-regulated. In this dissertation the genes involved in the biosynthesis of two secondary metabolites, depudecin and HC-toxin, from *Alternaria brassicicola* and *Alternaria jesenskae*, respectively, were identified. Both depudecin and HC-toxin are inhibitors of histone deacetylase (HDAC), and HC-toxin is a virulence factor for *Cochliobolus carbonum*.

The gene cluster responsible for depudecin biosynthesis was identified. The cluster has six genes (*DEP1-DEP6*). Mutation by targeted gene disruption of *DEP6*, which encodes a predicted transcription factor, demonstrated that the cluster is co-regulated. The polyketide synthase gene encoded by *DEP5* was also shown by gene disruption to be essential for depudecin production. *Alternaria brassicicola* wild type (depudecin +) and the *DEP5* knockout mutant (depudecin -) strains were tested for virulence on *Arabidopsis thaliana* Col-0 and *pad3* mutants. *Pad3* plants infected with the *A. brassicicola* depudecin mutant showed a 10% reduction in lesion size, indicating that depudecin plays a minor role in virulence in this pathosystem.

Alternaria jesenskae, a newly characterized species, is the first organism other than *Cochliobolus carbonum* shown to produce HC-toxin. Genome survey sequencing by pyrosequencing of *A. jesenskae* revealed a series of genes closely related to the HC-toxin biosynthetic genes of *C. carbonum*. The presence of the HC-toxin gene cluster in both *C.*

carbonum and *A. jesenskae* allowed us address the comparative evolutionary origins of HC-toxin biosynthesis in *A. jesenskae* compared to *C. carbonum*. The high degree of homology of the genes in the two clusters, the presence of multiple copies of each gene in both organisms, and the high conservation of intron/exon structures, indicates a close evolutionary relationship between the HC-toxin clusters in the two fungi. Analysis of a series of housekeeping proteins indicates that the two fungi are phylogenetically closely related. Therefore, evolution of the HC-toxin genes can be explained by evolution from a common ancestor. However, future work is necessary to eliminate the possible role of horizontal gene transfer.

A. jesenskae was tested for virulence on maize, cabbage, *Arabidopsis thaliana* and *Fumana procumbens* (from which *A. jesenskae* was originally isolated). No disease occurred on any host, indicating that if HC-toxin has a role in plant pathogenesis that none of these plants are hosts for *A. jesenskae*, or that HC-toxin has some other role in the biology of *A. jesenskae*.

The results presented in this dissertation indicate that HDAC inhibition by toxins produced by fungal pathogens is critical in some pathosystems but not others. Further work is needed to clearly identify if HDACs play a role in plant defense in dicotyledonous plants such as *Arabidopsis*.

DEDICATION

I dedicate this thesis to my parents, Dave and Liz Wight, and my significant other Christopher Piland, for all their love and support.

ACKNOWLEDGMENTS

I would like to begin by acknowledging Jonathan Walton for letting me be a part of the lab and for helping me throughout the years. I would also like to acknowledge my committee members, Dr. Ray Hammerschmidt, Dr. Min-Hao Kuo, Dr. Brad Day, and Dr. Frances Trail. They have been a wonderful committee and have helped me out tremendously with their advice and suggestions. I particularly want to thank Frances Trail for all of her advice and support over the years scientifically, professionally and personally as well. I would like to thank all the members of the Walton lab, past and present, for all of their help, guidance, and support. I especially want to thank John Scott-Craig for his friendship, wisdom, support, guidance, advice, and encouragement in good times and bad. I would like to thank all of the Plant Biology, CMB and PRL staff, faculty, postdocs, and graduate students for all of their help and advice. I would also like to thank the members of the MSU RTSF department for all of their help in sequencing Alternaria jesenskae and Dr. C. Robin Buell for assembling the genome survey.
A special thanks to Gerry Adams for his help with culturing the temperamental Alternaria jesenskae.

I would like to thank collaborator Dr. Christopher Lawrence and Dr. Kwang-Hyung Kim from the Virginia Bioinformatics Institute for their help and support with the Alternaria brassicicola project, including teaching me their transformation protocol and with their contribution to the manuscript.

Thank you to all of my friends that I met at Michigan State University for their help and support over the years whether it was scientific advice, support or dinner and beer, including Janet Paper, Heather Hallen-Adams, Hong Luo, Melissa Borrusch, and many others!!!

Finally, I would like to thank my family including my sisters, Carissa and Deanna, for all their love and support during these years as well as my parents, Liz and Dave, for all their love, encouragement and support, both emotionally and financially that allowed me to pursue this degree in the first place. Last but not least, I would like to thank my partner, Christopher, for putting up with me and supporting me through the good (success), the bad (the failed experiments), and the ugly (thesis writing stress). Without all of these people, this would not have been possible. Thank you so much.

TABLE OF CONTENTS

LIST OF TABLES ix

LIST OF FIGURES x

CHAPTER 1: LITERATURE REVIEW 1

HISTONE MODIFICATION 2

ALTERNARIA 6

Alternaria jesenskae 7

Alternaria brassicicola 7

SECONDARY METABOLITES 8

Classes of fungal secondary metabolites 9

Clustering of secondary metabolite genes 10

HORIZONTAL GENE TRANSFER IN PROKARYOTES 12

HORIZONTAL GENE TRANSFER IN EUKARYOTES 12

CHAPTER 2: BIOSYNTHESIS AND ROLE IN VIRULENCE OF THE HISTONE DEACETYLASE INHIBITOR DEPUDECIN FROM *ALTERNARIA BRASSICICOLA* 20

ABSTRACT 21

INTRODUCTION 22

MATERIALS AND METHODS 26

Fungal growth and depudecin analysis. 26

Gene disruption. 27

DNA extraction and analysis. 29

Virulence assays. 30

RESULTS 31

AbPKS9 encodes the depudecin polyketide synthase. 31

The depudecin biosynthetic cluster. 32

DEP6 regulates the depudecin cluster. 33

Genes of the depudecin cluster. 34

A related gene cluster in *Coccidioides immitis*? 36

Synteny of the depudecin cluster region between *A. brassicicola* and other Pleosporaceae. 37
Depudecin contributes to virulence on cabbage. 38
DISCUSSION 39
ACKNOWLEDGMENTS 41

CHAPTER 3: BIOSYNTHESIS OF HC-TOXIN FROM *ALTERNARIA JESENSKAE* 53
INTRODUCTION 54
MATERIALS AND METHODS 56
Fungal growth and HC-toxin analysis. 56
DNA extraction and analysis. 57
Virulence assays. 59
Bioinformatic Analysis. 60
RESULTS 61
Alternaria jesenskae produces HC-toxin 61
Alternaria jesenskae HC-toxin gene cluster 62
The genes of ajTOX2: 62
Organization of genes within the cluster 64
Virulence assays 64
Phylogenetic analysis. 65
DISCUSSION 66

CHAPTER 4: FUTURE DIRECTIONS 88
Evolutionary origin of the depudecin cluster from *Alternaria brassicicola* 89
Alternaria jesenskae 90

APPENDIX I 92

APPENDIX II 98

REFERENCES 99

LIST OF TABLES

Table 1. Restriction enzyme digest list...81

Table 2. *Cochliobolus carbonum* TOX gene accessions...82

Table 3. NCBI NRPS adenylation domain sequences used in Fig. 17...83

Table 4. Percent Identity between indicated gene and its ortholog in *C. carbonum* or the other copies in *A. jesenskae*...85

Table 5. Toxbox and toxbox like sequences of *C. carbonum* and *A. jesenskae*............................86

Table 6. Housekeeping protein analysis between *C. carbonum* and *A. jesenskae*.........................87

LIST OF FIGURES

Figure 1. Symptoms characteristic of northern corn leaf spot on maize leaves...........................15

Figure 2. *Alternaria brassicicola* conidia and spores..15

Figure 3. Life cycle of Alternaria infection..16

Figure 4. Cabbage leaf infected with *A. brassicicola*..17

Figure 5. Polyketide synthase...18

Figure 6. Non ribosomal synthase..19

Figure 7. Structure of the polyketide depudecin..42

Figure 8. Strategies for disrupting depudecin biosynthetic genes..43

Figure 9. Characterization of depudecin mutants and complemented strains in *A. brassicicola* ATCC 96836...44

Figure 10. Characterization of depudecin mutants in MUCL 20297 ……………….........….. 46

Figure 11. Mutational characterization of genes in the depudecin cluster ……….....................47

Figure 12. Analysis of *DEP6* mutants in MUCL 20297..49

Figure 13. The depudecin gene cluster of *A. brassicicola* ATCC 96836 …………....................50

Figure 14. Synteny between *A. brassicicola*, P. tritici-repentis and S. nodorum in the depudecin cluster region ……………………………………...…………………..........51

Figure 15. Virulence analysis of *A. brassicicola DEP5* (AbPKS9) mutants...............................52

Figure 16. Analysis of HC-toxin from *A. jesenskae* …………………………………….............71

Figure 17. Southern blot analysis of ajTOX genes………………………………………..............73

Figure 18. Gene structure of *C. carbonum* and *A. jesenskae* TOX2 orthologs.............................74

Figure 19. Differences in gene order and orientation of TOX genes in *C. carbonum* ccTOX2 and *A. jesenskae* ajTOX2 loci..76

Figure 20. Phylogenetic tree using adenylation domains from fungal NRPS proteins (See also Table 7)....78

Figure 21. Alignment of *ccTOXE* and the two copies of *ajTOXE*....79

Figure 22. Virulence assays....80

CHAPTER 1: LITERATURE REVIEW

Cochliobolus carbonum, phylum Ascomycota, family Pleosporaceae, is a necrotrophic fungal plant pathogen of maize. On susceptible varieties, it causes northern corn leaf spot, capable of infecting the plant at all stages of development. Symptoms characteristic of infection begin with small brown necrotic flecks on leaves and stems (Fig. 1) and within days progress to plant death. While there are several races of *Cochliobolus carbonum*, only race 1 produces the secondary metabolite HC-toxin (Scheffer 1965). Named for *Helminthosporium carbonum*, an older anamorph name for *Cochliobolus carbonum,* HC-toxin is a host-selective toxin that is a critical determinant of virulence; *tox* minus mutants are unable to cause disease (Scheffer 1965; Walton 2006).

From the standpoint of the host, only maize plants that are homozygous recessive at the nuclear *HM1* locus are sensitive to HC-toxin and susceptible to HC-toxin-producing isolates of *C. carbonum* (Meeley et al. 1992). *HM1* encodes HC-toxin reductase, an NADPH-dependent reductase required for the detoxification of HC-toxin (Meeley et al. 1991). As a determinant of host resistance, orthologs of *HM1* are present in other grasses, indicating that HC-toxin reductase activity is an ancient mechanism for detoxifying HC-toxin, and/or HC-toxin like compounds in grasses (Han et al. 1997; Multani et al. 1998; Sindhu et al. 2008). The only known mode of action of HC-toxin is as a histone deacetylase inhibitor (Walton 2006) leading to the favored hypothesis that histone deacetylase inhibition is an ancient, common strategy used by grass pathogens (Sindhu et al. 2008)

HISTONE MODIFICATION

As noted above, the mode of action of HC-toxin is as a histone deacetylase (HDAC) inhibitor, known to be involved in chromatin remodeling. Chromatin is comprised of an octomer

of four core histone proteins (H2A, H2B, H3 and H4) encircled with 147 base pairs of DNA. Each of these nuclear histone proteins contains a lysine-rich tail that extends out from the primary core structure. This tail is critical for histone posttranslational modifications, ultimately resulting in both structural and functional changes (Turner, 2007). One example of posttranslational modification is the acetylation of the lysine residues of histone tails. Acetylation is a reversible process and is often associated with changes in gene expression. There are two enzymes that play a role in acetylation, histone acetyl transferase (HAT) and histone deacetylase (HDAC). HATs catalyze the transfer of an acetyl group from acetyl-Coenzyme A onto lysine residues on histone tails. This activity affects the bonding properties between the histone and DNA resulting in relaxation of the chromatin or euchromatin. In this relaxed state genes become physically accessible by transcription factors and other cofactors or proteins associated with gene expression. HDACs catalyze the opposite reaction by removal of the acetyl group from the lysine residues resulting in compaction of the chromatin or heterochromatin, associated with gene repression.

The first enzymes with histone deacetylase activity, related to the yeast transcription factor regulator Rpd3p, were isolated 1996 using a trapoxin affinity matrix column (Taunton et al. 1996). Since then HDACs have been identified from nearly all eukaryotes. There are several recognized classes of HDACs which have been characterized, including Type I (RPD3/HDA1-like), Type II (HD2, plant-specific) and Type III (SIR2-like, sirtuin) classes (Pandey et al. 2002). Type I and Type II HDACs are zinc dependent and include catalytic channels and the presence of a Zn+ ion, which are required for their activity. Homology of the critical domains of HDACs, their role in chromatin remodeling, gene expression and their presence in nearly all eukaryotes indicates a conserved and ancient protein family (Leipe et al. 1997).

In addition to their role in chromatin remodeling, HDACs have also been shown to deacetylate lysine residues of non-histone proteins, such as transcription factors, cytoskeletal proteins, heat shock proteins, transcriptional co-repressors, and others (Tian et al. 2001; Choudhary et al. 2009; Alvarez et al. 2010; Rushton et al. 2010; Winkler et al. 2010; Karolczak-Bayatti et al. 2011)(See also Chapter 2). Due to their ability to deacetylate both histones and non-histone proteins, HDACs play a role in a number of processes in eukaryotic systems, such as the jasmonic acid and ethylene pathways (Zhou et al. 2005), embryo and flower development (He et al. 2003), cell cycle (Rossi et al. 2007) and alternative splicing (Hnilicova et al. 2011). HC-toxin is an inhibitor of histone deacetylases of the Rpd3/Hda1 class, but not Sir2 HDACs (Brosch et al. 1995). Consistent with this, maize histones 3 and 4 become hyper-acetylated during infection (Ransom et al. 1997) suggesting that HDACs play a role in plant defense in this pathosystem. However, the exact nature of the role of HDACs in plant defense in other pathosystems has not been determined.

HDAC inhibitors are chemicals that prevent the activity of HDACs by interacting with the catalytic channel of the enzyme. There are four classes of HDAC inhibitors: hydroxamates (trichostatin and suberoylanilide hydroxamic acid), cyclic peptides (HC-toxin, apicidin and trapoxin A), aliphatic acids (phenylbutyrates and valproic acid), and benzamides (MS-275 and CI-994)(Monneret 2005; Dokmanovic et al. 2007; Pontiki et al. 2010). Trichostatin A from *Streptomyces hygroscopicus* was the first HDAC inhibitor to be isolated (Yoshida et al. 1990). Since that time a number of other natural and synthetic HDAC inhibitors have been identified (Monneret 2005; Pontiki et al. 2010) including apicidin from *Fusarium sp*., Cyl-2 from *Cylindrocladium scoparium*, depudecin from *Alternaria brassicicola* (see Chapter 2) and HC-toxin (below and Chapter 3). HDAC inhibitors share some features such as a linker domain,

metal-binding domain and surface recognition domain which allows it to interact with HDACs and prevent their activity. HDAC inhibition may result in hyper-acetylation, apoptosis, and changes in gene expression. Because they tend to be cytostatic rather than cytotoxic, HDAC inhibitors are being intensively studied possible anti-cancer drugs (Pontiki et al. 2010; Robey et al. 2011).

HC-toxin is an HDAC inhibitor of the cyclic peptide class; it is a cyclic tetrapeptide with the structure cyclo (D-Pro-L-Ala-D-Ala-L-Aeo), where Aeo stands for 2-amino-8-oxo-9,10-epoxidecanoic acid (Fig. 6B) Seven genes involved in the biosynthesis of HC-toxin have been identified; they are clustered on a 600 kilobase region known as the *TOX2* locus. *HTS1* is a nonribosomal peptide synthetase. It has a 15.7-kb open reading frame and encodes a 570-kDa polypeptide, Hts1. Hts1 has four adenylation domains and one epimerase module (Panaccione et al. 1992; Scott-Craig et al. 1992). *TOXA* encodes a member of the Major Facilitator Superfamily (MFS) of transporters (Pitkin et al. 1996). Since MFS transporters are involved in the transport of substrates across membranes, the role of *TOXA* is probably to transport HC-toxin out of the fungal cells where it can enter plant cells. Export of HC-toxin might also be important for self-protection against HC-toxin (Pitkin et al. 1996). *TOXC* encodes a fatty acid synthase beta subunit. It is predicted to be involved in the biosynthesis of the backbone of Aeo (Ahn et al. 1997). *TOXD* encodes a putative dehydrogenase. Dehydrogenases are involved in oxidation/reduction reactions but the specific role of *TOXD* in HC-toxin biosynthesis has not been identified. *TOXE* encodes a transcription factor. It contains a basic leucine zipper domain and ankyrin repeats. It is required for expression of the other genes of *TOX2*, and binds to their promoters (Pedley et al. 2001). *TOXF* encodes a putative branched-chain amino acid aminotransferase (Ahn et al. 1996; Cheng et al. 1999). It is required for HC-toxin biosynthesis

but its exact function is not known (Cheng et al. 1999). *TOXG* encodes an alanine racemase. It produces the D-Ala that is incorporated into HC-toxin by *HTS1* (Cheng et al. 2000).

ALTERNARIA

The genus *Alternaria* was first described by Nees von Esenbeck in the 1800's using the isolate *Alternaria tenuis* (Tweedy et al. 1963; Thomma 2003). Since, hundreds of additional *Alternaria* species have been described (Kirk et al. 2008). *Alternaria* is a member of the family Pleosporaceae and is further classified as mitosporic pleosporacea because it lacks a known perfect stage. *Alternaria* composes a group of filamentous fungi with worldwide distribution and including saprophytic as well as pathogenic species (Tweedy et al. 1963; Thomma 2003). Several *Alternaria* species such as *A. alternata* and *A. infectoria* are human pathogens and are known to cause asthma, respiratory infections and cutaneous and subcutaneous infections (Wiest 1987; Pastor et al. 2008; Marcoux et al. 2009; Hamilos 2010). The genus *Alternaria* also includes a number of economically important plant pathogens including *A.dauci* (carrot leaf blight), *A. solani* (early blight on tomato and potato) *A. brassicicola* (dark spot or black spot on cabbage) and *A. alternata* which in addition to being a human allergen also causes leaf spot and rot on hundreds of agronomically important plant species (USDA fungal database http://nt.ars-grin.gov/fungaldatabases/) (Tweedy et al. 1963; Agrios 1997; Thomma 2003).

The key characteristics of the genus *Alternaria* is the production of large, darkly pigmented muriform spores and/or dictyospores often described as club shaped that are produced individually or as branched chains on conidiophores (Fig. 2) (Tweedy et al. 1963; Thomma 2003). Additionally septa are both longitudinal and transverse. *Alternaria* overwinters in the form of spores and/or mycelia on plant debris, seeds, or in the soil. In the spring, or when conditions are optimal, it grows and germinates and the resulting spores are dispersed by wind

and rain/moisture. After germination, the fungus can penetrate host tissue directly via an appressorium or through openings in the tissue. Ultimately, the fungus produces conidia on the surface of the tissue continuing the cycle (Fig. 3) (Agrios 1997). Symptoms characteristic of *Alternaria* infection are brown or grey necrotic lesions with a circular almost bull's-eye- like appearance caused by fluctuations in environmental conditions affecting growth (Fig.4) (Thomma 2003). Lesions are frequently surrounded by a chlorotic halo that moves ahead of lesion formation as a result of fungal secondary metabolites that diffuse through the plant cells (Fig.4) (Thomma 2003). Sporulation occurs in the center of the lesion (Thomma 2003).

Alternaria jesenskae

Alternaria jesenskae was first described in 2008, and was isolated from seeds of *Fumana procumbens* (Family Cistaceae) (Labuda et al. 2008). Morphological characteristics such as colony pigmentation and beak branching of *A. jesenskae* resemble *A. tomatophila*; however, based on analysis of molecular data (ITS1, 5.8x and ITS2 sequencing) *A. jesenskae* is a distinct taxon (Labuda et al. 2008). Nothing else is known about *A. jesenskae*, including geographical distribution, host specificity (if applicable), and ecological niche.

Alternaria brassicicola

Alternaria brassicicola is a necrotrophic plant-pathogen causing dark spot or black spot disease on members of the family *Brassicaceae* which includes important crops such as broccoli, cabbage, mustard, canola and the model organism *Arabidopsis thaliana* (Thomma 2003; Glazebrook 2005). It can infect plants at all stages of development and it is estimated to cause a loss of yield of 20%-50% annually. The *A. brassicicola* genome was sequenced by the Genome Institute, Washington University School of Medicine, and is available at the DOE Joint Genome

Institute website (http://genome.jgi-psf.org/Altbr1/Altbr1.home.html). The availability of the genome, in addition to the fact that *A. brassicicola* can infect *A. thaliana,* has made it a model system for studying pathogenesis and resistance (Cramer et al. 2004) (Gachon et al. 2004; Oh et al. 2005; Mukherjee et al. 2009).

Some plant factors believed to modulate resistance to *A. brassicicola* include the plant hormones jasmonic acid and ethylene. *Arabidopsis thaliana* mutants in either pathway showed enhanced susceptibility to *A. brassicicola* (Thomma et al. 1998; Ton et al. 2002). Autophagy and programmed cell death have also been implicated in resistance to *A. brassicicola* (Lai et al. 2011; Lenz et al. 2011; Su'udi et al. 2011). The phytoalexin camalexin has been reported to have a role in resistance to *A. brassicicola (Glazebrook 2005)*. Studies using *A. thaliana* phytoalexin biosynthetic mutant pad3 show a role of phytoalexins in resistance to *A. brassicicola* (Glazebrook 2005). However, a screen of *Arabdopsis* ecotypes with naturally varying phytoalexin production demonstrates that the role of phytoalexins in resistance to *A. brassicicola* varies by ecotype and that other factors may play a more central role in defense (Kagan et al. 2002).

Alternaria species, including *A. brassicicola,* produce a number of secondary metabolites, many of which have biological effects on animals, plants, and microbes (Chelkowski 1992; Walton 1996; Thomma 2003). Below I will give a short review of the structures, biosynthesis, and modes of action of microbial secondary metabolites with emphasis on those of *Alternaria* species

SECONDARY METABOLITES

Secondary metabolites are organic molecules, while not specifically required for the normal growth and development of an organism; they often play critical roles in numerous

processes related to lifestyle or response to environmental cues (e.g., biotic and abiotic stress). As such, several secondary metabolites have been shown to impart an advantage upon the organism, such as serving as signals in symbiosis, as virulence and pathogenicity factors, as well as in aiding in the expansion of host range through increasing virulence (Vining 1990; Vining 1992; Demain et al. 2000; Calvo et al. 2002; Keller et al. 2005). However, for the vast majority of secondary metabolites, a clear understanding of the importance and selective advantage they might confer on the producing organism in unknown. Below, I outline classes of secondary metabolites and gene clustering.

Classes of fungal secondary metabolites

There are four broad classes of secondary metabolites produced by fungi (Keller et al. 2005; Hoffmeister et al. 2007; Chiang et al. 2009). These are polyketides, non-ribosomal peptides, terpenoids, and alkaloids. As a group, polyketides are the largest class of secondary metabolites produced by fungi, and are synthesized by single multi-domain proteins called polyketide synthases (PKSs). Fungal type I PKSs are iterative meaning that biosynthetic reactions are repeated by a single multi-domain protein. Ketoacyl CoA synthase, acyltransferase and acyl carrier domains are essential PKS domains while number of other 'optional' domains may also be present (Fig. 5A). The number of iterative reactions, reduction steps, cyclization and further downstream reactions (Keller et al. 2005) contribute to a wide range of polyketide structures like small linear molecules such as depudecin from *Alternaria brassicicola* (See Chapter 2), to much larger and more complex structures such as aflatoxins from *Aspergillus spp*. (Fig. 5B) (Keller et al. 2005; Borkovich 2010).

The second class of secondary metabolites, nonribosomal peptides, are synthesized by a class of large multi-domain enzymes known as nonribosomal peptide synthetases (NRPS).

NRPSs are organized into modules, with each responsible for the incorporation of a single amino acid into the final product. Each module has a number of smaller catalytic domains, namely the adenylation, thiolation, and condensation domains (Fig.6A). The adenylation domain is responsible for recognizing and activating the amino acid monomers, the thiolation domain catalyzes thioester bond formation and the condensation domain catalyzes peptide bond formation and elongation. In fungi, NRPSs are responsible for synthesizing a wide range of complex structures such as HC-toxin (See Chapter 3), cyclosporine, peptaibols, and AM-toxin (Fig.6B) (Keller et al. 2005; Hoffmeister et al. 2007; Chiang et al. 2009).

The two final classes of secondary metabolites are the terpenoids (e.g., carotenoids and gibberellins) and the indole alkaloids (e.g., ergotamine). These classes are less relevant to this thesis and are reviewed in depth elsewhere (Keller et al. 2005; Hoffmeister et al. 2007).

Several fungal secondary metabolites are classified as host-specific toxins (HST). HSTs are a structurally diverse group of compounds and proteins. The production of HSTs by the pathogen are critical for causing disease, toxic only to specific plant species or genotypes (Walton 1996; Wolpert et al. 2002). Conversely in the absence of toxin production pathogens are unable to cause disease (Sweat et al. 2008). HSTs have been identified from over 20 pathogenic fungi and some examples include victorin from *Cochliobolus victoriae*, AK toxin and AF toxin from *Alternaria* spp, T-toxin from *Cochliobolus heterostrophus* race T, and HC-toxin from *Cochliobolus carbonum* (Walton 1996; Wolpert et al. 2002; Friesen et al. 2008).

Clustering of secondary metabolite genes

In regard to the biosynthesis of fungal secondary metabolites, although polyketide synthases and NRPSs are often the “central” enzymes, most secondary metabolites require many other enzymes for their biosynthesis. For example, the backbone of sterigmatocystin is

biosynthesized by a PKS, but is then modified by numerous other other enzymes, such as monooxygenases, dehydrogenases and ketoreductases (Brown et al. 1996; Hicks JK 2002). Most or all of the genes involved in the pathway of a particular secondary metabolite are frequently clustered into operons in bacteria and into co-regulated gene clusters in fungi. (Jacob 1961; Koonin et al. 2001; Zheng et al. 2002; Rocha 2008) (Keller et al. 1997; Keller et al. 2005; Hoffmeister et al. 2007; Osbourn 2010; Chu et al. 2011). The selective advantage of gene clustering in fungal (eukaryotic) secondary metabolite clusters is unclear (Keller et al. 2005; Fondi et al. 2009). However, it has been suggested that gene clustering could facilitate lateral or horizontal gene transfer of the secondary metabolite trait, enabling its spread and persistence and hence providing an evolutionary persistence. This "selfish cluster" hypothesis has been developed for bacterial operons and fungal gene clusters (Lawrence et al. 1996; Lawrence 1999; Rosewich et al. 2000; Walton 2000; Wong et al. 2005; Khaldi et al. 2011).

Organisms may possess shared traits for a number of reasons. Vertical transfer is the inheritance of genetic material from a parent to 'progeny" as described by Mendelian genetics. Convergent evolution is the when two unrelated organisms independently evolve a trait with the same or similar biological function (Slot et al. 2010; Pichersky et al. 2011). Evolution from a common ancestor by vertical transmission is based on the principal that there exists a last common ancestor for branches of a particular group of organisms, and therefore all branches share similar genes (Postberg et al. 2010; Desmond et al. 2011; Heitman 2011; Parfrey et al. 2011). Finally, horizontal gene transfer is the transfer or movement of genetic material from one organism to another across taxonomic boundaries (Doolittle 1998; Rosewich et al. 2000; Keeling 2009; Boto 2010; Richards et al. 2011). Horizontal gene transfer requires that the gene or genes

are integrated, survive and are maintained in the genome of the recipient. Below I review what is currently known about horizontal transfer in both prokaryotes and eukaryotes focusing on fungi.

HORIZONTAL GENE TRANSFER IN PROKARYOTES

Horizontal gene transfer among prokaryotes occurs frequently. Horizontal gene transfer of single genes as well as operons is accomplished by conjugation, transduction, transformation, and probably also by phagocytosis (Doolittle 1998; Kelly et al. 2009; Kelly et al. 2009; Mehrabi et al. 2011). Horizontal gene transfer in prokaryotes through the F pilus was visualized in *Escherichia coli* using fluorescent protein fusions (Babic et al. 2008). One recent example of horizontal gene transfer involving secondary metabolism between two prokaryotes is granaticin biosynthesis. Granaticin is an antibiotic produced by certain *Streptomyces* species. The biosynthetic gene cluster for granaticin (*gra*) was also found in the bacterium *Streptomyces vietnamensis* (Deng et al. 2011). Sequencing and phylogenetic analysis of this cluster indicates a high overall homology to the *gra* cluster from *Streptomyces violaceoruber* Tü22 (Deng et al. 2011). The distribution of the gra cluster in *Streptomyces* is scattered which authors suggest may be the result of horizontal transfer. Additionally, they indicate that the absence of a flanking gene, insertions and deletions indicate that rapid evolution of the *gra* cluster may have occurred (Deng et al. 2011).

HORIZONTAL GENE TRANSFER IN EUKARYOTES

Horizontal gene transfer involving eukaryotes is usually from prokaryotes to eukaryotes. However, there are a few exceptions to that rule. A well-studied case of horizontal gene transfer among eukaryotes includes P elements from *Drosophila melanogaster* to *Drosophila willistoni* (Engels 1997). The transfer of the lectin like antifreeze protein gene (which is not present across

all species) is likely a result of horizontal gene transfer although it is not entirely clear how it is happening. However several possibilities exist ranging from the involvement of bacteria, viruses and parasitic organisms to sperm-mediated horizontal gene transfer (Graham et al. 2008). Other examples of horizontal gene transfer in eukaryotes include the transfer of DNA from trypanosomes to human hosts (Hecht et al. 2010), transfer of carotenoid genes from fungi to aphids (Moran et al. 2010), and the likely transfer of the gene ShContig9483, which encodes a protein of unknown function, from its host to *Striga hermonthica* and *Striga gesnerioides* (Yoshida et al. 2010).

While horizontal transfer among fungi is not as widely documented as it is in prokaryotes, it is quickly becoming one of the fastest growing areas of fungal phylogenetics. Until recently the limited number of genome sequences available has presented the biggest obstacle in evolutionary studies. However, with the advent of inexpensive and high quality sequencing systems more genomes are being sequenced resulting in increased examples of horizontal gene transfer among fungi. One of first reported examples of fungal horizontal gene transfer involves the *ToxA* gene shown to be transferred from *Stagonospora nodorum* to *Pyrenophora tritici-repentis* (Friesen et al. 2006) Authors demonstrated that the genes are 99% identical and that acquisition of the *ToxA* gene by *P. tritici-repentis* confers expanded host range and increased virulence on wheat (Friesen et al. 2006). Another example of horizontal gene transfer in fungi involves the nitrate assimilation gene cluster (fHANT-AC). fHANT-AC consists of three genes and has been found in *Aspergillus nidulans, Pichia angusta, Hebeloma clindrosporum*, *and Phanerochaete chrysosporium.* Researchers used an intensive phylogenetic analysis to show that the fHANT-AC cluster was transferred from a basidiomycete to an ancestor of *Trichoderma reesei* (Slot et al. 2007).

Until recently, most reports of horizontal gene transfer in fungi have involved either single genes or fairly small gene clusters. However, the possibility of horizontal gene transfer of much larger clusters has been raised. Slot and Rokas demonstrated that the presence of the large sterigmatocystin clusters (23 genes extending over~54kb) found in *Aspergillus nidulans* and *Podospora anserina* is a result of horizontal gene transfer. Phylogenetic analysis of the cluster in both organisms showed that both clusters share not only the same gene number and size but sequence and microsynteny as well. Detailed phylogenetic analysis also indicates that the direction of HGT of the cluster was likely from *A. nidulans* to *P. anserina* (Slot et al. 2011).

Another possible example of horizontal gene transfer of a large secondary metabolite gene cluster is HC-toxin. The genes for this cyclic peptide were first described in *C. carbonum* (Walton 2006). HC-toxin was later reported from *A. jesenskae* (Roman Labuda, personal communication). A goal of this thesis research was to explore the evolutionary explanation for the presence of this trait in two unrelated fungi. Chapter 3 will address whether the presence of HC-toxin trait in these two organisms is due to horizontal gene transfer, descent from a common ancestor, or convergent evolution?

Figure 1. Symptoms characteristic of northern corn leaf spot on maize leaves. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

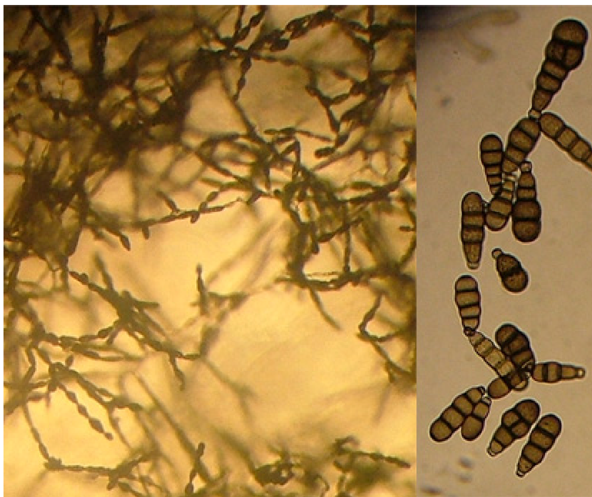

Figure 2. Alternaria brassicicola conidia and spores

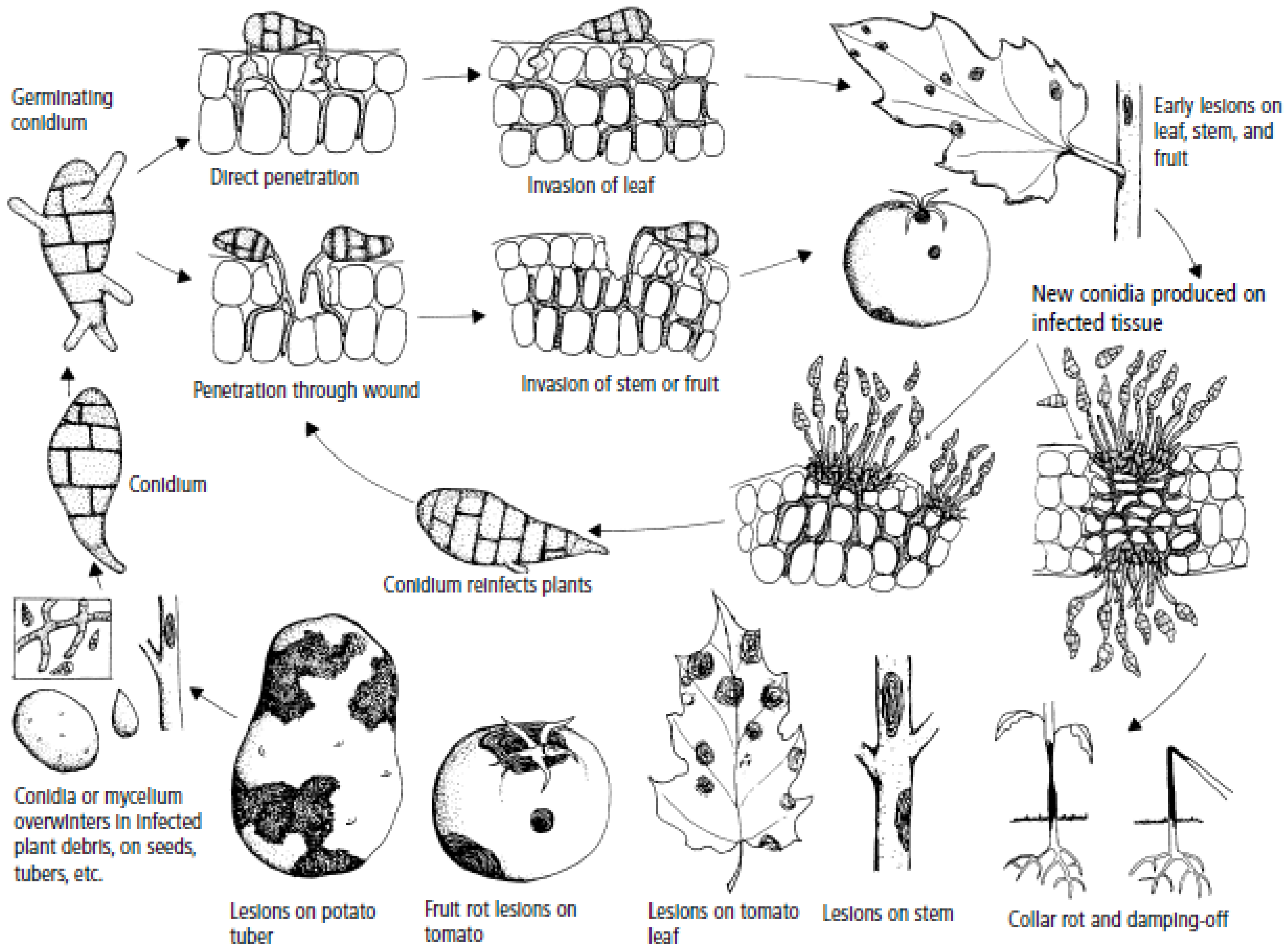

Figure 3. Life cycle of *Alternaria* infection (Agrios 1997)

Figure 4. Cabbage leaf infected with *A. brassicicola*.

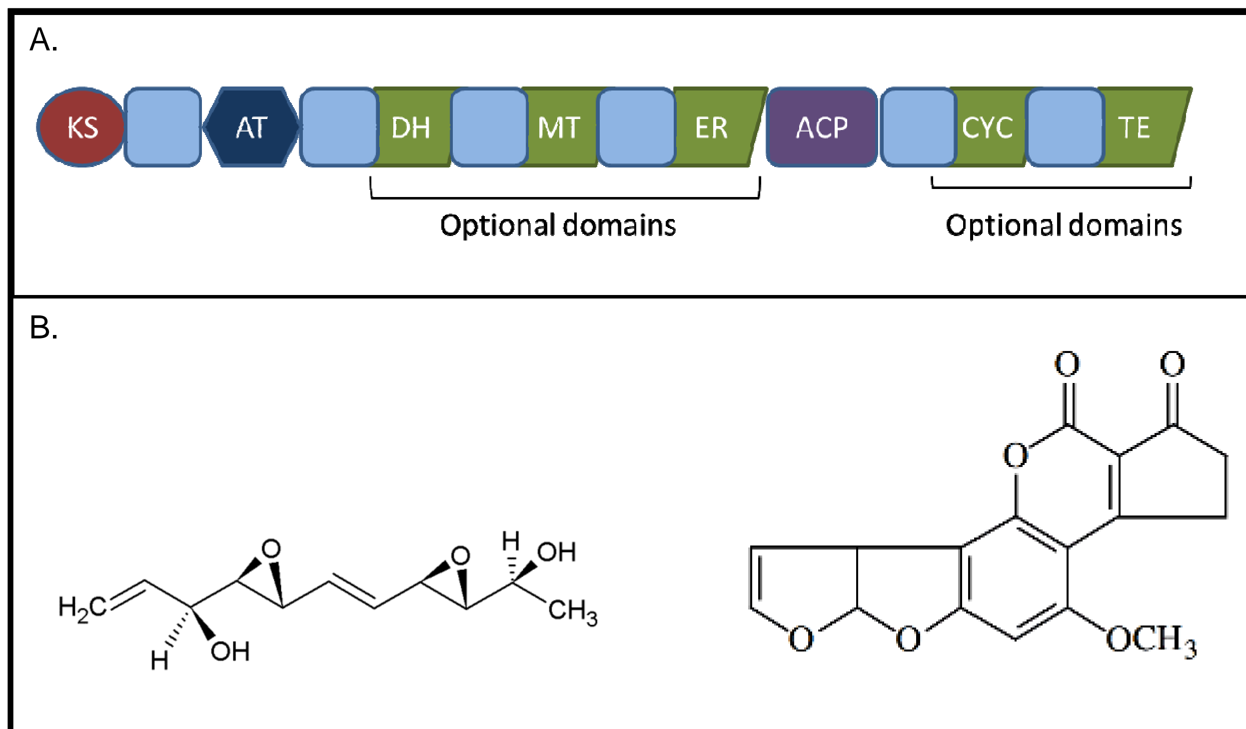

Figure 5. A) Polyketide synthase structure. Required domains include Ketoacyl CoA synthase (KS), acyltransferase (AT) and acyl carrier domains (ACP). Optional domains may include dehydratase (DH), methyltransferase (MT) enoyl reductase (ER), cyclase (CYC) and thioesterase (ET). B) Fungal polyketide metabolites depudecin (Left) and aflatoxin (Right).

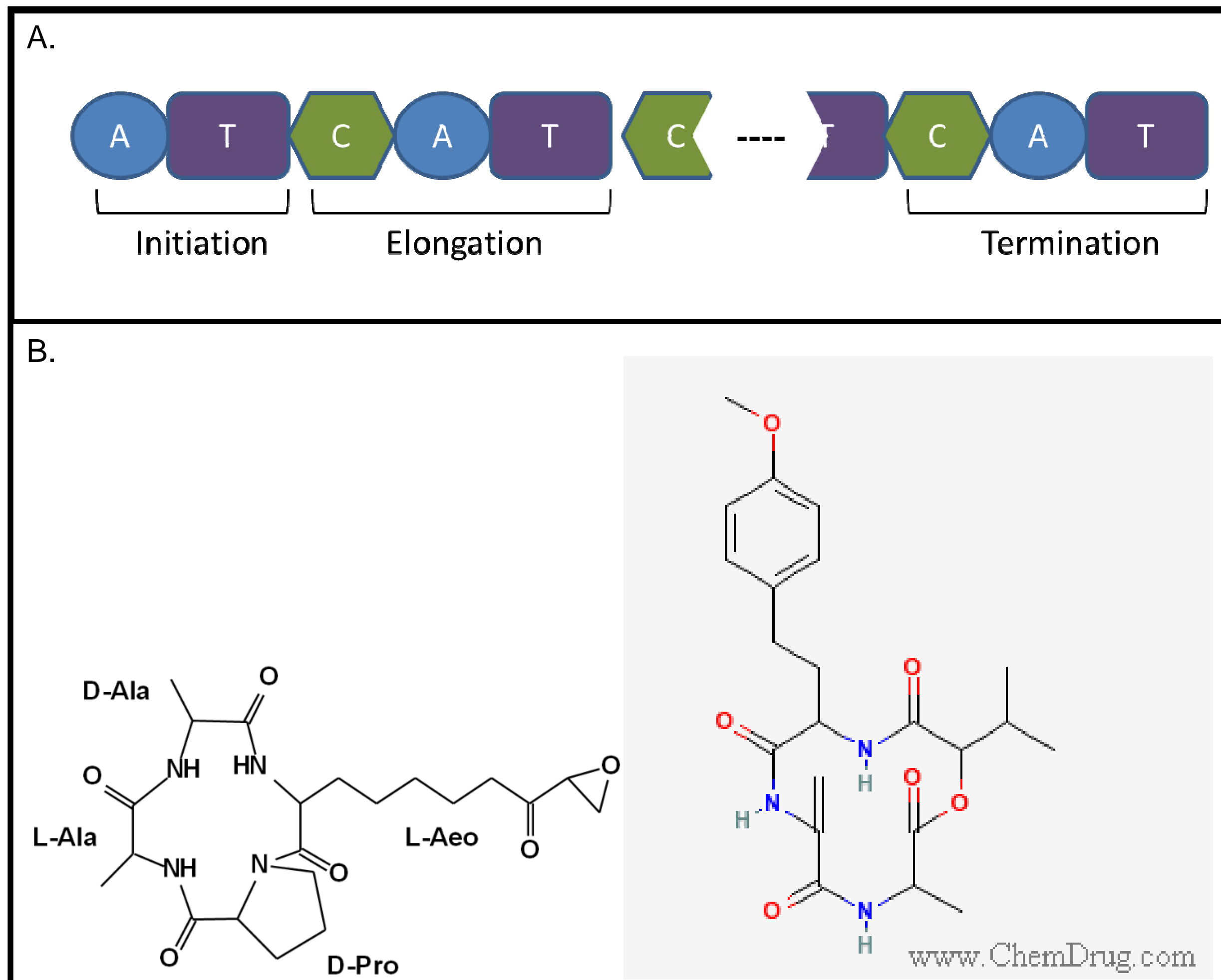

Figure 6. A) Non ribosomal synthase structure. Minimal domains include the adenylation (A), thiolation (T), and condensation domains (C). B) Fungal non ribosomal peptides HC-toxin (Left) and AM toxin (Right).

CHAPTER 2: BIOSYNTHESIS AND ROLE IN VIRULENCE OF THE HISTONE DEACETYLASE INHIBITOR DEPUDECIN FROM *ALTERNARIA BRASSICICOLA*

Wanessa Wight[1], Kwang-Hyung Kim[2], Christopher B. Lawrence [2],

and Jonathan D. Walton[1]

[1] Department of Energy - Plant Research Laboratory, Michigan State University,

East Lansing, MI 48824, U.S.A.

[2] Virginia Bioinformatics Institute and Department of Biological Sciences,

Virginia Tech, Blacksburg, VA 24061, U.S.A.

This work was published in MPMI in October 2009, Volume 22, Number 10

ABSTRACT

Depudecin, an eleven-carbon linear polyketide made by the pathogenic fungus *Alternaria brassicicola*, is an inhibitor of histone deacetylase (HDAC). A chemically unrelated HDAC inhibitor, HC-toxin, was earlier shown to be a major virulence factor in the interaction between *Cochliobolus carbonum* and its host, maize. In order to test whether depudecin is also a virulence factor for *A. brassicicola*, we identified the genes for depudecin biosynthesis and created depudecin-minus mutants. The depudecin gene cluster contains six genes (*DEP1-DEP6*), which are predicted to encode a polyketide synthase (AbPKS9 or *DEP5*), a transcription factor (*DEP6*), two monooxygenases (*DEP2* and *DEP4*), a transporter of the major facilitator superfamily (*DEP3*), and one protein of unknown function (DEP1). The involvement in depudecin production of *DEP2, DEP4, DEP5*, and *DEP6* was demonstrated by targeted gene disruption. *DEP6* is required for expression of *DEP1 - DEP5*, but not the immediate flanking genes, thus defining a co-regulated depudecin biosynthetic cluster. The genes flanking the depudecin gene cluster, but not the cluster itself, are conserved in the same order in the related fungi *Stagonospora nodorum* and *Pyrenophora tritici-repentis*. Depudecin-minus mutants have a small (10%) but statistically significant reduction in virulence on cabbage (*Brassica oleracea*), but not on *Arabidopsis*. The role of depudecin in virulence is therefore less dramatic than that of HC-toxin.

Abbreviations: PKS, polyketide synthase; HST, host-selective toxin; Sn, *Stagonospora nodorum*; Ptr, *Pyrenophora tritici-repentis*; JA, jasmonic acid; MFS, major facilitator superfamily; LME, linear minimal element; RACE, rapid amplification of cDNA ends

INTRODUCTION

Host-selective toxins (HSTs) are positive agents of specificity and virulence in a number of plant disease interactions (Walton et al. 1993; Walton 1996; Friesen et al. 2008). All known HSTs are made by fungi, and most of them are small, secondary metabolites. HC-toxin, a cyclic tetrapeptide made by *Cochliobolus carbonum*, is a critical determinant of virulence in the interaction between the pathogen and its host, maize. Isolates of *C. carbonum* that synthesize HC-toxin are extremely virulent on maize homozygous recessive at the nuclear HM1 and HM2 loci, killing plants in a few days (Walton 2006; Sindhu et al. 2008). HM1 encodes HC-toxin reductase, which detoxifies HC-toxin by reducing an essential carbonyl group in HC-toxin (Johal et al. 1992; Meeley et al. 1992; Multani et al. 1998). HC-toxin is an inhibitor of histone deacetylases (HDACs) of the Type I (RPD3/HDA1-like), and Type II (HD2, plant-specific) classes, but not of the Type III (SIR2-like, sirtuin) class (Brosch et al. 1995; Hollender et al. 2008). HC-toxin has been shown to inhibit Type I HDACs in maize, protozoans, yeast, and mammals both in vivo and in vitro, and has been used as a specific HDAC inhibitor in numerous studies (Brosch et al. 1995; Darkin-Rattray et al. 1996; Joung et al. 2004; Deubzer et al. 2008). There is no evidence that HC-toxin and chemically related compounds have any other sites of action.

Earlier studies on the role of HC-toxin in plant pathogenesis raise the question of the role of HDACs in plant disease – why does inhibition of HDACs facilitate the development of disease, i.e., what is the role of HDACs in disease resistance? Attempting to further our understanding of HDACs and disease is complicated by several factors. First, there are multiple sensitive HDACs in maize and other plants. Maize has 14 and *Arabidopsis* has 16 HDAC genes of the HC-toxin-sensitive classes (RPD3, HDA1, and HD2) (Gendler et al. 2008). Second, all of

the core histones are subject to reversible acetylation, and each has multiple lysine residues capable of being acetylated. HC-toxin affects acetylation of multiple core histones in maize, especially H3 and H4 (Ransom et al. 1997). There are thus many possible permutations of histone acetylation/ deacetylation, especially when combined with multiple possible permutations of acetylation-dependent methylation, which can lead to different physiological outcomes (Jenuwein et al. 2001). Third, many non-histone proteins have now been shown to be regulated by reversible acetylation, including tubulin, cell cycle regulators, transcription factors, DNA helicases, heat shock factors, and HDACs themselves (Brandl et al. 2009; Westerheide et al. 2009). Therefore, there are many possible relevant targets of HC-toxin, each in turn with multiple possible substrates.

Reversible histone acetylation is involved in many cellular processes (Brandl et al. 2009; Haberland et al. 2009), and as a result HDAC mutations in plants are pleiotropic. Mutational and inhibition studies indicate that HDACs control the expression of numerous plant genes and are involved in the regulation of processes such as embryo and flower development, the jasmonic acid (JA) and ethylene pathways, light responses, senescence, nucleolar dominance, silencing of transgenes and transposons, Agrobacterium transformability, leaf polarity, abscisic acid and abiotic stress responses, and root hair density (Hollender et al. 2008). In cereals, hda101 mutants of maize have pleiotropic effects on development and gene expression (Rossi et al. 2007). Overexpression of a rice HDAC gene causes changes in plant growth and architecture (Jang et al. 2003).

In regard to the role of histone acetylation in plant pathogenesis, HDA19 expression in *Arabidopsis* is induced by infection with the pathogen *Alternaria brassicicola*, and overexpression of HDA19 causes enhanced resistance and upregulation of ethylene and JA-

induced pathogenesis-related (PR)-proteins (Zhou et al. 2005). However, in light of the multiple developmental abnormalities of HDA19 mutants, it is not clear if HDA19 has a primary or secondary role in disease resistance (Wu et al. 2000; Tian et al. 2001; Wu et al. 2003). Mutants in a gene, *HUB1*, encoding a histone H2B mono-ubiquitinating enzyme, have earlier flowering, thinner cell walls, and increased susceptibility to *A. brassicicola* and *Botrytis cinerea* (Dhawan et al. 2009).

Another connection between histone acetylation and disease response comes from the finding that HDA6 of *Arabidopsis* interacts with COI1, an F-box protein required for JA signaling (Devoto et al. 2002; Thines et al. 2007). This is consistent with the known importance of JA in response to *A. brassicicola* (see below) and with transcription profiling experiments suggesting a link between COI1-regulated genes and resistance to *A. brassicicola* (van Wees et al. 2003). JA induces expression of HDA6 and HDA19, but not HDA5, HDA8, HDA9, HDA14, or HD2A (Zhou et al. 2005).

HDA19 interacts with WRKY38 and WRKY62, which are negative regulators of defense induced by salicylic acid or *Pseudomonas syringae* infection in an NPR1-dependent manner (Kim et al. 2008). Overexpression of HDA19 enhances resistance, and mutation enhances susceptibility; that is, HDA19 appears to act as a positive regulator of defense. HDA19 might work through *ERF1*, a gene that integrates JA and ethylene pathways, because HDA19 overexpression up regulates *ERF1* (Zhou et al. 2005).

HDACs and their target proteins can be both positive and negative regulators of gene expression (Brandl et al. 2009). If HDACs are necessary for induction of defense genes, then by inhibiting HDACs HC-toxin might suppress expression of those genes (Brosch et al. 1995). This model is consistent with the studies on HDA19 in the *P. syringae*/*Arabidopsis* pathosystem (Kim

et al. 2008), but does not exclude other models, e.g., that HDACs repress a negative regulator of defense. There are several additional major uncertainties, such as whether HDACs could have a different role in dicotyledons versus cereals such as maize, and to what extent the role of HDACs might be affected by the known differences in defense signaling pathways in response to bacterial biotrophic pathogens such as *P. syringae* as opposed to necrotrophs such as *C. carbonum* and *A. brassicicola*.

In order to expand our understanding of the role of HDACs in defense, we have considered the *A. brassicicola*/*Arabidopsis* pathosystem. *A. brassicicola* causes black spot of most cultivated Brassica species including broccoli, cabbage, canola, and mustard. Many species of *Alternaria* make host-selective toxins (Walton 1996). Although *A. brassicicola* is a weak pathogen on wild type *Arabidopsis* (Kagan et al. 2002), it causes significant disease on pad3 (phytoalexin-deficient) and DELLA mutants (Thomma et al. 1999; Navarro et al. 2008). Its genome has been sequenced and it is genetically tractable (Cho et al. 2006). Transcriptional changes during infection have been profiled in the fungus and in the host (Schenk et al. 2003; van Wees et al. 2003). Plant factors that modulate reaction to *A. brassicicola* include JA, gibberellins acting through the JA pathway, *BOS1* (encoding an R2R3MYB transcription factor), *BOS2, BOS3, BOS4, RLM3* (encoding a TIR-domain protein), and lipase (Mengiste et al. 2003; Veronese et al. 2004; Oh et al. 2005; Navarro et al. 2008; Staal et al. 2008).

A critical attribute of *A. brassicicola* from the point of view of elucidating the role of reversible histone acetylation in defense is that it makes an HDAC inhibitor called depudecin (Matsumoto et al. 1992; Kwon et al. 1998). Depudecin is a small linear polyketide (Fig. 7) (Tanaka et al. 2000). Depudecin is anti-parasitic and anti-angiogenic and, like HC-toxin, causes detransformation of oncogene-transformed mammalian cells and inhibits HDAC activity in vitro

and in vivo (Matsumoto et al. 1992; Oikawa et al. 1995; Kwon et al. 1998; Kwon et al. 2003). If depudecin is a virulence factor for *A. brassicicola* on *Arabidopsis* one could exploit the genetic resources of both partners to address the role of HDACs in disease resistance. Here we report the identification and characterization of the gene cluster responsible for depudecin biosynthesis in *A. brassicicola* and the disease phenotype of depudecin-minus strains. Although depudecin does contribute to virulence of this fungus, the effect is much less dramatic than the role of HC-toxin in the *C. carbonum*/maize interaction.

MATERIALS AND METHODS

Fungal growth and depudecin analysis.

A. brassicicola American Type Culture Collection (ATCC) 96836 and Mycothèque Université Catholique de Louvain, Belgium (MUCL) 20297 were maintained on potato dextrose agar (PDA) (Difco) or V8-juice agar. For depudecin production, the fungus was grown in still culture in one-liter flasks containing 125 ml potato dextrose broth (PDB) (Difco) for 7 to 10 d. The cultures were filtered through Whatman #1 paper and extracted twice with equal volumes of dichloromethane. The dichloromethane fractions were evaporated under vacuum at 40ºC and redissolved in 3 ml methanol. After concentration under vacuum, the residue was dissolved in 100 µl methanol. This crude extract was used for both TLC and HPLC. Depudecin standard was obtained from Sigma-Aldrich and dissolved in methanol at 1 mg/ml.

For TLC, crude extract (10 µl) was spotted onto 250-µm silica plates with adsorbent strip (Whatman). Plates were developed in 1:1 acetone:dichloromethane. Depudecin was detected using an epoxide-specific reagent (Hammock et al. 1974).

For HPLC, 20 µl of extract was combined with 60 µl of acetonitrile and 20 µl of sterile distilled water. The sample was injected onto a C18 reverse phase column (Agilent Eclipse XDB-C18 silica, 5 µm, 4.6 x 150 mm) and eluted with a linear gradient of 10% (v/v) acetonitrile in water to 100% acetonitrile in 30 min at a flow rate of 1 ml/min. The eluant was monitored at 210 nm.

Gene disruption.

To disrupt *DEP5* (*AbPKS9*) by the LME method (Cho et al. 2006), two primers were designed, one at the 954-bp position (in relation to the start codon) with an added HindIII enzyme site and the other at the 1442-bp position with an added XbaI site (see Fig. 8 and Appendix I, Table 7.1 for all primer sequences). These primers were used to amplify a 509-bp fragment from genomic DNA. PCR products were digested with HindIII and XbaI and ligated to the corresponding sites in pCB1636 (Sweigard 1997). The ligation was transformed into E. coli strain DH5α (Invitrogen). The plasmid was isolated and used as template for PCR amplification using M13 forward and M13 reverse primers. The PCR product was purified and concentrated to 1 µg/µl.

To disrupt *DEP6, DEP2*, and *DEP4* by the LME method, two primers for each gene (*DEP6*KOFor and *DEP6*KORev for *DEP6*, *DEP2*KOFor and *DEP2*KORev for *DEP2*, and *DEP4*KOFor and *DEP4*KORev for *DEP4*) were used to amplify 751-bp partial *DEP6*, 812-bp partial *DEP2*, and 807-bp partial *DEP2* sequences from genomic DNA. Another set of two primers for each was used to amplify cassettes of hph1 (encoding hygromycin phosphotransferase) from the plasmid pCB1636: *DEP6*HygFor and *DEP6*HygRev, *DEP2*HygFor and *DEP2*HygRev, or *DEP4*HygFor and *DEP4*HygRev. The resulting fragments for each gene were mixed and subjected to a second PCR reaction with primers *DEP6*KOFor and

*DEP6*HygRev, *DEP2*KOFor and *DEP2*HygRev, or *DEP4*KOFor and *DEP4*HygRev. The resulting final products were used to transform *A. brassicicola* to make *DEP6*, *DEP2*, or *DEP4* mutants, respectively.

In order to complement the *DEP5* mutant, the wild type *DEP5* allele from *A. brassicicola* genomic DNA was amplified using primer set P9comF and P9comR. To complement the *DEP6*, *DEP2*, and *DEP4* mutants, a 2.7-kb *DEP6* allele with primers TfcomF and TfcomR, a 2.6-kb *DEP2* allele with primers Mo1comF and Mo1comR, and a 3.4-kb *DEP4* allele with primers Mo2comF and Mo2comR were amplified from genomic DNA. Separately, a 1449-bp nourseothricin resistance (NAT) cassette carrying the nourseothricin acetyltransferase-encoding gene nat1 was amplified using primer set PNRcomF and PNRcomR from plasmid pNR1 (Malonek et al. 2004). The final two PCR products, each target gene fragment, and a NAT cassette were used to transform simultaneously the *DEP2*, *DEP4,* or *DEP6* mutant strains, and transformants were selected on PDA plates containing nourseothricin. All transformants were subjected to two rounds of single-spore isolation.

For disruption by gene replacement, primer pairs 1 and 2 were used to amplify 575-bp and 476-bp fragments from the 5’ and 3’ ends of *DEP5*, respectively, overlapping the transcriptional start and stop sites (see Fig. 8 and Appendix I, Table 7.1). Primer pairs 4 and 5 were used to amplify 476-bp and 507-bp fragments of *DEP6*. The internal primers had an additional 25-30 bp complementary to hph1 of pCB1003 (Carroll AM 1994). Primers 7 and 8 were used to amplify hph1 and had an additional 25 bp complementary to the target gene. The upstream and downstream flanking regions of *DEP5* and *DEP6* were combined with hph1 in a second round of PCR using the outside gene primers.

PCR products were ligated into pGem T-easy (Promega) and transformed into E. coli DH5α (Invitrogen). PCR products were purified with the QIAquick PCR purification Kit (Qiagen) and used directly in transformation of protoplasts (Cho et al. 2006). Transformants were purified by two rounds of single-spore isolation.

DNA extraction and analysis.

DNA isolation and blotting were performed as described previously (Kim et al. 2007). The DNA blots shown in Fig. 9B was performed and hybridized according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany) using digoxigenin (DIG)-labeled DNA probes. A total of 2-3 µg of genomic DNA was digested with EcoRI for analysis of *DEP5* (AbPKS9), with XhoI and BamHI for analysis of *DEP6*, with PstI for analysis of *DEP2*, and with BamHI for analysis of *DEP4*. The PCR DIG Probe Synthesis Kit (Roche) was used to label a 509-bp fragment of *DEP5*, a 751-bp fragment of *DEP6*, an 812-bp fragment of *DEP2*, and 807-bp fragment of *DEP4*. A 500-bp fragment from pCB1636 and a 1-kb fragment from pNR1 were used as probes for the selectable marker genes.

For the DNA blots shown in Fig. 10, DNA was extracted from lyophilized mycelial mats of 5-7 day-old *A. brassicicola* MUCL 20297 grown in potato dextrose broth in still culture (Pitkin et al. 1996). DNA (15 µg) was digested with NruI (*DEP5* mutants) or MluI (*DEP6* mutants) (Fig. 8B, C). DNA was transferred to Nytran SPC (Whatman) and hybridized with 32P probes (Panaccione et al. 1996).

RNA was extracted as described (Hallen et al. 2007). Reverse transcriptase (RT)-PCR followed by 5' and 3' RACE was done with the SMART RACE cDNA amplification kit (Clontech). Overlapping gene-specific primers were designed from available genomic sequence.

In most cases several gene-specific primers were utilized. PCR products were cloned into pGem T-easy (Promega) and transformed into E. coli DH5α (Invitrogen).

For RNA blotting, 15-20 μg of total RNA was used per lane. Internal gene-specific probes using primers given in Table S1 were generated directly from cDNA templates. rRNA bands on the blots were stained with 0.02% methylene blue in 0.5 M sodium acetate, pH 5.5.

Virulence assays.

Pathogenicity assays used wild type and mutant strains of ATCC 96836. Conidia were harvested from potato dextrose agar (PDA) plates incubated for 7 d at 25°C and suspended in sterile water at 2 x 104 conidia/ml. Conidial suspensions (10 μl) were applied as drops on the surface of leaves at the fifth through sixth leaf stages. Inoculated plants were placed in a plastic box at room temperature (21°C) and kept at 100% relative humidity for 24 h in the dark, followed by 4 d under fluorescent lights with a photoperiod of 16 hr light/8 hr dark. Lesion diameters were measured. The experiments were repeated four times with >15 plants per treatment. Results were analyzed using a pair-wise t-test using JMP (SAS, Cary, NC).

Arabidopsis plants (Col-0 and pad3) were grown in a growth chamber at 20°C, 70% relative humidity, and a 12-hr light/dark cycle. *A. brassicicola* spores were collected from PDA culture plates in 2 ml 0.1% Tween-20. The third through the seventh true leaves from 4-week-old plants were spot-inoculated with 10 μl of 10^5 spores/ml. Plants were covered to maintain high humidity. Lesions were measured and photographed 4 d after inoculation.

RESULTS

***AbPKS9* encodes the depudecin polyketide synthase.**

We identified nine genes encoding putative polyketide synthases (PKSs), designated *AbPKS1* through *AbPKS9*, in the genome of *A. brassicicola*. Disruption mutants for each PKS gene were generated in strain ATCC 96836 using the linear minimal element (LME) method (Cho et al. 2006). The strategies and expected results for homologous integration at *AbPKS9* are shown in Fig. 8A, B. Mutants of *AbPKS1* through *AbPKS8* still produce depudecin (Fig. 9A, and data not shown). Of three transformants obtained using LME targeted to *AbPKS9*, DNA blotting indicated that two are knockout mutants and one is an ectopic transformant (Fig. 9B, panel 1). Both the wild type and the *pks9-1* strains contain a 1.6-kb EcoRI fragment hybridizing to *AbPKS9* and strain *pks9-1* has a 3.6-kb band hybridizing to hph1, which together indicate that *pks9-1* is an ectopic transformant. In both *pks9-2* and *pks9-3* transformants, the 1.6-kb band is replaced by a ~12-kb band hybridizing to *AbPKS9* (Fig. 9B, panel 1), consistent with homologous tandem integration of five copies of the construct (see Fig. 8). The 1.6-kb wild type band is restored in the complemented mutant PKS9-2C (Fig. 9B, panel 1). Based on TLC and HPLC analysis, neither of the knockout mutants (*pks9-2* and *pks9-3*) produces depudecin, whereas the ectopic transformant (*pks9-1*) and the complemented strain do (Fig. 9C, D).

Additional mutants of *AbPKS9* were made by double-crossover gene replacement in a different strain of *A. brassicicola*, MUCL 20297. Two independent mutants of *AbPKS9*, called *pks9-4* and *pks9-5*, were verified by DNA blotting (Fig. 10A, B). Neither produces depudecin (Fig. 10C, D). Together, these results indicate that *AbPKS9* encodes the PKS responsible for depudecin biosynthesis. In light of subsequent characterization of the depudecin biosynthetic cluster, *AbPKS9* was renamed *DEP5* (see below).

The depudecin biosynthetic cluster.

The protein coding regions in 72 kb of genomic DNA surrounding *AbPKS9* were predicted with FGENESH (www.softberry.com) using the Alternaria-specific training matrix. The genes adjacent to *PKS9* are predicted to encode two monooxygenases, a membrane transporter of the major facilitator superfamily (MFS), and a transcription factor, all of which could have a plausible role in depudecin biosynthesis and regulation. The two monooxygenases and the transcription factor were chosen for mutational analysis. Disruption of the putative MFS transporter was not attempted because genes of this class are often necessary for self-protection, in which case a mutant would be lethal (Pitkin et al. 1996).

The LME strategy was used to construct mutants of the two monooxygenases (*DEP2* and *DEP4*). Two independent mutants of each gene were obtained (*DEP2*-2, *DEP2*-4, *DEP4*-4, and *DEP4*-5) (Fig. 9B, panels 2 and 3). Both *DEP2* mutants fail to produce depudecin as judged by TLC and HPLC, but do produce smaller amounts of an epoxide-containing metabolite of slightly higher Rf than native depudecin (Fig. 11A, lanes 3 and 4). This compound is not depudecin as judged by HPLC (Fig. 11B). Both *DEP4* mutants also produce a trace of an epoxide-containing compound of the same Rf as native depudecin (Fig. 11A, lanes 5 and 6). Again, however, this is not native depudecin as judged by HPLC, in which no compound of the same retention time as depudecin is seen (Fig. 11B). Complementation of the *DEP2-4* and *DEP4-4* mutants (*DEP2-4C* and *DEP4-4C*) restores depudecin synthesis (Fig. 11B). The mutants have no other distinguishable phenotypes (growth, color, morphology, and sporulation). These results indicate that the two putative monooxygenase genes are also required for, and dedicated to, depudecin biosynthesis.

Two engineered mutants of the putative transcription factor gene, called *DEP6*, made with the LME method (*DEP6*-1 and *DEP6*-2), do not produce depudecin (Fig. 9B, panel 4, and Fig. 11A, lanes 7 and 8, and Fig. 11B). Complementation of *DEP6*-1 restores depudecin production (*DEP6-1C*) (Fig. 11B). Three independent *DEP6* mutants (*DEP6*-3, *DEP6-4*, and *DEP6-5*) constructed using double-crossover gene replacement in MUC 20297 also fail to synthesize depudecin (Fig. 12). *DEP6* mutants have no discernible phenotype other than loss of depudecin production. These results indicate that *DEP6*, like *AbPKS9*, *DEP2*, and *DEP4*, is required for, and dedicated to, the biosynthesis of depudecin.

***DEP6* regulates the depudecin cluster.**

DEP6 is required for biosynthesis of depudecin (Figs. 11, 12). Its sequence shows some similarity to known fungal transcription factors (see below). Therefore, it might be a pathway-specific regulator of the expression of the genes involved in depudecin biosynthesis and could be used to help define the depudecin cluster. In order to test this, expression of the putative depudecin cluster genes was analyzed by RNA blotting. As shown in Fig. 7, RNA expression of *DEP2* and *DEP4* (the monooxygenases), *DEP3* (the MFS transporter), *DEP5* (*AbPKS9*), and *DEP6* itself are dependent on *DEP6*. Furthermore, expression of another gene upstream of *DEP2*, called *DEP1*, is also dependent on *DEP6* (Fig. 13). On this basis, *DEP1* is part of the depudecin gene cluster. Expression of the genes immediately upstream of *DEP1* and downstream of *DEP6* (genes 4 and 11) are not affected in the *DEP6* strain, and therefore are not part of the co-regulated gene cluster (Fig. 13).

Genes of the depudecin cluster.

Reverse transcriptase PCR and RACE were used to determine the structures and protein products of *DEP1* through *DEP6*. *DEP1* has no introns. *DEP1*, the protein encoded by *DEP1*, has 580 amino acids and a molecular mass of 40.3 kDa. Regulation by *DEP6* (Fig. 13) and synteny analysis (see below) suggest a role in depudecin biosynthesis. It has no detectable conserved domains. The best match by BLASTP in GenBank is to a predicted protein (CIMG_02399) from *Coccidioides immitis* (score 303, expect 2e-80, 48% amino acid identity). The second best hit, a predicted protein from *Talaromyces stipitatus*, is considerably less similar (score 132, expect 5e-29, 28% identity). Several additional hypothetical proteins from ascomycetes give weaker scores (>1e-22). There are no strong orthologs in other Pleosporaceae (Fig. 14). None of the putative orthologs has a known function.

DEP2 has three introns. Its predicted product has 528 amino acids (59.1 kDa). BLASTP results indicate that it has conserved domains corresponding to the pyridine nucleotide-disulfide oxidoreductase superfamily. It has an FAD-binding domain (pfam family 01494). *DEP2* is also a member of COG0654 (UbiH) containing 2-polyprenyl-6-methoxyphenol 4-monooxygenase and related FAD-dependent oxidoreductases. The best BLASTP hit against the NR database is to a hypothetical protein (CIMG_01450) from *C. immitis* (expect score 1e-135, 53% identity). *DEP2* is also similar (expect scores <1e-50) to a number of hypothetical FAD-dependent monooxygenases from other ascomycetes. By BLASTP against SwissProt, *DEP2* shows weak amino acid similarity to salicylate monooxygenase (EC 1.14.13.2) and zeaxanthin epoxidase (EC 1.14.12.4). These monooxygenases are all in class A (van Berkel et al. 2006). A number of monooxygenases of this class have been shown to be involved in secondary metabolite biosynthesis. For example, atmM of Aspergillus flavus and paxM of *Penicillium paxilli* are

predicted monooxygenases involved in the biosynthesis of the indole-diterpenes aflatrem and paxilline, respectively. These compounds contain hydroxyl groups and aflatrem contains an epoxide (Young et al. 2001; Zhang et al. 2004). In the biosynthesis of depudecin, *DEP2* might be responsible for the epoxidations or hydroxylations of depudecin (Fig. 7). The novel product made in the *DEP2* mutants might be depudecin with one less hydroxyl and/or epoxide (Fig. 11A).

DEP3 has eight introns. Its product is 564 amino acids (60.1 kDa). *DEP3* encodes a membrane transporter of the major facilitator superfamily (MFS). MFS genes are found in many fungal secondary metabolite clusters and presumed to be responsible for exporting secondary metabolites and/or to provide self-protection (Pitkin et al. 1996).

DEP4 has four exons and its product is 581 amino acids in length (66.0 kDa). Its best hit is CIMG_02397 of *C. immitis* (expect score 0.0, 66% identity), followed by a large number of hypothetical proteins in other ascomycetes. Conserved domains include COG2072 (TrkA, predicted flavoprotein involved in K+ transport), and pfam00743 (flavin-binding monooxygenase). Although *DEP2* and *DEP4* are both predicted to encode monooxygenases, they are of different classes. Whereas *DEP2* is in class A, *DEP4* is in class B (van Berkel et al. 2006). *DEP4* is related to cyclohexanone 1,2-monooxygenase (EC 1.14.13.22), 4-hydroxyacetophenone monooxygenase, and dimethylaniline monooxygenase. Like monooxygenases of class A, enzymes related to *DEP4* can catalyze epoxidations (Colonna et al. 2002; van Berkel et al. 2006).

DEP5 (also known as *AbPKS9* and as AB01916 in the recently released annotation of *A. brassicicola* at the Department of Energy Joint Genome Institute [DOE-JGI] and at the Virginia Bioinformatics Institute [www.alternaria.org], has five exons, and its product has 2376 amino

acids (259 kDa). *DEP5* is a polyketide synthase (PKS), the central enzyme in depudecin biosynthesis. Of characterized PKSs in the SwissProt database, *DEP5* shows high overall amino acid similarity to the lovastatin nonaketide synthase of *Aspergillus terreus* (Hendrickson et al. 1999). *DEP5* is a Type I reducing PKS with modules for ketoacyl synthase (KS), acyltransferase (AT), enoyl reductase (ER), and terminal acyl carrier protein (ACP). There is also probably a dehydratase (DH) module.

DEP6 has four exons and encodes a 646-amino acid protein (72.6 kDa). At its amino terminus *DEP6* contains a GAL4-like Zn2Cys6 binuclear cluster DNA-binding motif typical of fungal transcription factors. The best hit against GenBank NR is to SNOG_06678, a putative transcription factor in *Stagonospora nodorum*. The dependence of expression of *DEP1* through *DEP5* on *DEP6* is consistent with it encoding a pathway-specific transcription factor (Pedley et al. 2001).

A related gene cluster in *Coccidioides immitis*?

The best BLASTP hits against GenBank NR for three of the depudecin cluster genes are proteins from *Coccidioides immitis*, even though GenBank contains many complete genomes of fungi more closely related to *A. brassicicola* (Dothidiomycetes) than *C. immitis* (Eurotiomycetes). Furthermore, the two best hits to the products of *DEP1* and *DEP4* are *C. immitis* proteins closely linked to each other (CIMG_02399 and CIMG_02397, respectively). This raises the possibility that *C. immitis* has a gene cluster related to the depudecin cluster of *A. brassicicola*.

To test this possibility further, additional BLASTP queries were performed, especially specifically against the genome of *C. immitis* RS. The best match of *DEP1* to any protein in GenBank NR is CIMG_02399 (see above). *DEP3*, encoding the MFS transporter, has numerous

very strong hits in GenBank NR. However, the third best hit in GenBank, and the best hit within *C. immitis*, is CIMG_02396, which is now re-annotated as CIMG_10938 (score 676, expect 0.0, identity 63%). The best hit of *DEP4* (monooxygenase) to any protein in GenBank is CIMG_02397 (score 769, expect 0.0, 66% identity), which is also clustered with CIMG_02399.

PKS genes are common in fungi, and *DEP5* (*AbPKS9*) aligns with more than 50 proteins in GenBank NR with expect scores of 0.0. However, the fourth best hit of *DEP5* against any protein is CIMG_02398 (score 2390, expect 0.0, identity 52%). *DEP2* and *DEP6* do not continue the same pattern of clustering in *C. immitis*. The best match to *DEP2* of any protein in GenBank NR is clearly also a *C. immitis* protein (CIMG_01450), but this is not clustered with the others. Finally, the best hit of *DEP6* in *C. immitis* is CIMG_10246, which has relatively poor similarity (score 103, expect 8e-22, 24% identity).

In conclusion, four of the six genes of the depudecin cluster (*DEP1*, *DEP3*, *DEP4*, and *DEP5*) have as their best, or among their best, BLASTP hits four clustered genes in *C. immitis* (CIMG_2399, CIMG_2396, CIMG_2397, and CIMG_2398, respectively). This suggests that *C. immitis* has a gene cluster that is evolutionarily or functionally related to the depudecin cluster of *A. brassicicola*, and raises the possibility that *C. immitis* makes a secondary metabolite that is chemically related to depudecin.

Synteny of the depudecin cluster region between *A. brassicicola* and other Pleosporaceae.

The predicted protein sequences flanking the depudecin cluster and the experimentally deduced proteins for *DEP1* through *DEP6* were used to search the genomes of *S. nodorum* (*Phaeosphaeria nodorum*) (Sn) and *Pyrenophora tritici-repentis* (Ptr), which are also Dothideomycetes in the family Pleosporaceae. The four contiguous genes on the left flank of the depudecin cluster of *A. brassicicola* (numbered 1-4 in Fig. 14) are contiguous in Ptr and Sn. The

seven genes on the right side (genes 12-18) are also contiguous in all three fungi, although there is one local rearrangement in Sn (inversion of SNOG_8336 and SNOG_8334) (Fig. 14). In both Ptr and Sn, the syntenic regions on the right and on the left are linked on the same supercontig but are not contiguous, i.e., there is a 396-gene gap in Ptr and a 51-gene gap in Sn between the left and right flanks. The closest hits to the depudecin genes themselves are scattered throughout the genomes of Ptr and Sn, representing other members of the same gene families (i.e., other PKSs, MFS transporters, monooxygenases, etc.). There is no evidence for a gene cluster similar to the depudecin cluster in either Sn or Ptr.

These results indicate that the genomes of these three fungi are syntenous in the region surrounding the depudecin cluster, but not the cluster itself. The depudecin cluster thus appears as an indel of ~25 kb in the genomes of *A. brassicicola*, Sn, and Ptr (Fig. 14). These results also support the conclusion drawn from analysis of the *DEP6* mutant that *DEP1* through *DEP6* constitute the depudecin gene cluster (Fig. 13). In this regard, gene 11 is an anomaly; it is not regulated by *DEP6* (Fig. 13), nor is it syntenic with Ptr and Sn (Fig. 14). It is unlikely to be involved in depudecin biosynthesis because it is predicted to encode an α-1,3-mannosyltransferase. This gene is a single copy in Ptr and Sn. One possible explanation for how this situation arose is that the presence of gene 11 is related to the indel event, i.e., it moved by chance with the cluster to its present location in the genome of *A. brassicicola*, either from elsewhere in the genome or from another organism.

Depudecin contributes to virulence on cabbage.

Virulence of the wild type, two *DEP5* (*PKS9*) mutants, an ectopic insertion mutant (which had wild type production of depudecin), and a complemented mutant of *DEP5* were compared on *Brassica oleracea* (green cabbage) leaves (Fig. 15). We observed a small but

statistically significant ($p < 0.01$) reduction of ~10% in lesion size between wild-type and mutant strains (Fig. 15B). Depudecin is thus a minor virulence factor of *A. brassicicola* on cabbage. No statistically significant difference in virulence among the strains could be seen on *Arabidopsis*; however, even the pad3 mutant of *Arabidopsis* is more resistant to *A. brassicicola* than cultivated Brassica species and detection of statistically significant changes in disease development may require much larger experimental sample sizes (Fig. 15C).

DISCUSSION

In this study we have identified a gene cluster involved in depudecin biosynthesis and have constructed multiple depudecin-minus strains of *A. brassicicola*. The depudecin cluster appears to comprise six genes, four of which we have shown by gene disruption to be required for depudecin biosynthesis. We cannot exclude that additional, unclustered genes are also required for depudecin biosynthesis. With the exception of *DEP1*, the genes of the depudecin cluster have plausible roles in depudecin biosynthesis based on our knowledge of similar genes in other secondary metabolite pathways. *DEP6*, a putative transcription factor, is a positive regulator of the other genes of the cluster.

Based on the pathogenicity assays it appears that while depudecin does play a minor role in virulence of *A. brassicicola* on cabbage, it is not a major virulence factor like HC-toxin is for *C. carbonum* on maize. It was not possible to detect a statistically significant effect of depudecin on virulence on *Arabidopsis pad3* plants.

There are several possible reasons why HC-toxin but not depudecin is a strong virulence factor in their respective pathosystems. One possibility is that depudecin is not made in sufficiently high concentrations, or penetrates insufficiently well, to effectively inhibit HDACs

during infection of cabbage or *Arabidopsis*. Compared to HC-toxin, depudecin is a relatively weak HDAC inhibitor; whereas Aeo-containing cyclic peptides such as HC-toxin cause half-maximal inhibition of in vitro HDAC activity at <10 nM, depudecin is active only at ~50 µM (Kwon et al. 1998; Monneret 2005; Deubzer et al. 2008).

Another possible explanation is that HDAC inhibition is an effective virulence strategy against grasses but not against other plants, including *Arabidopsis* (Sindhu et al. 2008). That is, HDACs might be required for defense against pathogens in some plants but not others. Despite many lines of data suggesting that HDACs have at least an indirect role in defense signaling in *Arabidopsis*, none of the evidence is as strong as that from the *C. carbonum*/maize pathosystem (Devoto et al. 2002; Zhou et al. 2005; Walton 2006; Thines et al. 2007; Kim et al. 2008; Dhawan et al. 2009). A possibly relevant consideration in this regard is the apparent specialization of the genera *Alternaria* and *Cochliobolus*. Although both make a number of host-selective toxins, Alternaria tends to pathogenize dicotyledonous plants, whereas *Cochliobolus* is specialized on cereals (Walton 1996). It is possible that defense in cereals differs fundamentally from other plants in the involvement of histone acetylation.

A third possible explanation for our results is that *A. brassicicola* makes additional HDAC inhibitors, which mask the loss of depudecin production. In addition to at least five known cyclic tetrapeptide inhibitors related to HC-toxin, there are a number of other natural products that are HDAC inhibitors (Monneret 2005; Walton 2006). Depudecin itself is known to be made by two fungi other than *A. brassicicola* (Tanaka et al. 2000; Amnuaykanjanasin et al. 2005). If *A. brassicicola* makes additional HDAC inhibitors, the mutant strains generated in this study will be useful for finding them.

ACKNOWLEDGMENTS

We thank Bart Thomma for MUCL 20297, and Dan Jones and Lijun Chen of the MSU Mass Spectrometry Facility for analysis of depudecin. This work was supported by award DE-FG02-91ER20021 from the Chemical Sciences, Geosciences and Biosciences Division, Office of Basic Energy Sciences, Office of Science, U.S. Department of Energy (J.D.W); and by the Virginia Bioinformatics Institute, National Science Foundation award number DBI-0443991, and the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service, grant number #2004-35600-15030 (C.B.L.).

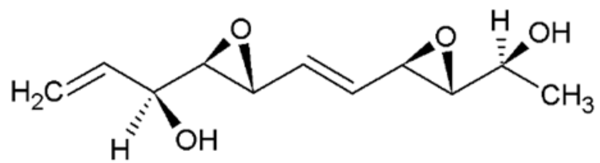

Figure 7. Structure of the polyketide depudecin (Tanaka et al. 2000).

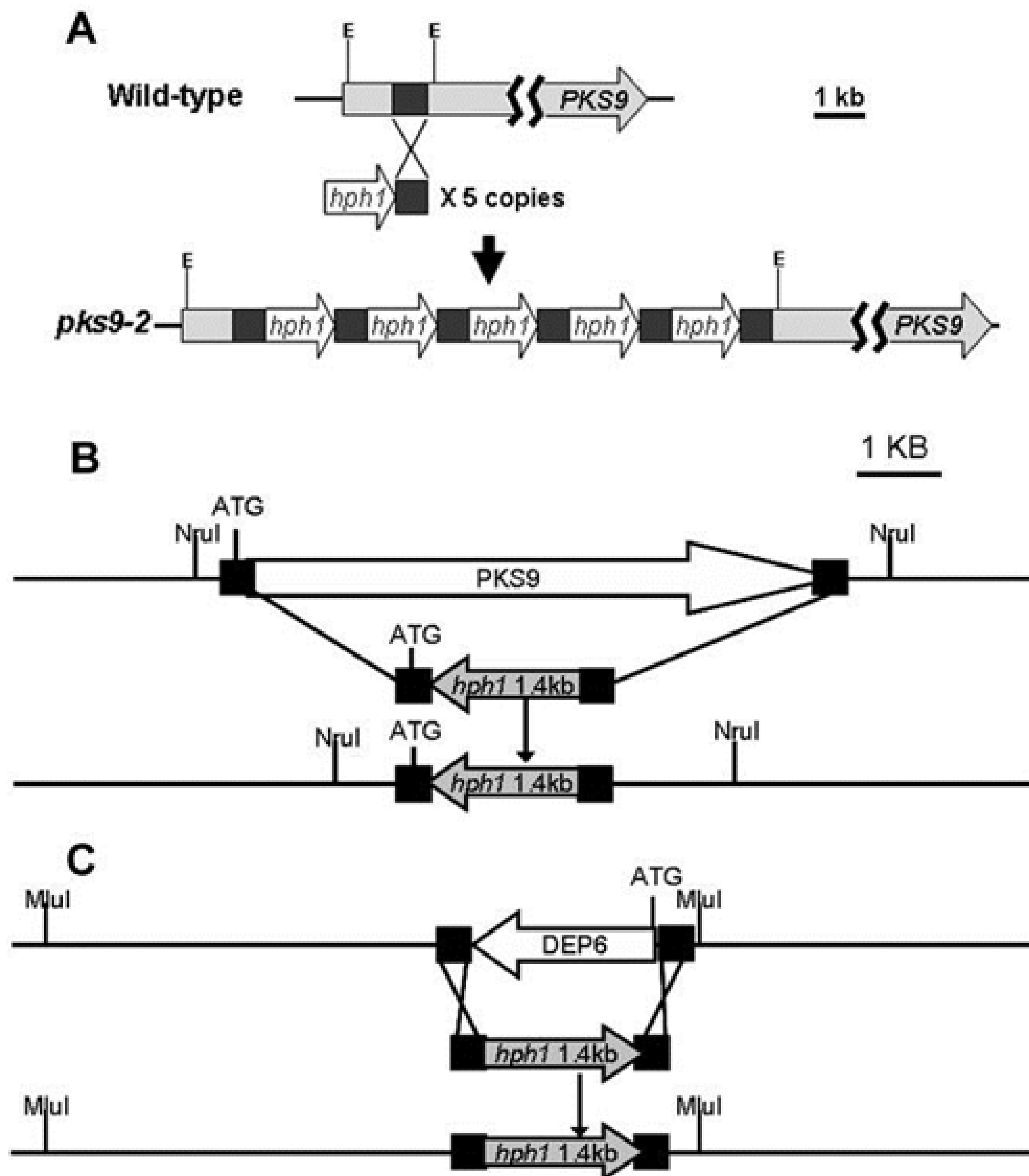

Figure 8. Strategies for disrupting depudecin biosynthetic genes. A, Linear minimal element (LME) strategy and predicted outcome (shown for *PKS9-2* in ATCC 96836). B, Gene replacement strategy and predicted outcome for *DEP5* (*AbPKS9*) in MUCL 20297. C, Gene replacement strategy and predicted outcome for *DEP6*. *hph1* designates the gene encoding hygromycin phosphotransferase, and E designates *Eco*RI sites.

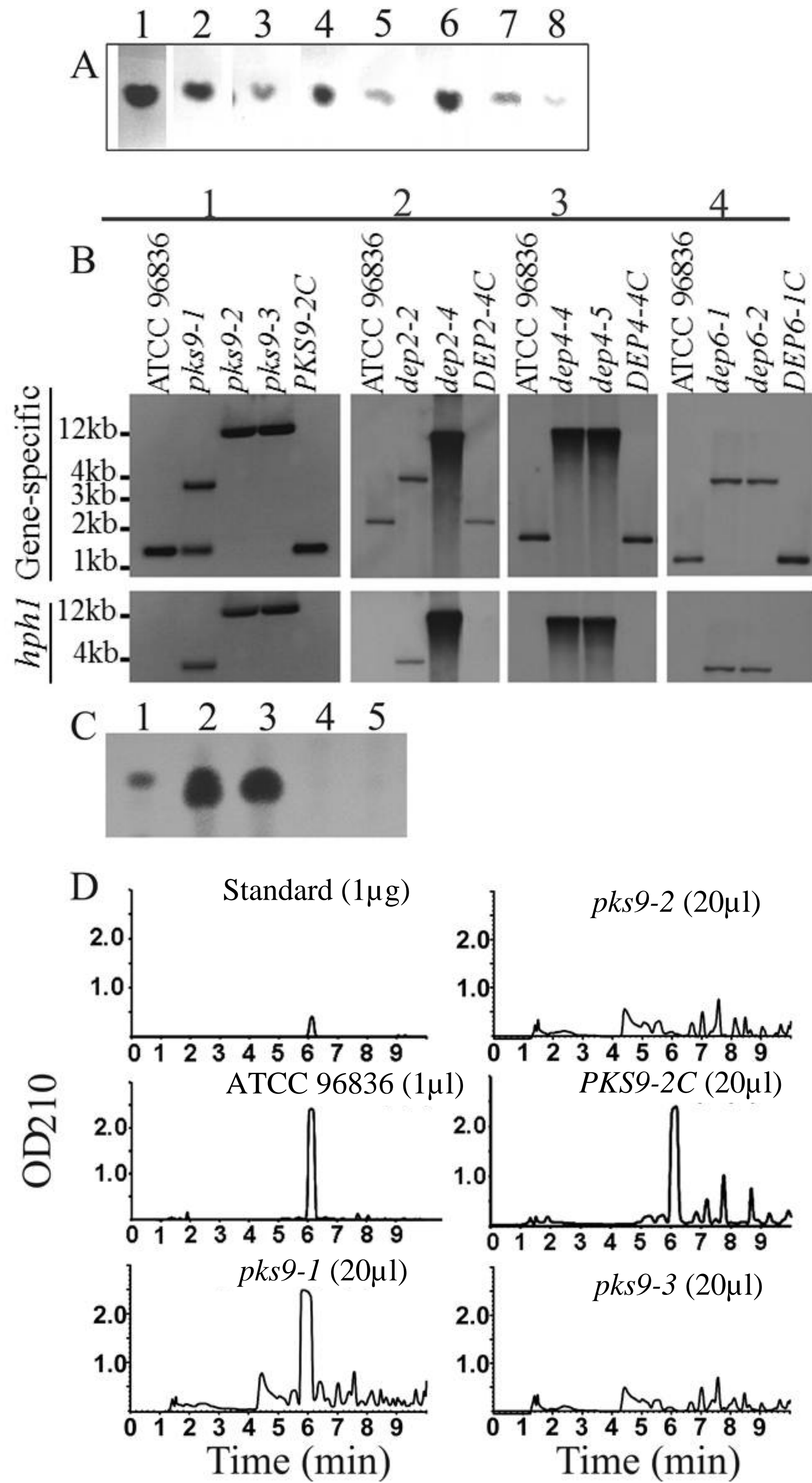

Figure 9. Characterization of depudecin mutants and complemented strains in *A. brassicicola* ATCC 96836.

Figure 9. Cont'd A, Lanes 1-8: TLC analysis of culture filtrates of *Abpks1-Abpks8* mutants. Depudecin was detected with an epoxide-specific reagent. B, DNA blot analysis of transformants. In each pair of panels labeled 1-4, the upper panel was hybridized with a gene-specific probe and the lower panel with a fragment of the *hph1* gene. Panel 1 shows three independent *AbPKS9* transformants (*PKS9-1*, *PKS9-2*, and *PKS9-3*) and a complemented strain (*PKS9-2C*) of mutant *PKS9-2*. DNA was cut with *Eco*RI. Panel 2 shows two independent mutants (*DEP2-2* and *DEP2-4*) and a complemented strain (*DEP2-4C*) of *DEP2*. DNA was cut with *Pst*I. Panel 3 shows two independent mutants (*DEP4-4* and *DEP4-5*) and a complemented strain (*DEP4-4C*) of *DEP4*. DNA was cut with *Bam*HI. Panel 4 shows two independent mutants (*DEP6-1* and *DEP6-2*) and a complemented strain (*DEP6-1C*) of *DEP6*. DNA was cut with *Xho*I and *Bam*HI. C, TLC analysis of ATCC 96836 wild type, *PKS9-1* ectopic mutant, and *PKS9-2* and *PKS9-3* disruption mutants. Lane 1, 5µg depudecin standard; lane 2, 10µl extract of ATCC 96836; lane 3, 10µl *PKS9-1* ectopic extract; lane 4, 10 µl *PKS9-2* mutant; and lane 5, 10 µl *PKS9-3* mutant. D, HPLC analysis of transformants. Note that 20-fold more culture filtrate was injected of the four transformants than the wild type. Depudecin was eluted from the column at ~6 min and was detected at 210 nm. The identities of the compound eluted at 6 min and the compound with the same R_f on TLC as authentic depudecin were confirmed by mass spectrometry (data not shown).

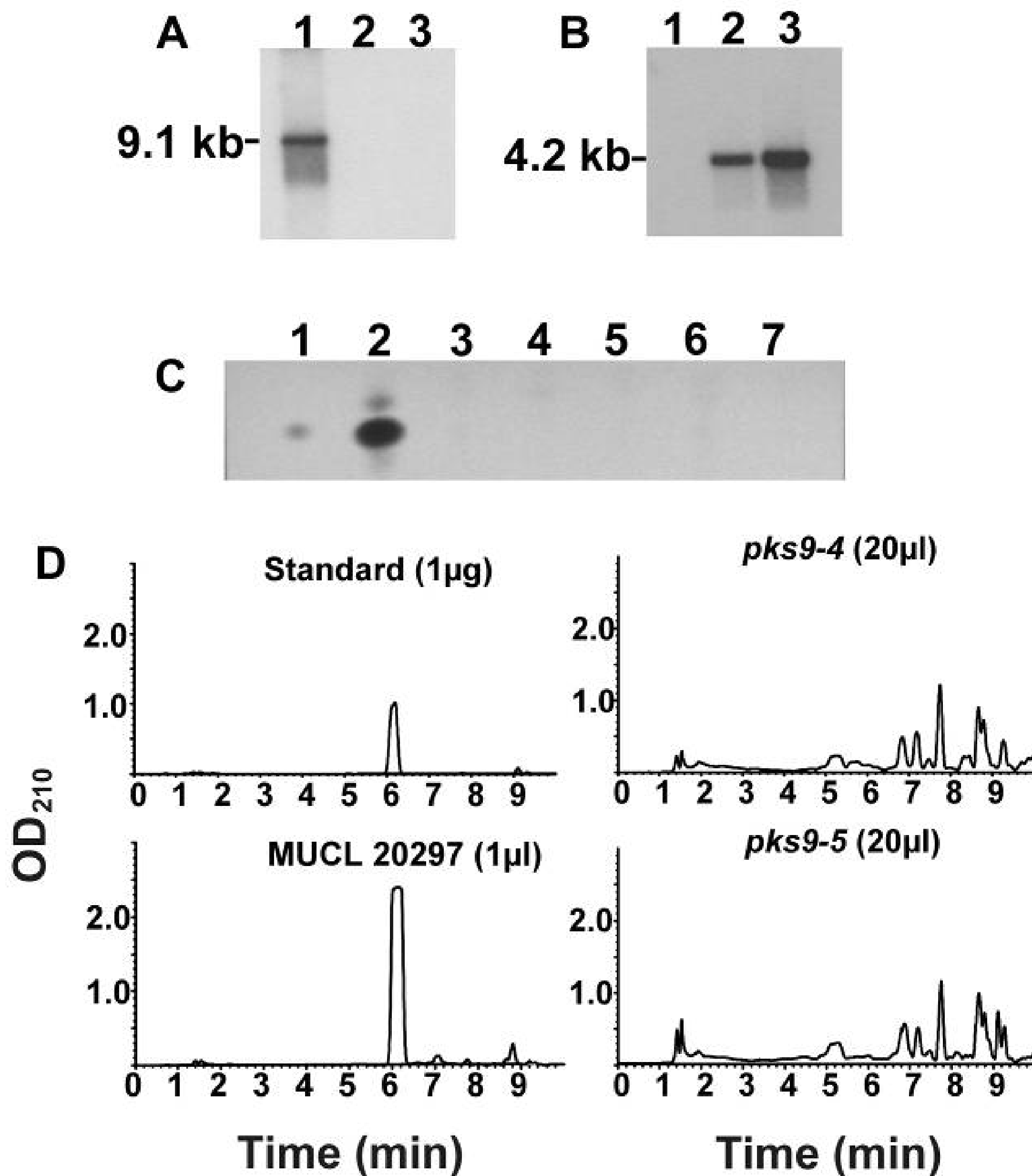

Figure 10. Characterization of depudecin mutants in MUCL 20297. A, DNA blot analysis of wild type (lane 1) and two disruption transformants (lane 2, *PKS9-4*; lane 3, *PKS9-5*) probed with the deleted fragment of *AbPKS9* (*DEP5*) (see Fig. 2). B, DNA blot analysis of the same three strains probed with a fragment of the *hph1* gene. C, TLC analysis of crude extracts of wild type and *PKS9-4* and *PKS9-5* replacement mutants. Lane 1, 5µg depudecin standard; lane 2, 10 µl wild type; lane 3, 10 µl *PKS9-4*; lane 4, 10 µl *PKS9-5*. D, HPLC analysis of wild type and replacement mutants. Note that 20-fold more of the two mutants than the wild type was injected.

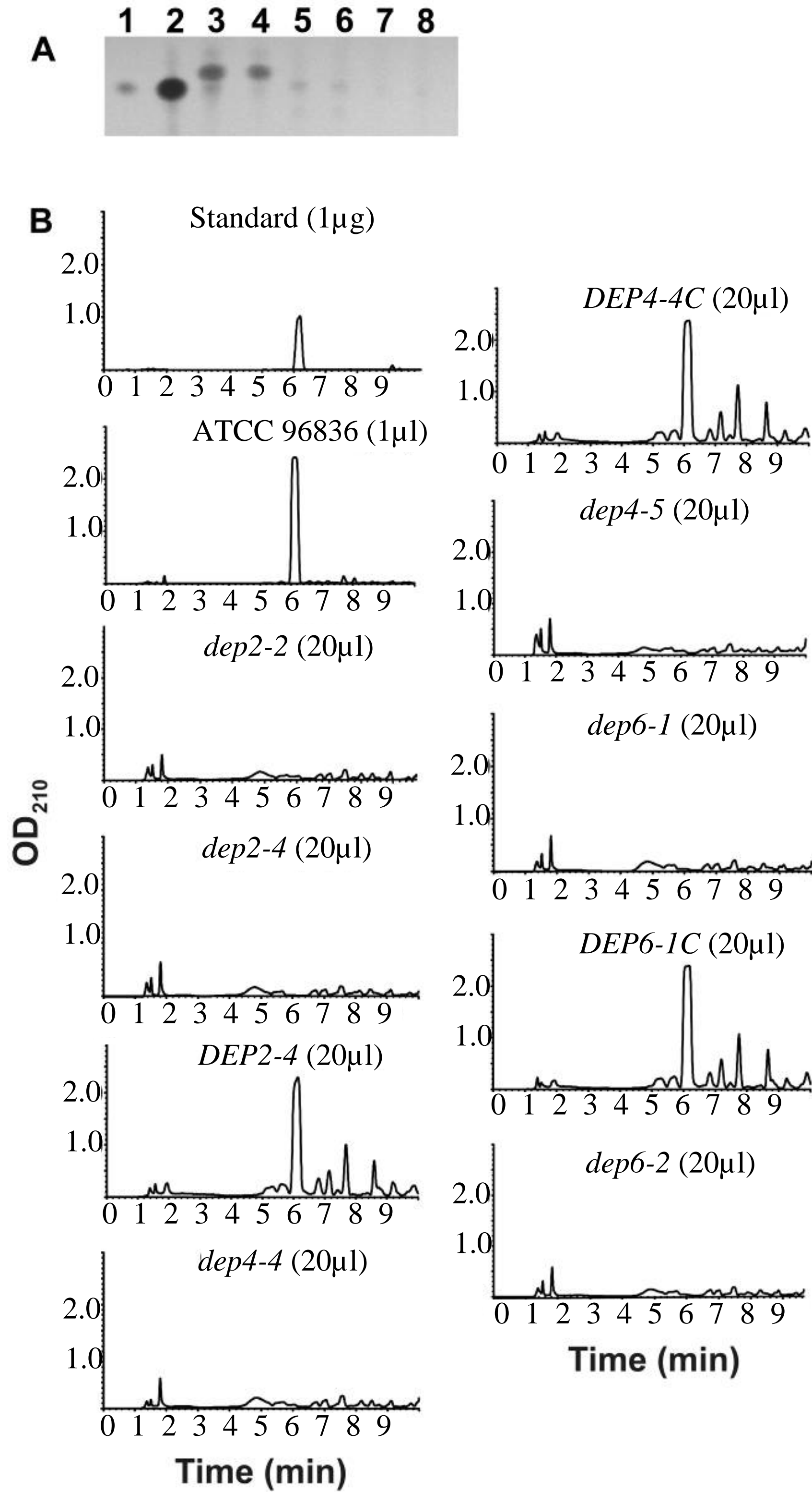

Figure 11. Mutational characterization of genes in the depudecin cluster.

Figure 11 Cont'd, A, TLC analysis of depudecin production. Ten µl crude extract were applied to each lane. Lane 1, depudecin standard; lane 2, wild type ATCC 96836; lane 3, *DEP2-2*; lane 4, *DEP2-4*; lane 5, *DEP4-4*; lane 6, *DEP4-5*; lane 7, *DEP6-1*; lane 8, *dep 6-2*. B, HPLC analysis of wild type, depudecin mutants (*DEP2-2*, *DEP2-4*, *DEP4-4*, *DEP4-5*, *DEP6-1*, and *DEP6-2*), and complemented mutants (*DEP2-4C*, *DEP4-4C*, and *DEP6-1C*). Depudecin is the peak eluted at ~6 min.

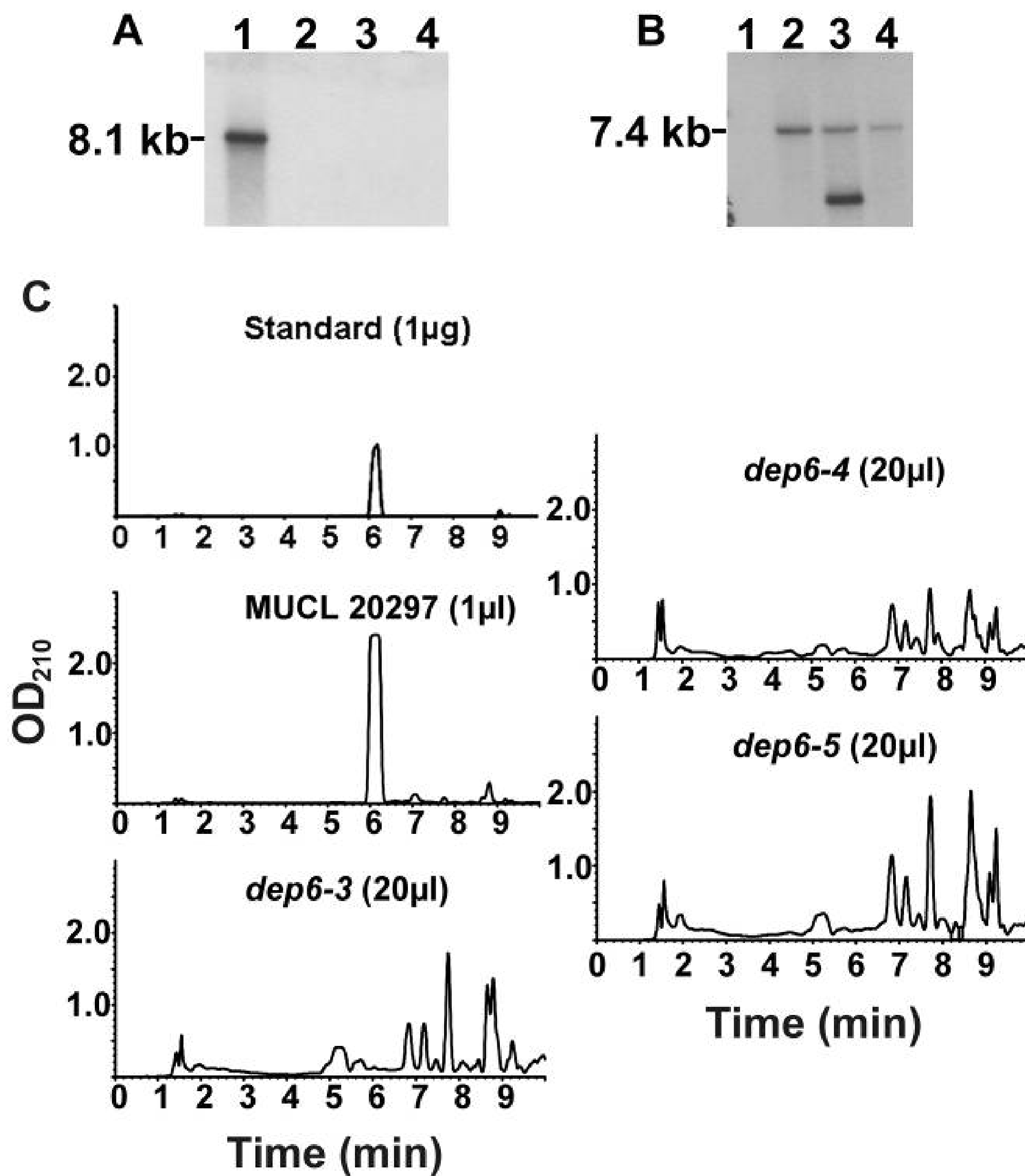

Figure 12. Analysis of *DEP6* mutants in MUCL 20297. A, DNA blot analysis of wild type (lane 1) and three mutants (lane 2, *DEP6-3*; lane 3, *DEP6-4*; lane 4, *DEP6-5*). Blot was probed with a fragment of *DEP6*. B, DNA blot analysis of the same strains except probed with *hph1*. *DEP6-4* shows multiple bands hybridizing to *hph1*, apparently due to a multiple integration event or to an additional ectopic integration. C, HPLC analysis of depudecin production by the wild type and the mutant strains.

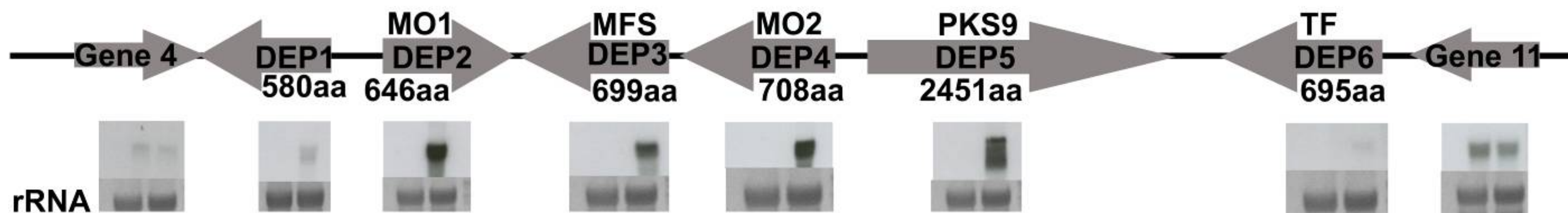

Figure 13. The depudecin gene cluster of *A. brassicicola* ATCC 96836. Arrows indicate the directions of transcription. The sizes of the mature proteins are indicated in amino acids (aa). RNA analysis of each gene in the *dep6* mutant are shown under the map. In each panel, the upper two panels show RNA hybridization to the indicated gene. Wild type is on the right and the *dep6* mutant on the left. The two lower panels show the major ribosomal band stained on the blot with methylene blue; wild type is on the right and the *dep6* mutant on the left. MO1, monooxygenase 1; MFS, major facilitator superfamily transporter; MO2, monooxygenase 2; PKS9, polyketide synthase 9; TF, transcription factor. "Gene 4" and "gene 11" refer to two flanking genes that are not regulated by *dep6* (see Fig. 8). The depudecin gene cluster (*DEP1 - DEP6*) has been submitted to GenBank with accession number FJ977165.

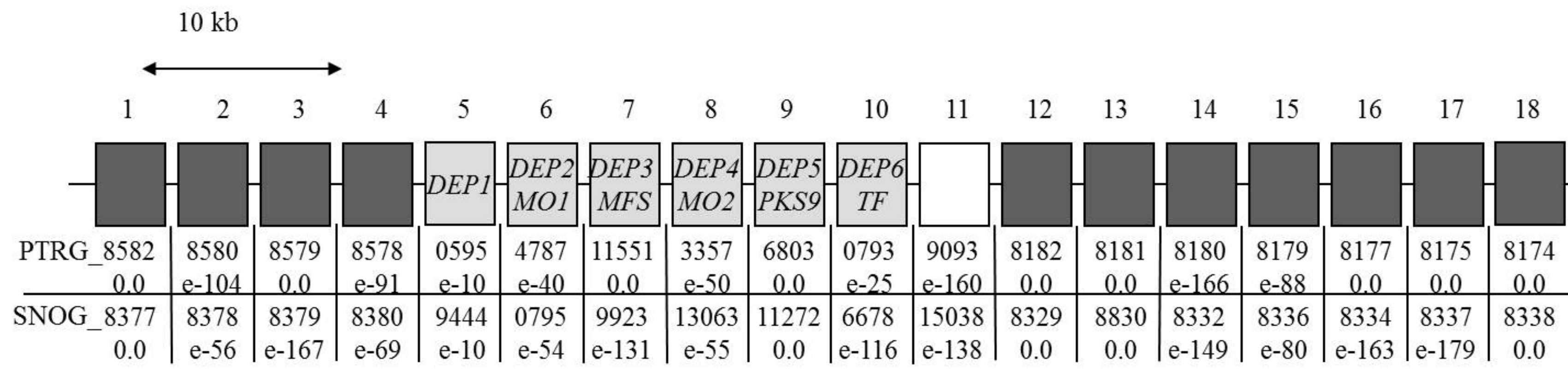

Figure 14. Synteny between A. brassicicola, P. tritici-repentis, and S. nodorum in the depudecin cluster region. The gene numbers indicate the best BLASTP hit of each A. brassicicola protein in the genomes of Ptr (PTRG) or Sn (SNOG). The numbers underneath the gene numbers are the expect scores of the BLASTP hit. Syntenic genes are shown in dark gray, the genes of the depudecin cluster in light gray, and the “anomalous” gene 11 in white.

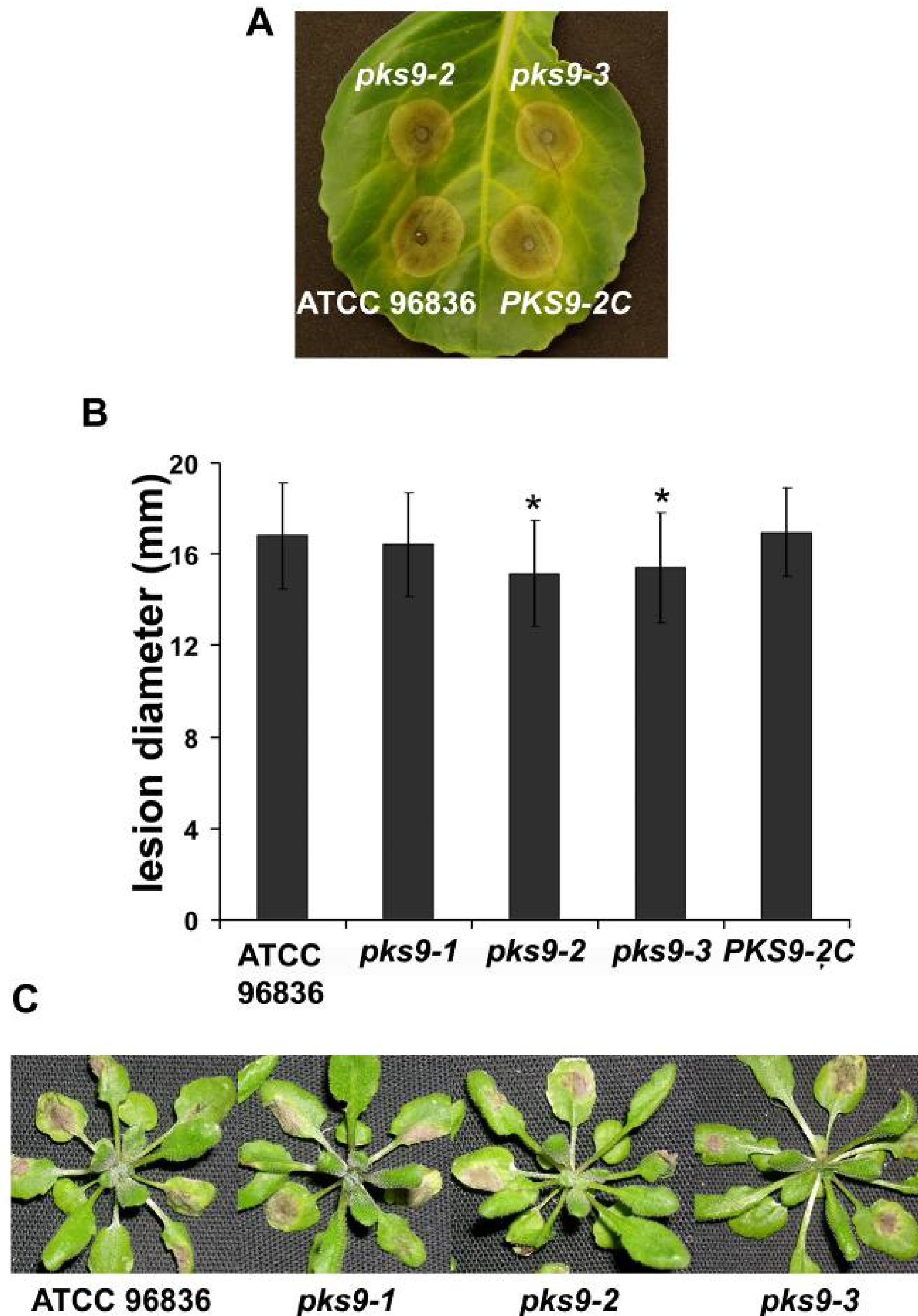

Figure 15. Virulence analysis of *A. brassicicola DEP5* (*AbPKS9*) mutants. A, Conidial suspension (10 µl of 2 x 10^4 conidia/ml) of ATCC 96836 wild type, two mutants (*PKS9-2* and *PKS9-3*), or a complemented strain (*PKS9-2C*) were inoculated on green cabbage leaves. Five days after inoculation, disease severity was calculated based on the lesion diameter. B, Quantitation of virulence. Column heights and error bars represent average and standard deviation, respectively, of four independent experiments, each experiment measuring at least 15 lesions. Stars indicate lesion diameters statistically significant ($p<0.01$) from the others. *PKS9-1* is an ectopic transformant (see Fig. 3). C, Virulence of the ectopic transformant and two mutants on *Arabidopsis pad3* plants.

CHAPTER 3: BIOSYNTHESIS OF HC-TOXIN FROM *ALTERNARIA JESENSKAE*

INTRODUCTION

Fungal secondary metabolites are complex molecules not specifically required for the normal growth and development of an organism; however, they often play critical roles in lifestyle-related processes and to response to environmental cues (e.g., biotic and abiotic stress). Secondary metabolites usually require multiple genes for biosynthesis and regulation, which are frequently clustered together in the genome and are often co-regulated (Keller et al. 1997; Shwab et al. 2008). Clustering of genes has led to speculation that the ability to produce secondary metabolites may be a result of horizontal transfer of gene clusters from one species to another (Walton 2000).

The first reports of horizontal gene transfer (HGT) were in the 1990's in bacteria (Hilario et al. 1993; Boto 2010). Since that time evidence of HGT between prokaryotes, and from prokaryotes to eukaryotes has been reported (Dunning Hotopp 2011). HGT between eukaryotic organisms has been harder to establish. However, with an increase in the number of eukaryotic genomes available and the ease with which large scale phylogenetic analyses can be performed, evidence for HGT between eukaryotes has been on the rise. Some of the first examples of HGT among fungi include *TOXA* between *Stagonospora nodorum* and *Pyrenophora tritici-repentis* (Friesen et al. 2006) and the *ACE1* cluster from *Magnaporthe grisea* to *Aspergillus clavatus* (Khaldi et al. 2008). More recently, evidence was provided for the horizontal transfer of the large sterigmatocystin biosynthetic gene cluster (23 genes in a 54KB region) from *Aspergillus nidulans* to *Podospora anserina* (Slot et al. 2011).

The plant pathogenic fungus *Cochliobolus carbonum* causes northern corn leaf spot on maize (Ullstrup 1941; Scheffer 1965; Walton 2006). While there are several races of *C. carbonum*, only race 1 produces HC-toxin, a critical virulence factor (Walton 1996). HC-toxin is

a cyclic tetrapeptide that is an inhibitor of the RPD3 class of histone deacetylases (Walton et al. 1982; Kawai et al. 1983; Brosch et al. 1995; Brosch et al. 2001; Baidyaroy et al. 2002; Walton 2006). Like many secondary metabolites, the genes involved in HC-toxin biosynthesis are clustered together in the TOX2 locus (Ahn et al. 1996; Ahn et al. 2002). There are at least seven genes involved in HC-toxin biosynthesis, and each gene has multiple, nearly identical, copies. (Walton 2006).

TOXA is a member of the Major Facilitator Superfamily of transporters believed to be involved in transporting HC-toxin out of the fungus. *HTS1* is a 5233-amino acid non-ribosomal peptide synthetase encoded by a 15.7-kb open reading frame, and it contains four core adenylation domains (Panaccione et al. 1992; Scott-Craig et al. 1992). *TOXC* is likely involved in the biosynthesis of the decanoic acid backbone of 2-amino-9,10-epoxi-8-oxodecanoic acid (Aeo) (Ahn et al. 1997). *TOXD* encodes a putative dehydrogenase, which may be involved in oxidation/reduction reactions, but the specific role in HC-toxin biosynthesis has not been identified (Pedley and Walton, 2001). *TOXE* is a fungal specific transcription factor that contains four ankyrin repeat domains at the C-terminus and a b-zip DNA binding motif at the N-terminus (Pedley and Walton, 2001). *TOXF* encodes a putative branched-chain amino acid aminotransferase. It is required for HC-toxin biosynthesis but its exact function is not known (Cheng et al. 1999). *TOXG* encodes an alanine racemase. It produces the D-Ala that is incorporated into HC-toxin by HTS1 (Cheng et al. 2000).

Natural toxin nonproducing strains of *C. carbonum* completely lack all of the genes of the cluster (Baidyaroy et al. 2002). How did *C. carbonum* acquire the ability to synthesize HC-toxin? Or did other strains of *Cocholiobolus* lose the genes? Based on increasing reports of HGT of gene clusters, the TOX2 cluster in *C. carbonum* may be the result of HGT (Walton 2000).

However, the absence of any other known HC-toxin producing microorganism has prevented testing of this hypothesis.

Alternaria jesenskae is a newly described species of *Alternaria* (Labuda et al. 2008). It is a large-spored filamentous fungus morphologically similar to *A. tomatophila* and phylogenetically related to *A. multirostrata* (Labuda et al. 2008). It was isolated from seeds of *Fumana procumbens*, a shrubby perennial with a wide geographic distribution (Labuda et al. 2008). *A. jesenskae* was suggested to produce HC-toxin (Roman Labuda, personal communication) making it the second organism after *C. carbonum* known to do so. The goal of this study was to determine if *A. jesenskae* contains a gene cluster similar to TOX2 of *C. carbonum*, and if so, to determine the evolutionary relationship between the two clusters and the fungi themselves.

MATERIALS AND METHODS

Fungal growth and HC-toxin analysis.

Cochliobolus carbonum strains tox+ and tox- (strains 367-2A and 164R1 respectively) were maintained on potato dextrose agar (PDA) in 100 mm petri dishes, 26°C for 7-10 d under 32 watt fluorescent light (Philips 432T8/TL741 Universal/ Hi-Vision Hg). *C. carbonum* spores were collected from PDA culture plates in 2 ml 0.1% Tween-20. *Alternaria jesenskae* was obtained from Dr. Emory Simmons (Wabash College, Crawfordsville, Indiana). The fungus was grown and maintained on V8-juice agar petri dishes at 4°C. Spore suspensions were stored frozen in 25% glycerol at -80 °C. Sporulation was obtained by growing unsealed agar dishes at 26°C approximately10 cm from a 32 watt fluorescent light (Philips 432T8/TL741 Universal/ Hi-

Vision Hg). *A. jesenskae* spores were collected from V8 culture plates in 2 ml 0.1% Tween-20. For HC-toxin production, the fungus was grown in stationary culture in 1-liter flasks containing 125 ml of potato dextrose broth (PDB; Difco) for 7 to 10 d at 26°C in ambient light. At harvest, the culture fluid was filtered through Whatman #1 paper and then extracted twice with equal volumes of dichloromethane. The dichloromethane fractions were evaporated under vacuum at 40°C and the residues were resuspended in 3 ml methanol. After concentration of the 3µl of methanol under vacuum; the residue was dissolved in 10-50 µl of water. This crude extract was used for analysis by thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and massspectrometry. HC-toxin from *Cochliobolus carbonum* was used as a standard.

For TLC, concentrated crude extract in water (10 µl) was spotted onto 250-µm silica plates with an adsorbent strip (Whatman). Plates were developed in 1:1 acetone/dichloromethane. HC-toxin was detected using the epoxide-specific reagents 4-(p-nitrobenzyl)-pyridine and tetraethylenepentamine (Hammock et al. 1974).

For HPLC, 20 µl of extract was combined with 60 µl of acetonitrile and 20 µl of sterile distilled water. The sample was injected onto a C18 reverse phase column (Agilent Eclipse XDB-C18 silica, 5 µm, 4.6 × 150 mm; Agilent, Santa Clara, CA) and was eluted with a linear gradient of 10% (vol/vol) acetonitrile in water to 100% acetonitrile in 30 min at a flow rate of 1 ml/min. The eluant was monitored at 210 and 230 nm. HC-toxin eluted from the column at 9 min. HC-toxin standard and *A. jesenskae* fractions eluting at 8 and 9 min, were collected and analyzed by the MSU Mass Spectrometry Facility by Electrospray Ionization.

DNA extraction and analysis.

DNA was extracted from lyophilized mycelial mats of 5- to 7-d-old *A. jesenskae* grown in PDB in stationary culture using the Gentra Puregene Tissue DNA extraction kit (Qiagen,

Valencia, CA) using the mouse tail protocol (Pitkin et al. 1996) with the following modifications: Approximately 5 mg Lyophilized tissue was ground to a fine powder under liquid nitrogen in a small mortar and pestle and transferred to 1.5 ml eppendorf tube. 150 ul of Cell Lysis Solution was added and tissue was resuspended by vortexing. One ul of Proteinase K solution was added and lysate was incubated at 50°C overnight. 1 ul of RNase A solution was added and incubated at 37°C for 45 minutes. Sample was cooled on ice for one minute. 50 ul of Protein Precipitation Solution was added, vortexed for 30 seconds at 12K rpm and incubated on ice for 10 minutes. Sample was centrifuged at room temperature in microfuge at full speed for 3 minutes. Supernatant was decanted into a new microfuge tube and 150 ul of isopropanol (2-propanol) was added. Sample was mixed by inversion approximately 50 times and centrifuged at full speed in microfuge for 10 minutes. Pour off supernatant and wash pellet by inversion with 150 ul of 70% ethanol. Spin again in microfuge for 10 minutes. Pour off ethanol, invert tube over paper towel and air dry for 5 minutes. Pellet was resuspended in 50 ul of DNA Hydration Solution at room temperature overnight.

For Southern analysis, purified DNA was digested with restriction endonucleases selected specifically to evaluate each gene copy based on genomic sequence (Table 1). For DNA electrophoresis, 1-2 μg of DNA was loaded per lane. DNA blotting was done using internal gene-specific probes generated based on assembled gene sequence (Appendix I, Table 7.2). DNA was transferred to Nytran SPC (Whatman, Maidstone, England) and was hybridized with ^{32}P-labeled DNA probes (Appendix I, Table 7.2)(Pitkin et al. 1996).

RNA was extracted as described (Hallen et al. 2007). RT-PCR followed by 5′ and 3′ RACE was performed with the SMART RACE cDNA amplification kit (Clontech, Mountain View, CA.). Overlapping gene-specific primers were designed from available genomic sequence

(Appendix I, Table 7.2). In most cases, several gene-specific primers were used. PCR products were sequenced directly or cloned into pGem T-easy (Promega); transformed into *E. coli* DH5α (Invitrogen) and sequenced using M13 forward and reverse primers.

Virulence assays.

Seeds of maize were germinated in soil in greenhouse in ambient light. Six- week old maize plants (genotype *hm1*/*hm1* [susceptible to HC-toxin] and *Hm1*/*Hm1* [resistant to HC-toxin]) were spray-inoculated with ~ $1x10^5$ spores/ml of *C. carbonum* tox+, *C. carbonum* tox-, or *A. jesenskae*. Plants were covered with plastic bags overnight to maintain humidity. Observations of disease progression were made beginning 3 d post inoculation. Photographs were taken 4 d post inoculation.

Brassica oleracea (Capitata group) seeds were germinated in clear scintillation vials on Whatman filter paper or in trays on moist paper towels for three to four days. Germinated seeds were transferred to soil and plants were grown in seed trays in a growth chamber at 20°C, 70% relative humidity, and a 12-hr light and dark cycle. Leaves from 4-week-old plants were spot-inoculated with 10 µl of ~ $1x10^5$ spores/ml inoculum. Plants were covered overnight with clear tray covers to maintain humidity. Plants were observed for signs of infection beginning 4 d after inoculation. Photographs were taken after 4 days.

Arabidopsis thaliana plants (Col-0, *pad3* and a DELLA quad mutant [Dr. Fumiaki Katagiri, University of Minnesota] were grown in a growth chamber at 20°C, 70% relative humidity, and a 12-hr light and dark cycle. The third through the seventh true leaves from 4-week-old plants were spot-inoculated with 10 µl of spores at a concentration of ~ $1x10^5$ spores/ml. Plants were covered with clear tray covers to overnight to maintain humidity. Plants were observed for signs of infection 4 d after inoculation. Photographs were taken after 4 days.

Seeds of *Fumana procumbens,* a perennial shrubby plant in the family Cistaceae (Hardyplants, MN, USA), were scarified with a razor blade and germinated in clear scintillation vials on Whatman filter paper. Seven to ten day old seedlings were transferred to soil and grown at room temperature under a 32 watt fluorescent light (Philips 432T8/TL741 Universal/ Hi-Vision Hg) fluorescent light. *A. jesenskae* spores were collected from V8 culture plates in water. Conidial suspensions (10 µl of ~ $1x10^5$ spores/ml) were applied as a drop on the surface of leaves of 5-6 month old plants. Plants were covered with a standard clear dome lid and kept at 100% relative humidity for 48 hours. Observations were made beginning 3 d after inoculation. Photographs were taken on day 5.

Bioinformatic Analysis.

454-pyrosequencing (Roche Applied Science) of *A. jesenskae* was performed at the MSU Research Technology Support Facility (RTSF) facility. A single large format (70×75) sequencing run was completed. BLASTN and TBLASTN analysis with the genes of the *C. carbonum* TOX2 cluster (hereafter referred to as ccTOX2)(Table 2). against the *A. jesenskae* genome were performed using stand-alone BLAST version 2.2.15 [Oct-15-2006] downloaded from NCBI Default parameters were used. Alignments and manual annotation of genes and proteins were achieved using DNASTAR Lasergene software (SeqBuilder, MegAlign, and SeqMan) versions 7 and 8 (DNASTAR, Inc) and ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). SPIDEY was used for alignments of cDNA to genome sequences (NCBI). Predicted protein sequences were assembled manually in addition to computational prediction using DNASTAR Lasergene software (SeqMan and MegAlign) versions 7 and 8 (DNASTAR, INC.) and FGENESH (www.softberry.com) with *Alternaria* as the

training reference. BLASTP of the final assembled protein sequences was executed using the NCBI nonredundant database.

For NRPS tree, a range of NRPS adenylation domains were selected from the NCBI protein database. Additionally, BLASTP of the *C. carbonum* and *A. jesenskae* adenylation domains performed against the *C. heterostrophus* and *A. brassicicola* genomes (respectively) using the JGI Genome Portal. NRPS adenylation domain sequences from both NCBI and JGI were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). All protein sequences were obtained from Genbank at NCBI (Table 3). *C. carbonum* protein sequences were used as BLASTP queries against the *A. jesenskae* genome survey sequence to obtain orthologs. *Neurospora crassa*, *Cochliobolus heterostrophus*, and *Alternaria brassicicola* proteins were obtained by BLASTP with predicted *A. jesenskae* proteins and *C. carbonum* proteins against their respective predicted transcript databases. Proteins were aligned with ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

RESULTS

Alternaria jesenskae produces HC-toxin

TLC analysis confirmed the presence of an epoxide-containing compound in *Alternaria jesenskae* culture filtrates at the same R_f as HC-toxin (Fig. 16A). An additional spot at a different R_f was also detected, but remains unidentified (Fig. 16A). Dichloromethane extracts, analyzed by reverse-phase HPLC (Fig. 16B), and fractions analyzed by mass spectrometry (Fig. 16C), indicate that *A. jesenskae* synthesizes HC-toxin, as reported (Roman Labuda, personal communication).

***Alternaria jesenskae* HC-toxin gene cluster**

Pyro-sequencing resulted in 483,907,758 bases of sequence from the genome of *A. jesenskae*. An assembly of 34,399,400 bases resulted from Newbler software analysis of the sequences. The final coverage was estimated to be ~ 10x based on the known genome sizes of other sequenced ascomycetes. BLASTN and TBLASTN analysis of the assembled genome indicated that high-scoring orthologs of each of the known seven TOX2 genes from *C. carbonum* were present in *A. jesenskae* (Table 4). These will be hereafter collectively referred to as ajTOX2 genes. Southern analysis confirmed the presence of all seven genes in *A. jesenskae* (Fig. 17).

The genes of ajTOX2:

Both BLASTN and TBLASTN identified at least two copies of *TOXA (ajTOXA-1 and ajTOXA-2)* (Table 4) with strong homology to *C. carbonum TOXA* (*ccTOXA)*. The two copies of aj*TOXA* are 95% (nt) and 94% (amino acid) identical to each other within *A. jesenskae* and 81.5% (nt) and 79% (amino acid) between *C. carbonum* and *A. jesenskae* (Table 4). The manually assembled predicted intron/exon structure of *ajTOXA* is the same as *ccTOXA* with both *ccTOXA* and *ajTOXA* having four exons (Fig. 18).

Similar to *TOXA*, BLASTN and TBLASTN results indicated that there are at least two copies of *HTS1* in *A. jesenskae* (Table 4). The two copies of *HTS1* in *A. jesenskae* are 99% identical (nt) and 98% (amino acid) and between *A. jesenskae* and *C. carbonum* they are 84% (nt) and 82% (amino acid) identical (Table 4). The core four adenylation domains are 84% (amino acid) identical to each other between organisms. *HTS1* has no introns in either organism (Fig. 18). Phylogenetic analysis was done using the adenylation domains from several NRPS

proteins. The results indicate that *HTS1* from *C. carbonum* and *A. jesenskae* are most closely related to each other (Fig. 20).

While there are three copies of *ccTOXC* in *C. carbonum* both BLASTN and TBLASTN identified a single copy of *TOXC* in *A. jesenskae* (*ajTOXA)*(Table 4). Although only one copy of *TOXC* was identified in *A. jesenskae*, we cannot exclude that two identical copies exist in the genome that were artifactually merged during genome assembly. *AjTOXC* is 83% (nt) and 78% (amino acid) identical to *ccTOXC* (Table 4). *ajTOXC* has two predicted exons of 5.163 kb and 1.062 kb and a single 57-bp intron (Fig. 18). *CcTOXC* has a single 53 bp intron and its exons are 5.165 kb and 1.078 kb in length (Ahn et al. 1997) (Fig. 18). To demonstrate that this cluster is responsible for HC-toxin biosynthesis in *A. jesenskae*, a gene replacement mutant is currently being constructed for *ajTOXC* but has been unsuccessful to date (See Appendix II for details).

BLASTN and TBLASTN results indicated at least two copies each of *TOXD, TOXF* and *TOXG* clustered together on two distinct contigs in *A. jesenskae* (Table 4, Fig. 18 and 19). Blast scores for all three genes averaged 81-86% (nt) and 81-85% (amino acid) identity (Table 4). Gene structures and organization were experimentally verified by sequencing 5' and 3' RACE products. The intron/exon structures of all *aj*TOX genes are identical to *C. carbonum* (Fig. 18).

Two copies of *TOXE* were identified in *A. jesenskae* the two copies averaged 73% (nt) and 61% (amino acid) between organisms (Table 4). This degree of conservation between *ccTOXE* and *ajTOXE* is much lower than for any of the other TOX2 genes. The bZIP domain and the ankyrin repeats are more highly conserved than the intervening sequence (Fig. 21). Within *A. jesenskae* the two copies of *ajTOXE* are 85% (nt) and 76% (amino acid) identical (Table 4).

In *C. carbonum*, *TOXE* binds to promoters of the TOX2 genes containing the TOX BOX consensus (ATCTCNCGNA) (Pedley et al. 2001). A scan of the contigs containing the *ajTOX* genes indicates the presence of six TOX BOX motifs (Table 5). However, their location in relation to the genes themselves is unclear at this time, because the organization of the TOX2 locus in *A. jesenskae* has not been determined.

Organization of genes within the cluster

TOXA is clustered tightly with *HTS1*. The ATG start sites of the two genes are 681 bases apart in *A. jesenskae*, compared to 695 bases in *C. carbonum* (Fig. 19) (Pitkin et al. 1996). The two genes are transcribed from opposite strands. The nucleotide sequences of the four introns are 64% identical between the two species indicating some divergence. While *TOXA* and *HTS1* are tightly clustered in *A. jesenskae* and *C. carbonum* the order of the *TOXD/TOXF/TOXG* genes are not the same. In *C. carbonum*, *TOXF* and *TOXG* are clustered within ~300 bases, while at least 20 kb separates *TOXD* from *TOXF* and *TOXG* (Cheng et al. 1999; Cheng et al. 2000) (Fig. 19). In *A. jesenskae*, *TOXD*, *TOXF* and *TOXG* are within 300 bases of each other (Fig. 19).

Virulence assays

While there is no evidence to suggest that *A. jesenskae* is a pathogenic fungus, to test a possible biological role for HC-toxin in the interaction between *A. jesenskae* and plants, virulence screens were done with *Arabidopsis,* cabbage, *maize* and *Fumana procumbens*. *Arabidopsis* and cabbage were selected because of their known susceptibility to other species of *Alternaria*, namely *A. brassicicola* and *A. brassicae*. In neither case were visible symptoms observed on inoculated plants compared to control plants (Fig. 22 A and B). These results suggest that *A. jesenskae* is not a pathogen of tested *Brassicaceae* species.

Since HC-toxin is a determinate of virulence in host pathogen interactions for *C. carbonum*, *A. jesenskae* was also tested on maize plants. Lines known to be susceptible and resistant to HC-toxin-producing isolates of *C. carbonum* were assayed. Plants were observed for two weeks. While susceptible lines inoculated with toxin producing *C. carbonum* resulted in plant death, *A. jesenskae* did not produce any visible disease symptoms (Figure 22C). While microscopic analysis indicates that *A. jesenskae* is able to penetrate the leaf surface these results suggest that it is not a pathogen of maize lines tested (data not shown).

A. jesenskae was originally isolated from seeds of *Fumana procumbens* (Labuda et al. 2008). Six month old *F. procumbens* seedlings were tested for susceptibility to *A. jesenskae*. Saprophytic growth was observed by day four and death (not shown) resulted by week 2, whereas control plants were unaffected by the same conditions (Figure 22D). The experiment was repeated and although some minor symptoms (i.e., chlorosis and necrosis) at inoculation sites were observed, the symptoms did not progress through the plant and plant death did not result. Therefore, we conclude that *Alternaria jesenskae* is not a pathogen of *F. procumbens*.

Phylogenetic analysis.

To more fully understand the evolutionary relationship between *C. carbonum* and *A. jesenskae* and of their respective TOX2 genes, alignments were performed with five highly conserved (housekeeping) proteins (Table 6). The percent identity of all the proteins range from 76% to 96% (Average 84.2%, standard deviation 8.49)(Table 6).

DISCUSSION

We have shown that *A. jesenskae* produces HC-toxin, making this the first organism other than *C. carbonum* known to do so. Additionally, we have shown that *A. jesenskae* has orthologs of all of the known TOX genes of *C. carbonum* suggesting that the same biosynthetic pathway is used between these distantly related organisms to produce the HC-toxin trait. Is the HC-toxin trait a result of convergent evolution, descent from a common ancestor or horizontal gene transfer?

There are three hypotheses for evolution of a specific trait: convergent evolution, vertical transmission, and horizontal transmission. Convergent evolution is when two unrelated organisms independently evolve a trait with the same or similar biological function. *Alternaria jesenskae* and *Cochliobolus carbonum* are both in the family Pleosporaceae and therefore not unrelated organisms. Additionally, the biosynthetic pathway between two unrelated organisms would have to be independent of each other. The presence of orthologous TOX genes between *A. jesenskae* and *C. carbonum* and the high degree of similarity suggest that this is not the case, making convergent evolution of the HC-toxin trait unlikely.

Descent from a common ancestor is defined as evolution from a common ancestor by vertical transmission with all taxonomic branches sharing similar genes for the trait. The taxonomic relationship between *A. jesenskae* and *C. carbonum,* confirmed by the high degree of similarity of the housekeeping proteins analyzed, indicates that descent from a common ancestor is a possibility. However, for the HC-toxin trait to be a result of descent from a common ancestor, it would have had to be lost from all other *Cochliobolus* and *Alternaria species*.

Another factor in support of descent from a common ancestor is the presence of a similar cluster found in another related fungus, *Fusarium semitectum*. Apicidin is a secondary metabolite

produced by *Fusarium semitectum* (Park et al. 1999). Like HC-toxin, apicidin is a cyclic tetrapeptide that inhibits histone deacetylases (Darkin-Rattray et al. 1996; Park et al. 1999). The structure of apicidin is cyclo(N-O-methyl-L-Trp-L-Ile-D-Pip-L-amino-8-oxodecanoyl), which is very similar to HC-toxin in that it contains four amino acids, one of which is Pro or its chemical homolog Pip, and an aminodecanoic acid. The biosynthetic pathway of apicidin includes *APS1*, a non-ribosomal peptide synthetase (NRPS); *APS2*, a transcription factor; *APS4*, an aminotransferase; and *APS11*, a putative efflux carrier (Jin et al. 2010). All of these are orthologous to genes in the TOX2 locus of *C. carbonum* (Jin et al. 2010). The APS genes are tightly clustered in *F. semitectum* and each is present in a single copy. In this regard, the APS cluster is more typical of fungal gene clusters unlike the HC-toxin clusters of *A. jesenskae* and *C. carbonum*. In BLAST searches, the TOX2 genes and the orthologous genes in the apicidin cluster are often their best matches. Of particular interest is the presence of an ortholog of *TOXE* in the apicidin gene cluster, because this type of transcription factor is rare. In both organisms, the central NRPS (*HTS1* or *APS1*) is clustered with the transporter (*TOXA* or *APS11*) and divergently transcribed. The start site of *APS1* is approximately 800 bases from *APS11,* similar to what we see in the ccTOX2 locus. The two NRPS genes are 41% identical overall and *APS11* and *TOXA* are 46% identical. While this suggests that there is likely an ancestral evolutionary relationship, possibly from a common ancestor, between the genes for HC-toxin and those for apicidin, they are less related to each other than the two HC-toxin gene clusters in *C. carbonum* and *A. jesenskae*.

Horizontal gene transfer (HGT) is the transfer of genetic material from one organism to another across taxonomic boundaries. Frequently in the case of HGT, trait genes are absent in closely related species but present in more distantly related species. In the case of the HC-toxin

trait, out of the literally hundreds of known *Cochliobolus* or *Alternaria* species, none are known to have the TOX genes, making the production of these toxins specific to *A. jesenskae* and *C. carbonum*. Furthermore it could also be expected that in the event of horizontal transmission of a trait certain isolates of a species would lack the TOX gene(s). While there are several identified races of *C. carbonum* only Race 1 has the TOX genes while non-toxin producing races do not have any of the TOX genes present. This suggests that the TOX2 locus has either been lost from other races or was horizontally transferred to Race 1 from a different organism. Since *A. jesenskae* was only characterized in 2008 it is not known if other isolates exist making detailed comparative analysis of the presence of the TOX genes in other isolates impossible at this time.

Another factor supporting HGT of a trait is that genes involved frequently have similar intron/exon structures and that the arrangement or order of those genes within cluster could be similar in both organisms. All of the orthologous *C. carbonum* and *A. jesenskae* TOX genes have similar intron/exon numbers and sizes. We have also shown that *TOXA* and *HTS1* are tightly associated with each other and divergently transcribed in both organisms. While the orientation of *TOXD, TOXF,* and *TOXG* in both clusters appears to be the same between both organisms, they have a different arrangement in *A. jesenskae* and *C. carbonum.* However, divergence in specific gene order has been reported in other cases of HGT and does not refute the hypothesis of HGT (Slot et al. 2007; Slot et al. 2011).

The presence of *TOXE* is noteworthy as it is an unusual transcription factor that has only been identified in three filamentous fungi. TBLASTN of *ajTOXE* against the NCBI non-redundant database indicates that the strongest hits are three transcription factors. These are *TOXE* from *Cochliobolus carbonum* (Score 538, e-value of 0.0, 64% percent identity) required for expression of the TOX2 genes, *APS2* from *Fusarium semitectum* (score 178, e-value of 1e-

28, 44% percent identity) required for expression of the apicidin biosynthetic genes (Jin et al. 2010), and a hypothetical protein from *Pyrenophora tritici-repentis* Pt-1C-BFP (score 149, e-value 2e-17,33% percent identity). The rarity of the *TOXE* class of transcription factors and the high degree of homology between *A. jesenskae* and *C. carbonum* could support either the hypothesis that the two organisms either obtained the transcription factor by horizontal transfer or from a common ancestor.

Housekeeping protein analysis indicates that the housekeeping proteins share a similar percent identity to the *TOX* genes themselves (84%). This is unexpected if the trait were a result of horizontal gene transfer. However, these two fungi are both in the same family (Pleosporaceae) and a high degree of homology is to be expected. Without a deeper examination of the genomes the current analysis is inconclusive and could support either HGT or descent from a common ancestor.

While there is no evidence of the mechanism that drives HGT, or that movement favors particular genes or clusters, maintenance of the transferred cluster in the recipient is believed to occur only if the product confers a selective advantage (Lawrence et al. 1996; Lawrence 1997; Lawrence 1999; Slot et al. 2007). While the biological role of HC-toxin has been demonstrated in *C. carbonum*, it is possible that HC-toxin from *A. jesenskae* provides a role other than virulence, which may include protection against competitors in its niche, involvement in a signaling pathway, or as a self-defense mechanism to protect from pathogens.

Future work on this project should include a more detailed microscopic study of how *A. jesenskae* interacts with plants, to determine if the fungus is a saprophyte or if it can penetrate the leaf surface, which would suggest that it might be a pathogen of a different host. Additionally, we previously demonstrated that the depudecin cluster in *A. brassicicola* is embedded in a region

of synteny between three fungi (Wight et al. 2009). A more detailed analysis of genes surrounding the TOX2 clusters to look for synteny between the two organisms could be critical to determining the evolutionary origins of this gene cluster.

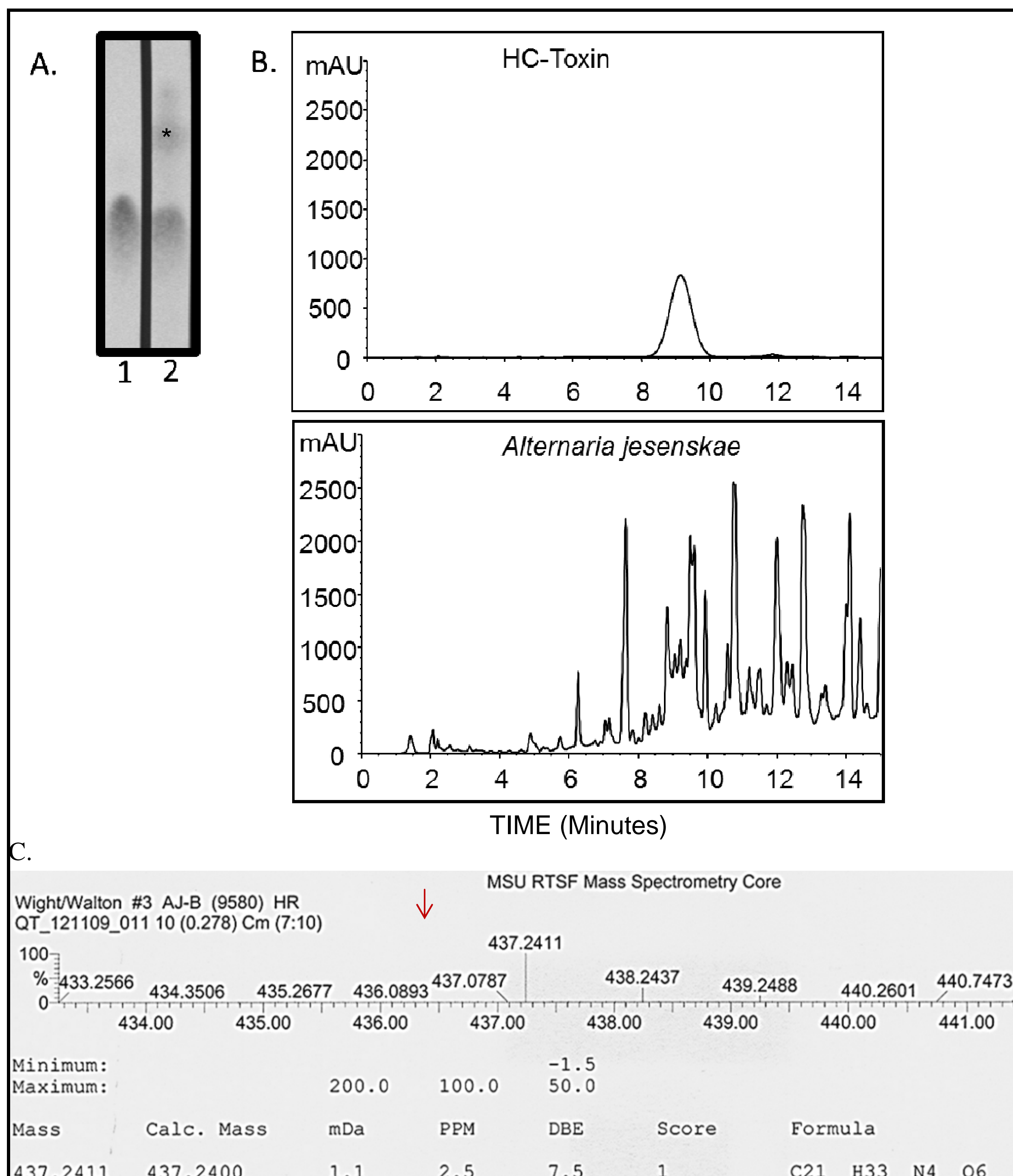

Figure 16. Analysis of HC-toxin from *A. jesenskae*. A) TLC followed by epoxide detection. Lane 1) HC-toxin standard (20 µg); Lane 2) 10 µl of 7 d- old *A. jesenskae* culture extract. The asterisk indicates an uncharacterized epoxide-containing compound. B) HPLC analysis of HC-toxin with detection at 230 nm.10 µg HC-toxin from *Cochliobolus carbonum (*upper trace). 20 µl of 7 d- old *A. jesenskae* culture extract (lower trace). HC-toxin elutes from the column at ~9 min. C) High resolution mass spectrometry of *A. jesenskae* fractions.

Figure 16. Cont'd Note major [MH+] peak at 437.2411 (arrow). HC-toxin has a molecular mass of 436 and molecular formula of $C_{21}H_{32}N_4O_6$.

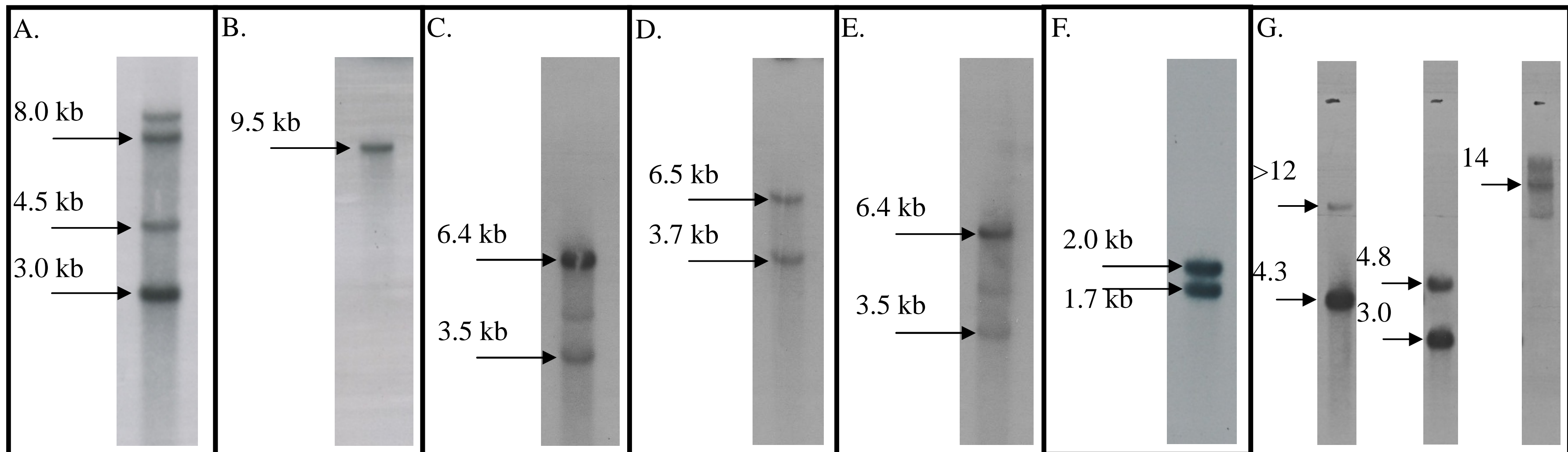

Figure 17. Southern analysis of ajTOX genes. A) DNA was digested with BamHI, which is predicted to give hybridizing bands at 5.7 kb and 2.9 kb. B) DNA was digested with NheI. One band expected at 9.5 kb. For C), D) and E) DNA was digested with a combination of AatII, BclI and KpnI and probed with *ajTOXD*, *ajTOXF*, and *ajTOXG* respectively. Hybridization expected at 3.8 kb and 7.0 kb for both C) and D) and 4.2kb and 7.0 kb for E). F) DNA was digested with NruI and EcoRI. Hybridization expected at 2.1 kb and 1.7 kb. G) DNA was digested with eight base cutters NaeI (left lane), EagI (middle lane) and AatII (right lane). Hybridization size was unknown for all three. Additional enzymes and enzyme combinations were tested (see Table 3) and in all cases no evidence for additional copies were found.

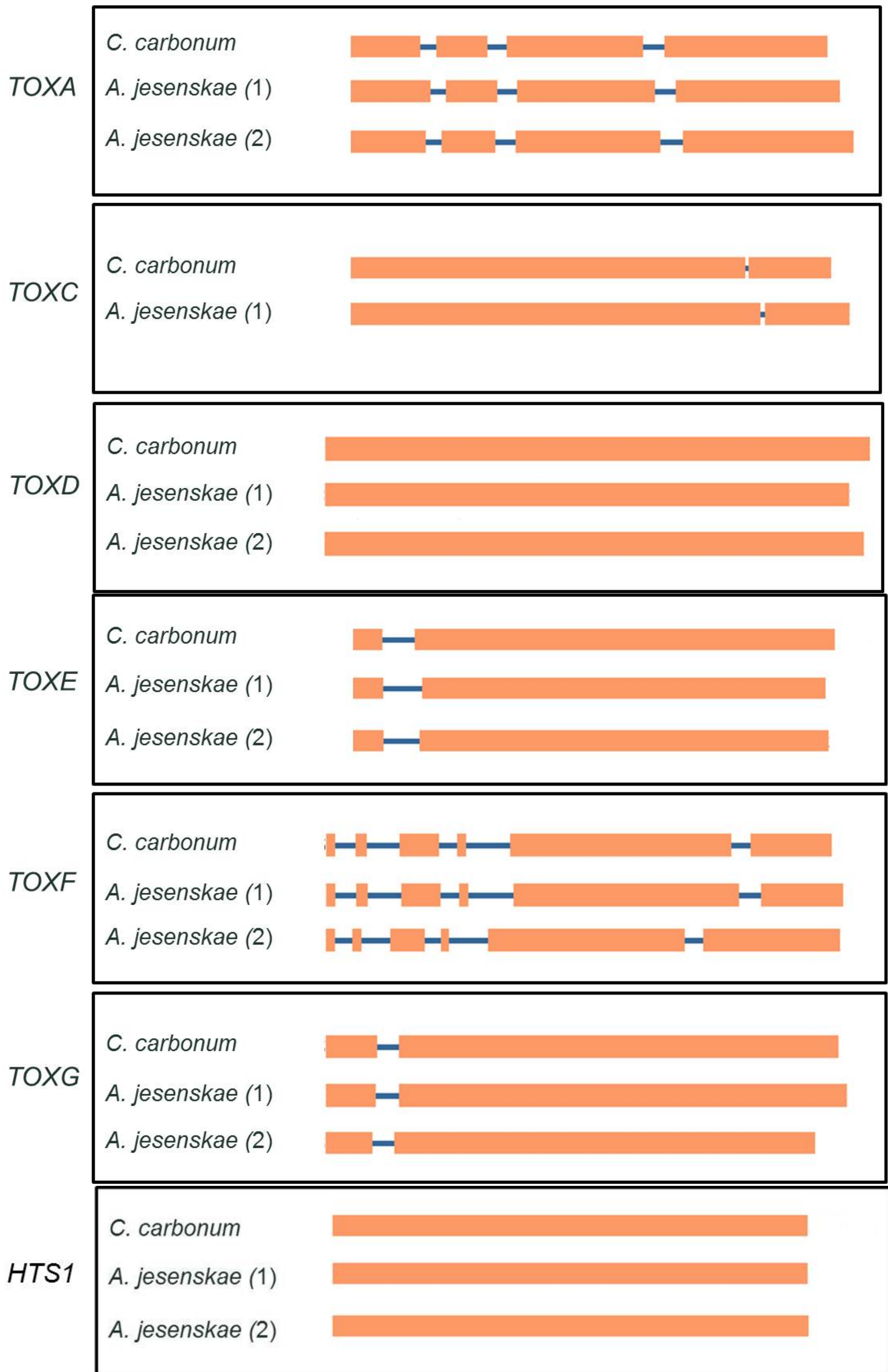

Figure 18. TOX gene structures in *C. carbonum* and *A. jesenskae*.

Figure 18. Cont'd Gene structures for *ajTOXA*, *ajTOXC* and *ajTOXE* are based on mRNA predictions using SPIDEY from NCBI. Structures for *ajTOXD*, *ajTOXF* and *ajTOXG* have been experimentally verified. (1) and (2) indicate copy number.

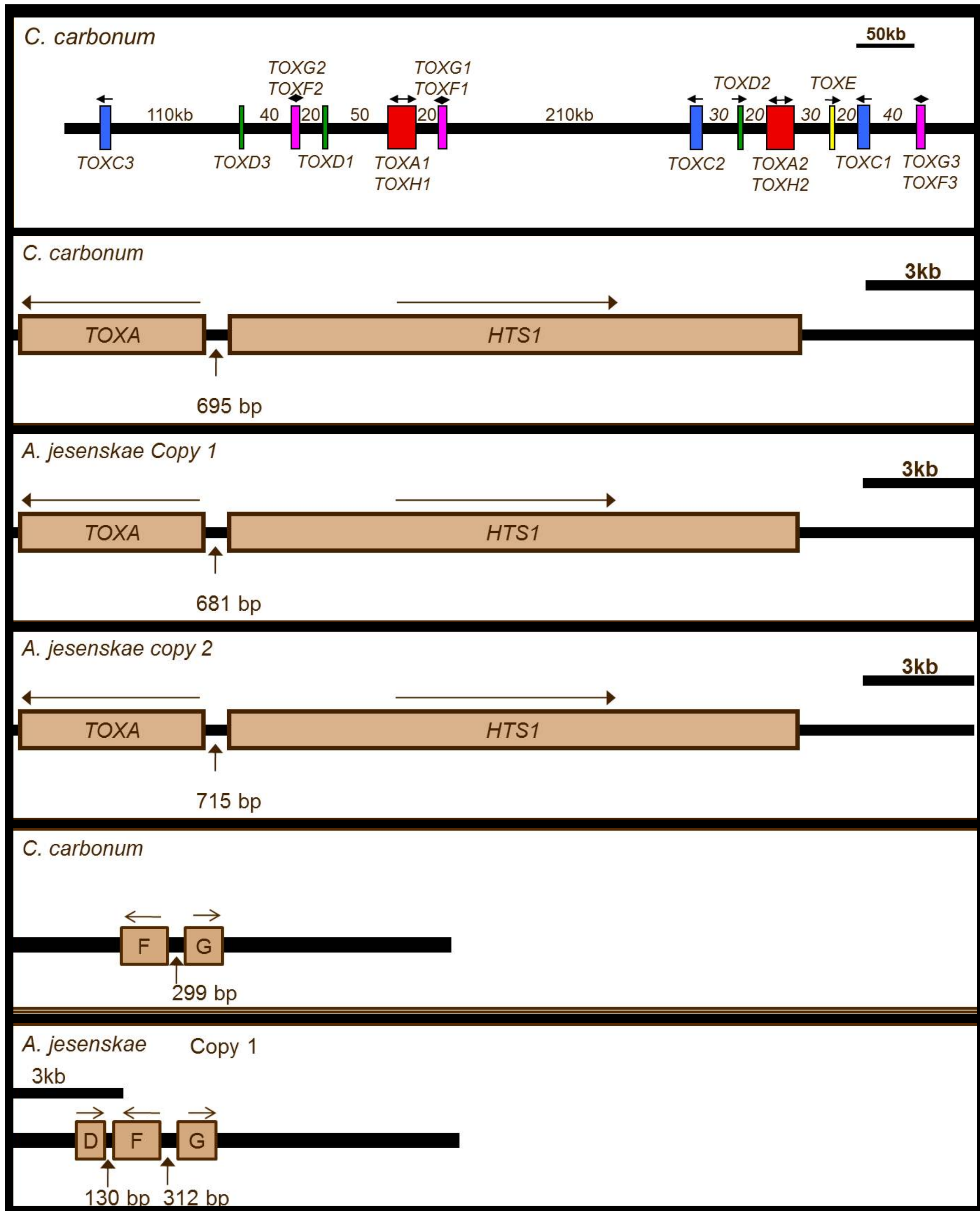

Figure 19. Differences in gene order and orientation of *TOX* genes in *C. carbonum ccTOX2* and *A. jesenskae*

Figure 19. Cont'd Differences in gene order and orientation of *TOX* genes in *C. carbonum ccTOX2* and *A. jesenskae ajTOX2* loci. D = *TOXD*, F= *TOXF* and G= *TOXG*. Map of *ccTOX2* is from (Ahn et al. 2002).

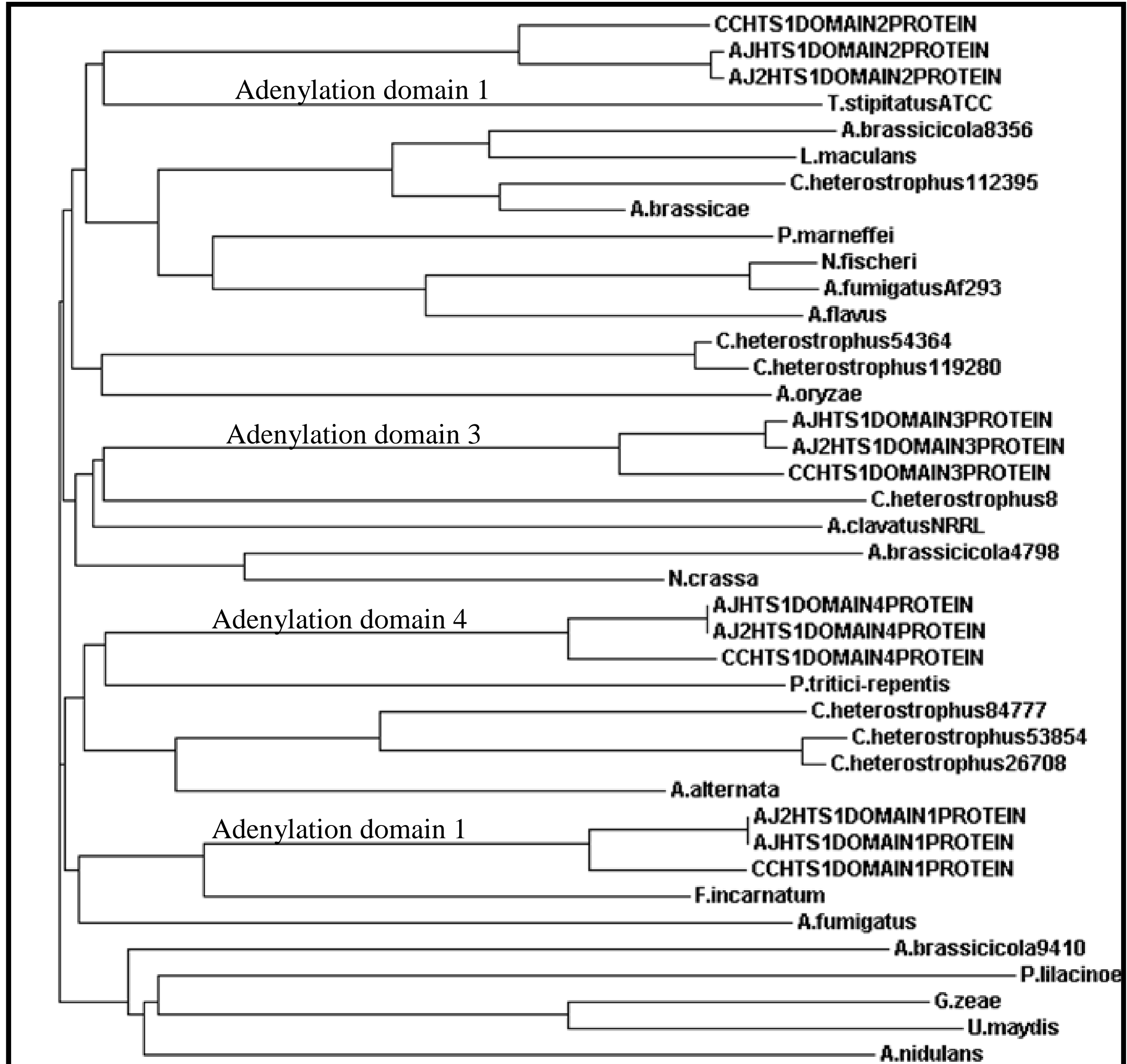

Figure 20. Phylogenetic tree using adenylation domains from fungal NRPS proteins (See also Table 7).

```
ajTOXE120       MNVDSSSSERHQPTDINERRKLQNRVAQKKYRTRQKTRMKLAEAVLNDISYFHPAFATIP 60
ajTOXE188       MNVASSSSEGHQTTDMNERRKLQNRVAQKKYRTRQKTRIKLAEAVLNDISYLHPAFAPHQ 60
ccTOXE          MGTTSPNSEKRQITDINERRKLQNRVAQRKYRTRQKTRMKLAEAVLNDYTYIHPTLGTIQ 60
                *.. *..** :* **:************:*********:********* :*:**::..

ajTOXE120       SKKKSPSAAEGDLLGDSCPDLSTATETRPETHPETRAKTHSTRARQSSNHLTYSSESVDN 120
ajTOXE188       SKKKSPLAAEDDLLGDSYPGFTPATETLPG--------TKSTHARQRSSQLTYSGESVDK 112
ccTOXE          SKKKSPLTMECDRSSASYPDLSSYAEICSETR----SETQATRARQLTSQRTCFRESVDN 116
                ****** : * *  . * *.::. :*  .         *::*:*** :.: *   ****:

ajTOXE120       NQADSGVQPSQCLDRQEVFCGFSGESEFSEGDPQNRMKCIDPVLTHGWLDMDFYSSTPKS 180
ajTOXE188       NQAHSGTRPVQCLDGQETLCGFSGEPQFSEDAPQNGMECIDPVLTHDWLDMDFHSGTPKP 172
ccTOXE          NQADSHAQLSRCLNRQEMFYGISGETEFSEGDTRDRVECIDPNLTRGWLDMDLRSGTPNS 176
                ***.* .:  :**: ** : *:***.:***. .:: ::**** **:.*****: *.**:.

ajTOXE120       STVIDCGLYTMGANGQLQTKPNFQEAVENLELCESNDRRVRGDLHEVAPGTRSTSSRGCS 240
ajTOXE188       PTVIDCSLYTVGADSQLQTRPNFREAVENLESCRLNGQRERGDLHEVAPGTCPASSRSCS 232
ccTOXE          STVVDCGLCTVGANSQPPTRTNVQEAIETLELFEPNDQRKTENLPREPCGSCPSSSHGYS 236
                .**:**.* *:**:.*  *:.*.:**:*.**  . *.:*   :* . . *: .:**:. *

ajTOXE120       PTLRNPSTLLLTPSESALSLNIVTSNSSLPAALDESLDDPVMSGVTTPGTMENQSTPLMT 300
ajTOXE188       PTPRNPPTLLLTSLESSLGVNTVNSDSPLPAALDESPDDPVTSGATIRGTVKNQSTPVMT 292
ccTOXE          PTSGNPSTLLLTPSESLMNSVIVTSDSPLLAADDKSPGDLVISEANTHGPKEDQFSPLMT 296
                **  **.*****. ** :.   *.*:*.* ** *:* .* * * ..  *. ::* :*:**

ajTOXE120       AISLGRLDIAKILLKSGATLDTPDDSGKTALHRAVGRGELHVVETLLELGADMLATDHKG 360
ajTOXE188       AVSLGRLDIARILLDSGATLDTPDDSGKTALHTAVGRGELHTVEALLELGADMLTTDHKG 352
ccTOXE          AISLGRLDIARILLQSGAPLDIPDDSGKTALHRAVGRRELHMVEALLNLGAEMLATDHEG 356
                *:********:***.***.** ********** **** *** **:**:***:**:***:*

ajTOXE120       NSLLHVAVKTNSLNMTKMLLERYESGKELGETQHRRGCRRHGNQLCDELWINFRNKDGMT 420
ajTOXE188       DSLLHVAVKTNSLNMTKMLLELYGSGKEPKETQRRRGCRRHSNQPRDELWINYRNKDGMT 412
ccTOXE          NSLLHIAVKTNSLSITRLLLERYKSCRELKDAQLGHGCRQHGNQVHSESWIDLRNREGMT 416
                :****:*******.:*::*** * * :*  ::*  :***:*.**  .* **: **::***

ajTOXE120       AVHLSVLWNRVEILGLLVKHGADVN 445
ajTOXE188       AVHQAVLWNRIEILTLLVERGADVN 437
ccTOXE          AVHLSVIFNRPEILQLLVKYSANVN 441
                *** :*::** *** ***: .*:**
```

Figure 21. Alignment of *ccTOXE* and the two copies of *ajTOXE*. The b-ZIP DNA binding region is indicated by the black box and the ankyrin repeats by the green box.

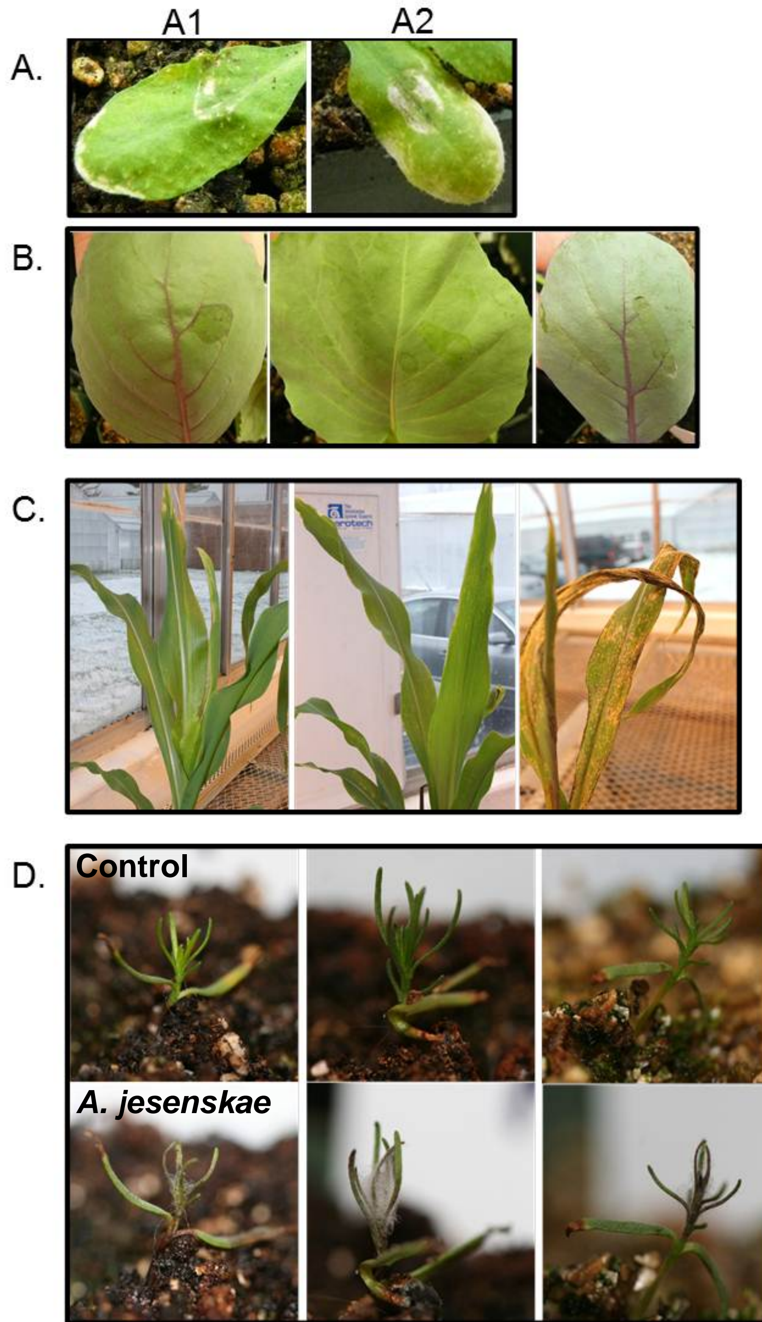

Figure 22. Virulence assays. A) *Arabidopsis thaliana* 4 days after inoculation. A1) mock inoculation control; A2) *Alternaria jesenskae*, 10^5 spores/ml. B) Cabbage leaves 4 d after inoculation. Mock inoculation (left side of vein), and 10 µl *A. jesenskae* was applied on the right side of the vein. C) Left) Susceptible maize sprayed with *Alternaria jesenskae*; Middle) Susceptible maize sprayed with *C. carbonum* Tox-; Right) Susceptible maize *C. carbonum* Tox+. Pictures taken four days after inoculation. D) *Fumana procumbens*. Control plants (top) were mock- inoculated; treated plants (bottom) were inoculated with spores of *A. jesenskae.* See text for details. Photographed 5 d after inoculation.

TABLE 1. List of restriction enzymes used for Southern analysis

Gene	Restriction Enzyme
AjTOXA	BamHI (Fig. 11), Double digest with HpaI and BclI (Not shown)
AjTOXC	NheI (Fig. 11), AatII (Not shown), double digest with BamHI and AatII (Not shown)
AjTOXD	Triple digest with AatII, BclI and KpnI (Fig. 11), KpnI (not shown)
AjTOXE	double digest with NruI and EcoRI (Fig. 11), NruI (not shown)
AjTOXF	Triple digest with AatII, BclI and KpnI (Fig. 11), KpnI (not shown)
AjTOXG	Triple digest with AatII, BclI and KpnI (Fig. 11), KpnI (not shown)
AjHTS1	NaeI (Fig. 11), EagI (Fig. 11), AatII (Fig. 11), HpaI(not shown)

TABLE 2. NCBI accession list for *Cochliobolus carbonum* TOX genes

TOX GENE	NCBI ACCESSION NUMBER Genomic /cDNA	NCBI ACCESSION NUMBER Protein
ccHTS1	M98024.2	AAA33023.2
ccTOXA	L48797.1	AAB36607.1
ccTOXC	U73650.1	AAC62818.1
ccTOXD	X92391.1	CAA63129.1
ccTOXE	AF038874.1	AAD13811.1
ccTOXF	AF157629.1	AAD45321.1
ccTOXG	AF169478.1	AAD47837.1

TABLE 3. NCBI NRPS adenylation domain sequences used in Fig. 20

Organism	Accession #
Leptosphaeria maculans	CBY02117.1
Pyrenophora tritici-repentis	XP_001939433.1
Cochliobolus heterostrophus	AAX09990.1
Aspergillus fumigatus Af293	EAL91342.1
Aspergillus fumigatus A1163	EDP52010.1
Penicillium marneffei	EEA22218.1
Neosartorya fischeri	EAW22836.1
Aspergillus clavatus	EAW15286.1
Aspergillus flavus	EED50111.1
Talaromyces stipitatus	EED20874.1
Aspergillus oryzae	XP_001825738.1
Penicillium lilacinoechinulatum	ABV48729.1

TABLE 3 CONT'D

Organism and Protein	Accession #
Emericella nidulans	AAA03914.1
Gibberella zeae	XP_386683.1
Fusarium incarnatum	ACZ66258.1
Ustilago maydis	XP_759255.1
Neurospora crassa	XP_963411.2
Alternaria alternate	BAI44759.1
Alternaria brassicae	AAP78735.1

TABLE 4. Percent Identity between indicated TOX gene and its ortholog in *C. carbonum* or the other copies in *A. jesenskae*

	C. carbonum		*A. jesenskae*	
Gene	DNA	Protein	DNA	Protein
TOXA (1)	81	80	95.2	94.5
TOXA (2)	82	80		
TOXC	83.3	80.5		
TOXD (1)	85	81	95	93
TOXD (2)	86	82		
TOXE (1)	74	64	85	76
TOXE (2)	72	58		
HTS1 (1)	85	82	99	98
HTS1 (2)	84	82		
TOXF (1)	84	84	97	94
TOXF (2)	85	85		
TOXG (1)	80	81	92	93
TOXG (2)	81	82		
Average	81.71	78.57	93.86	85.80

TABLE 5. TOXBOX and TOXBOX like sequences of *C. carbonum* and *A. jesenskae*

A. jesenskae-Sequence and contig/s they are associated with		*C. carbonum* – Sequence and associated gene (Pedley et al. 2001)	
TOXA (1)	ATCTCACGCA	*TOXA/HTS1*	ATCTCACGTA
TOXA (2)	ATCTCACGCA	*TOXA/HTS1*	ATCTCACGCA
TOXD (1)	ATCTCTCGTA ATCTCCCGTA	*TOXF/TOXG*	ATCTCGCGTA
HTS1 (1)	ATCTCACGGA ATCTCGCGGA	*TOXF/TOXG*	ATCTTACGTA
		TOXC	ATCTCTCGTC
		TOXC	ATCTCTCGAA
		TOXD	ATCTCTAGGC

TABLE 6. Housekeeping protein analysis between *C. carbonum* and *A. jesenskae*

HOUSEKEEPING PROTEIN	PERCENT IDENTITY between *C. carbonum* and *A. jesenskae*
Cellobiohydrolase (*CBH1*) Accession AAC49089.1	85%
Exo-beta 1,3 glucanase (*EXG1*) Accession AAC71062.1	76%
Glyceraldehyde 3-phosphate dehydrogenase (*GAPD*) Accession AAD48108	96%
Endo polygalacturonase (1) (*EPG*) Accession AAA79885.1	76%
Serine threonine protein kinase (*SNF1P*) Accession AAD43341.1	88%
Average	84.2%

CHAPTER 4: FUTURE DIRECTIONS

Evolutionary origin of the depudecin cluster from *Alternaria brassicicola*

Secondary metabolites, by definition, have a disjunct taxonomic distribution: some of these complex metabolites are made by taxonomically unrelated organisms, whereas in other cases, certain metabolites are not made by strains even within the same species. HC-toxin, for example, is made by *A. jesenskae* and *C. carbonum*, but not by other isolates of *C. carbonum* or other species of *Alternaria*. Another example of this might be the depudecin cluster. Only *A. brassicicola* within the genus *Alternaria* makes this compound. On the other hand, *Coccidioides immitis* has a cluster of at least four clustered genes *(CIMG_2399, CIMG_2396, CIMG_2397, and CIMG_2398)* that are highly related to four genes in the DEP cluster of *A. brassicicola*, *DEP1*, *DEP3*, *DEP4*, and *DEP5* respectively (Wight et al. 2009). These results suggest that *C. immitis* may produce a metabolite similar to depudecin. Biochemical analysis was done using thin layer chromatography followed by epoxide detection. My results indicate that there may be an epoxide containing compound in culture filtrates of *C. immitis*. Further biochemical and mass spectrometry analysis of culture filtrates will be needed to confirm the presence of such a compound.

Nimbya scirpicola has also been reported to produce depudecin (Tanaka et al. 2000). However the biosynthetic pathway has not been characterized. From an evolutionary perspective it would be interesting to determine if depudecin biosynthesis in these two unrelated fungi is a result of HGT or convergent evolution.

Alternaria jesenskae

The discovery that *Alternaria jesenskae* produces HC-toxin has the potential to help our understanding of the evolutionary origins of the TOX2 locus of *C. carbonum*. However, further research is needed. First, the HC-toxin cluster needs to be more fully defined. There are likely other genes involved in the biosynthetic pathway of this metabolite that need to be characterized. This would help a complete comparison of the *A. jesenskae* and *C. carbonum* TOX2 loci. For instance, *TOXC* is a putative fatty acid synthase (FAS) beta subunit. FAS requires both an alpha and a beta subunit. Genome sequencing of the TOX2 locus discovered an FAS alpha subunit gene linked to the other TOX2 genes, but it has not been characterized (J. Walton, unpublished results). Additionally, at least two P450 monooxygenase genes and an isoamyl alcohol oxidase gene have been found linked to other genes of TOX2, but they are also uncharacterized for a role in HC-toxin biosynthesis. Second, an examination of the genes surrounding the TOX2 locus to look for synteny would be important to determine if the gene cluster may be the result of convergent evolution, descent from a common ancestor, or horizontal gene transfer. Such an analysis should include not only determination of the ends of the cluster itself but also the sequences flanking the cluster. These flanking sequences might provide information, such as the presence of transposable elements which could support mobility of the cluster.

An advantage of working with *A. jesenskae* is that we have the 10x genome survey which allows for comparative analysis of gene sequences. However, since it is not complete coverage, additional sequencing may be necessary for full analysis of the *aj*TOX2 locus. An additional obstacle is that we do not have the genome of *C. carbonum.* Lastly, very little is known about *A. jesenskae* that in order to determine the potential role of HC-toxin more work needs to be done to characterize its ecological niche. Microscopic analysis to determine if *A. jesenskae* is able to

penetrate and colonize cells of healthy plants would help to determine if it is facultative saprophyte or if it has the potential to act as a pathogen.

APPENDIX I

TABLE 7.1: Supplementary TABLE S1. Wight et al. PCR primers used in this study. All primer sequences are shown 5' to 3'.		
	5'	3'
LME primers for *DEP5*	ATGCAAGCTTTTCAACAGCT ATGCCAACGGC	CGACTCTAGAGGCACA TTGATCTGCCATCCT
*DEP6*KOFor	CTTTACGCCAAATTCGTGCT	
*DEP6*KORev	ATCAGTTAACGTCGACCTCG CTCACGGTTCTGCATTCCTT	
*DEP2*KOFor	TACTGGCTTCATGCGATGTC	
*DEP2*KORev	ATCAGTTAACGTCGACCTCG ACCCCTTCCTCGAGTGAGAT	
*DEP4*KOFor	TGGCGCTGGTATGTATGGTA	
*DEP4*KORev	ATCAGTTAACGTCGACCTCG CCACAGCATTACGCTGAAGA	
*DEP6*HygFor	AAGGAATGCAGAACCGTGA GCGAGGTCGACGTTAACTGA T	
*DEP6*HygRev	CGTCGACGTTAACTGGTTCC	
*DEP2*HygFor	ATCTCACTCGAGGAAGGGGT CGAGGTCGACGTTAACTGAT	
*DEP2*HygRev	CGTCGACGTTAACTGGTTCC	
*DEP4*HygFor	TCTTCAGCGTAATGCTGTGG CGAGGTCGACGTTAACTGAT	
*DEP4*HygRev	CGTCGACGTTAACTGGTTCC	
P9comF	CGCCCACTAACACCTTCAAT	
P9comR	CCGATAAGACCCTGACGAAA	
TfcomF	AACAGAAGTAGCGCCGAAG A	

TABLE 7.1: Supplementary TABLE S1. CONT"D

Wight et al. PCR primers used in this study. All primer sequences are shown 5' to 3'.

TfcomR	GGTGTTATCCCGACTTCACCT	
Mo1comF	AAGGAGCGTGAGTTGAGCAT	
Mo1comR	GCAAGCCTCATCCTAATTGC	
Mo2comF	GCATTCCTCCTCAACGTAGC	
Mo2comR	TTTACGGAGTTGCAGCAAGA	
PNRcomF	GAGCGGAGACATGTGAGACC	
PNRcomR	TCATTCTAGCTTGCGGTCCT	
***DEP5* replacement primers**		
5' end of gene	AACATATTCCCGAAAACTCA GACA	AAAACCCTGGCGGTAT GGATATGGCAGAACCT
3' end of gene	GTTATCCGCTAGACATGCCA GCAAAACGAA	CGATACCCTGCGCCCC ACAC
DEP5 DNA blot probe	ATGATAGTGGCAAGCAATGG TG	TCGGCAAGCTCAACAA AAGTA
***DEP6* replacement primers**		
5' end of gene	AGTAGCGCCGAAGATAGC	CGTTTTACAA GATAGTCTGGCGGTTA GC
3' end of gene	GTTTCCTGTG CGGACAGTGTTTAGGTTG	GCTCCCACAGTATTTG AC
DEP6 DNA blot probe	GCGACGAAGCTCACAGTATC AG	TTCACACCTTTCAGCG TAGTTT

TABLE 7.1: Supplementary TABLE S1. Wight et al. PCR primers used in this study. All primer sequences are shown 5’ to 3’.		
***hph1* amplification primers for constructs**		
M13 hyg/*DEP5* primer	ATATCCATACCGCCAGGGTT TTCCCAGTCA	TGGCATGTCTAGCGGA TAACAATTTCACAC
M13 hyg/*DEP6* primer	CCAGACTATC TTGTAAAACGACGGCCAGT	ACACTGTCCGCACAGG AAACAGCTATGACC
Primers for probes for RNA blots		
Gene 4 probe (see Fig. 7)	TGAAGCGCGGCGACATCGAA GACATTGAC	TTCCTCTTCTCGACAG CCTCTTTGCGTTCC
DEP1 probe	CGATACGATAACCCGCACTC TCCACTTT	CGAATGACGCCGCACC TCCTG
DEP2 probe	GACCATCAAAACCCCCAGTA TT	GCATTGTAGCCCCGAG TTTT
DEP3 probe	TGGCGAGAAGCATGTTGAAG CAGTC	CCCCGATGAGAATGTT GTAGCCGTAGAT
DEP4 probe	CATACCTGGAGCTGGCCCCC GAGACTAA	GTTCACGCCATCCAAG CATCCAAGAGAC
DEP5 probe	ATGATAGTGGCAAGCAATGG TG	TCGGCAAGCTCAACAA AAGTA
DEP6 probe	GTAAAGCCAACCGAGGACCA CT	TCAGACGACGAGCTTC AACACG
Gene 11 probe (see Fig. 7)	GCCCGTCGTTACTGCAATCC CCAATCC	TCTTCTTTGCGGCCTG GTCACTGTATTC

TABLE 7.2. CHAPTER 3 PRIMER LIST All primer sequences are shown 5' to 3'.		
AjTOXC KNOCKOUT CONSTRUCT		
Name	Forward Primer	Reverse Primer
Primer Pair 1	CGCTCAAGTCAGAGCTCCTT	tgactgggaaaaccctggcgCACGCTGTGTCTTCCATGTT
Primer Pair 2	GtgtgaaattgttatccgctGCTGGCTTGAGGAGTATTGG	TTGAACGTAGATGGGCAACA
Primer Pair 3	AACATGGAAGACACAGCGTGcgccagggttttcccagtca	CCAATACTCCTCAAGCCAGCagcggataacaatttcacac
SEQUENCING PRIMERS		
GENE/ CONTIG	Forward Primer	Reverse Primer
ajTOXA	CGTTCAACAAGCATTTATTGGTGCT	AACATGACTACCAACAGGCC
	ATGGCAGCAACCAAACTGAG	ACGCCCAGCTTAGAAAAGGC
ajTOXC	TTCTCCTGGAAGCCTTCGATTCCACGTTTC	AGATGGCGGCTGTGAAAAGGTGCA
		TTGAACGTAGATGGGCAACA
ajTOXD	ATGGCCTTTCAAAACGCAGT	AAGTTTCTCTCCGCTAACGA
		TCAGACATTATACACAAGTTTCTCTCCGCT
ajTOXF	GACTTCCAGCAAACGAGATA	GCACGATCAGTGATACTCGA
		CTACGGTTGAGAGCAAAACC
ajTOXG	CACTTCAATCGCTAAGTGGA	TCATGGCCTCATTGTAGCTA
CONTIG 509 (*ajTOXA*)		ATGAAATAGAGGGCCTGGAACAGC
		TATCCATCACACAAACC
		CGATAGTATCCATCACACAAACC

TABLE 7.2. CHAPTER 3 PRIMER LIST All primer sequences are shown 5' to 3'.SEQUENCING PRIMERS CONTINUED		
CONTIG 740 (*ajTOXA*)	TGCTACTCTCGTGAGAAAGCC	
CONTIG 738 (ajTOXA)	CGCTACTCTCGTGAGAAAGCT	
CONTIG 819 (*ajTOXA*)		GTGAAATAGAGGGCCTGGAACAG T
		ACAGGATCCATCACACAAAAA
Name	Forward Primer	Reverse Primer
CONTIG 130 (*ajTOXD/ F/G*)	GTGTCGATAGATCACGTGAG	TATGGCCAAATTCATGAGCA
CONTIG 962 (*ajTOXD/ F/G*)	CGAAGCATGAGTGCATTTAC	ATCATCCAGCCATCAACTTT
ajHTS1	ATGCCACGCGAATCGCCA	GTGCAACTCGTGGACACA
	GCGATTGATGCATGGGAC	AGGAGACACGAGCAAGTCTT
SOUTHERN PROBES		
Name	Forward Primer	Reverse Primer
ajTOXA	TTGCTCATGAGCGGAACTATG CAGCCCCTT	AAAGCTCCGATCATACCAGTGAAG GTGGCT
ajTOXC	GCTGGCTTGAGGAGTATTGG	AATGTGAGTTGCTCCAGG
ajTOXD	TGAAAACTCAAGTTCTCTGGT ATCCATGCCTGA	GCGACTTCATGAGCTCAAAATTGT GGGGAG
ajTOXF	CTTGCGGGCACTGATAGCGGA GAAATTTGA	GCTCATCCTCTTCTCCGGCCTTTTT CATTC
ajTOXG	CATGCTATACACTATGAGGCA GGTGGCCCT	AGTGTGTCTTGACGTTGTTTTCCTT TGCCC
ajHTS1	GAGATTCTGTACACAGGGGTA GGAGGT	TCTTTGCCCCTCACCACTAACAAT GCCGTT

APPENDIX II

Gene disruption.

The *TOXC* ortholog in *A. jesenskae* is currently being disrupted by homologous recombination (Chapter 2, Fig. 2B). The list of PCR primers used for construction of the knockout vector can be found in the Appendix. Primer pairs 1 and 2 were used to amplify 770- and 811-bp fragments from the 5′ and 3′ ends of *TOXC*, respectively. The internal primers included an additional 28-30 bp complementary to *hph1* (for hygromycin resistance) of pCB1003 (Carroll AM 1994). Primer pair 3 was used to amplify *hph1* and included an additional 25 bp complementary to the target gene. The up- and downstream flanking regions of *TOXC* were combined with *hph1* in a second round of PCR, using the outside gene primers. PCR products were ligated into pGem T-easy (Promega, Madison, WI), transformed into E. coli DH5α (Invitrogen), and sequenced. Amplification of the knockout construct was done using Primer pair 1 forward primer and Primer pair 2 reverse primer. PCR products were purified with the QIAquick PCR purification kit (Qiagen, Valencia, CA) and used directly in transformation of protoplasts using a protocol modified from Cho et al. 2006 (Cho et al. 2006) .

REFERENCES

REFERENCES

Agrios, G. N. (1997). Plant Pathology (4th ed.) Academic Press, NY, NY.

Ahn, J. H., Y. Q. Cheng, et al. (2002). An extended physical map of the TOX2 locus of Cochliobolus carbonum required for biosynthesis of HC-toxin. Fungal Genet Biol 35(1): 31-38.

Ahn, J. H. and J. D. Walton (1996). Chromosomal organization of TOX2, a complex locus controlling host-selective toxin biosynthesis in Cochliobolus carbonum. Plant Cell 8(5): 887-897.

Ahn, J. H. and J. D. Walton (1997). A fatty acid synthase gene in Cochliobolus carbonum required for production of HC-toxin, cyclo(D-prolyl-L-alanyl-D-alanyl-L-2-amino-9, 10-epoxi-8-oxodecanoyl). Mol Plant Microbe Interact 10(2): 207-214.

Alvarez, M. E., F. Nota, et al. (2010). Epigenetic control of plant immunity. Mol Plant Pathol 11(4): 563-576.

Amnuaykanjanasin, A., J. Punya, et al. (2005). Diversity of type I polyketide synthase genes in the wood-decay fungus Xylaria sp. BCC 1067. FEMS Microbiol Lett 251(1): 125-136.

Babic, A., A. B. Lindner, et al. (2008). Direct visualization of horizontal gene transfer. Science 319(5869): 1533-1536.

Baidyaroy, D., G. Brosch, et al. (2002). Characterization of inhibitor-resistant histone deacetylase activity in plant-pathogenic fungi. Eukaryot Cell 1(4): 538-547.

Borkovich, K. A. a. E., Daniel J. (2010). Cellular and Molecular Biology of Filamentous Fungi. ASM Press.

Boto, L. (2010). Horizontal gene transfer in evolution: facts and challenges. Proc Biol Sci 277(1683): 819-827.

Brandl, A., T. Heinzel, et al. (2009). Histone deacetylases: salesmen and customers in the post-translational modification market. Biol Cell 101(4): 193-205.

Brosch, G., M. Dangl, et al. (2001). An inhibitor-resistant histone deacetylase in the plant pathogenic fungus Cochliobolus carbonum. Biochemistry 40(43): 12855-12863.

Brosch, G., R. Ransom, et al. (1995). Inhibition of maize histone deacetylases by HC toxin, the host-selective toxin of Cochliobolus carbonum. Plant Cell 7(11): 1941-1950.

Brown, D. W., J. H. Yu, et al. (1996). Twenty-five coregulated transcripts define a sterigmatocystin gene cluster in Aspergillus nidulans. Proc Natl Acad Sci U S A 93(4): 1418-1422.

Calvo, A. M., R. A. Wilson, et al. (2002). Relationship between secondary metabolism and fungal development. Microbiol Mol Biol Rev 66(3): 447-459, table of contents.

Carroll AM, S. J., Valent B (1994). Improved vectors for selecting resistance to hygromycin. . Fungal Genet. Newsl. 41(22).

Chelkowski, J. V., A. (1992). Alternaria: Biology, Plant Diseases and Metabolites (Topics in Secondary Metabolism) Elsevier Science; 1 edition

Cheng, Y. Q., J. H. Ahn, et al. (1999). A putative branched-chain-amino-acid transaminase gene required for HC-toxin biosynthesis and pathogenicity in Cochliobolus carbonum. Microbiology 145 (Pt 12): 3539-3546.

Cheng, Y. Q. and J. D. Walton (2000). A eukaryotic alanine racemase gene involved in cyclic peptide biosynthesis. J Biol Chem 275(7): 4906-4911.

Chiang, Y. M., K. H. Lee, et al. (2009). Unlocking fungal cryptic natural products. Nat Prod Commun 4(11): 1505-1510.

Cho, Y., J. W. Davis, et al. (2006). A high throughput targeted gene disruption method for Alternaria brassicicola functional genomics using linear minimal element (LME) constructs. Mol Plant Microbe Interact 19(1): 7-15.

Choudhary, C., C. Kumar, et al. (2009). Lysine acetylation targets protein complexes and co-regulates major cellular functions. Science 325(5942): 834-840.

Chu, H. Y., E. Wegel, et al. (2011). From hormones to secondary metabolism: the emergence of metabolic gene clusters in plants. Plant J 66(1): 66-79.

Colonna, S., N. Gaggero, et al. (2002). First asymmetric epoxidation catalysed by cyclohexanone monooxygenase. Tetrahedron Letters 43(10): 1797-1799.

Cramer, R. A. and C. B. Lawrence (2004). Identification of Alternaria brassicicola genes expressed in planta during pathogenesis of Arabidopsis thaliana. Fungal Genet Biol 41(2): 115-128.

Darkin-Rattray, S. J., A. M. Gurnett, et al. (1996). Apicidin: a novel antiprotozoal agent that inhibits parasite histone deacetylase. Proc Natl Acad Sci U S A 93(23): 13143-13147.

Demain, A. L. and A. Fang (2000). The natural functions of secondary metabolites. Adv Biochem Eng Biotechnol 69: 1-39.

Deng, M. R., J. Guo, et al. (2011). Granaticins and their biosynthetic gene cluster from Streptomyces vietnamensis: evidence of horizontal gene transfer. Antonie Van Leeuwenhoek.

Desmond, E., C. Brochier-Armanet, et al. (2011). On the last common ancestor and early evolution of eukaryotes: reconstructing the history of mitochondrial ribosomes. Res Microbiol 162(1): 53-70.

Deubzer, H. E., V. Ehemann, et al. (2008). Anti-neuroblastoma activity of Helminthosporium carbonum (HC)-toxin is superior to that of other differentiating compounds in vitro. Cancer Lett 264(1): 21-28.

Devoto, A., M. Nieto-Rostro, et al. (2002). COI1 links jasmonate signalling and fertility to the SCF ubiquitin-ligase complex in Arabidopsis. Plant J 32(4): 457-466.

Dhawan, R., H. Luo, et al. (2009). HISTONE MONOUBIQUITINATION1 interacts with a subunit of the mediator complex and regulates defense against necrotrophic fungal pathogens in Arabidopsis. Plant Cell 21(3): 1000-1019.

Dokmanovic, M., C. Clarke, et al. (2007). Histone deacetylase inhibitors: overview and perspectives. Mol Cancer Res 5(10): 981-989.

Doolittle, W. F. (1998). You are what you eat: a gene transfer ratchet could account for bacterial genes in eukaryotic nuclear genomes. Trends Genet 14(8): 307-311.

Dunning Hotopp, J. C. (2011). Horizontal gene transfer between bacteria and animals. Trends Genet 27(4): 157-163.

Engels, W. R. (1997). Invasions of P elements. Genetics 145(1): 11-15.

Fondi, M., G. Emiliani, et al. (2009). Origin and evolution of operons and metabolic pathways. Res Microbiol 160(7): 502-512.

Friesen, T. L., J. D. Faris, et al. (2008). Host-specific toxins: effectors of necrotrophic pathogenicity. Cell Microbiol 10(7): 1421-1428.

Friesen, T. L., E. H. Stukenbrock, et al. (2006). Emergence of a new disease as a result of interspecific virulence gene transfer. Nat Genet 38(8): 953-956.

Gachon, C. and P. Saindrenan (2004). Real-time PCR monitoring of fungal development in Arabidopsis thaliana infected by Alternaria brassicicola and Botrytis cinerea. Plant Physiol Biochem 42(5): 367-371.

Gendler, K., T. Paulsen, et al. (2008). ChromDB: the chromatin database. Nucleic Acids Res 36(Database issue): D298-302.

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu Rev Phytopathol 43: 205-227.

Graham, L. A., S. C. Lougheed, et al. (2008). Lateral transfer of a lectin-like antifreeze protein gene in fishes. PLoS One 3(7): e2616.

Haberland, M., R. L. Montgomery, et al. (2009). The many roles of histone deacetylases in development and physiology: implications for disease and therapy. Nat Rev Genet 10(1): 32-42.

Hallen, H. E., M. Huebner, et al. (2007). Gene expression shifts during perithecium development in Gibberella zeae (anamorph Fusarium graminearum), with particular emphasis on ion transport proteins. Fungal Genet Biol 44(11): 1146-1156.

Hamilos, D. L. (2010). Allergic fungal rhinitis and rhinosinusitis. Proc Am Thorac Soc 7(3): 245-252.

Hammock, L. G., B. D. Hammock, et al. (1974). Detection and analysis of epoxides with 4-(p-Nitrobenzyl)-pyridine. Bull Environ Contam Toxicol 12(6): 759-764.

Han, F., A. Kleinhofs, et al. (1997). Cloning and mapping of a putative barley NADPH-dependent HC-toxin reductase. Mol Plant Microbe Interact 10(2): 234-239.

He, Y., Michaels, S.D., and Amasino, R.M. and . (2003). Regulation of flowering time by histone acetylation in Arabidopsis. Science 302: 1751–1754.

Hecht, M. M., N. Nitz, et al. (2010). Inheritance of DNA transferred from American trypanosomes to human hosts. PLoS One 5(2): e9181.

Heitman, J. (2011). Microbial Pathogens in the Fungal Kingdom. Fungal Biol Rev 25(1): 48-60.

Hendrickson, L., C. R. Davis, et al. (1999). Lovastatin biosynthesis in Aspergillus terreus: characterization of blocked mutants, enzyme activities and a multifunctional polyketide synthase gene. Chem Biol 6(7): 429-439.

Hicks JK, S. K., Keller NP. (2002). Genetics and biosynthesis of aflatoxins and sterigmatocystin. In The Mycota XI, ed F Kempken, JW Bennett Berlin: Springer-Verlag: 55-69.

Hilario, E. and J. P. Gogarten (1993). Horizontal transfer of ATPase genes--the tree of life becomes a net of life. Biosystems 31(2-3): 111-119.

Hnilicova, J., S. Hozeifi, et al. (2011). Histone deacetylase activity modulates alternative splicing. PLoS One 6(2): e16727.

Hoffmeister, D. and N. P. Keller (2007). Natural products of filamentous fungi: enzymes, genes, and their regulation. Nat Prod Rep 24(2): 393-416.

Hollender, C. and Z. Liu (2008). Histone deacetylase genes in Arabidopsis development. J Integr Plant Biol 50(7): 875-885.

Jacob, F. a. M., J. (1961). Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. 3: 318-356.

Jang, I. C., Y. M. Pahk, et al. (2003). Structure and expression of the rice class-I type histone deacetylase genes OsHDAC1-3: OsHDAC1 overexpression in transgenic plants leads to increased growth rate and altered architecture. Plant J 33(3): 531-541.

Jenuwein, T. and C. D. Allis (2001). Translating the histone code. Science 293(5532): 1074-1080.

Jin, J. M., S. Lee, et al. (2010). Functional characterization and manipulation of the apicidin biosynthetic pathway in Fusarium semitectum. Mol Microbiol 76(2): 456-466.

Johal, G. S. and S. P. Briggs (1992). Reductase activity encoded by the HM1 disease resistance gene in maize. Science 258(5084): 985-987.

Joung, K. E., D. K. Kim, et al. (2004). Antiproliferative effect of trichostatin A and HC-toxin in T47D human breast cancer cells. Arch Pharm Res 27(6): 640-645.

Kagan, I. A. and R. Hammerschmidt (2002). Arabidopsis ecotype variability in camalexin production and reaction to infection by Alternaria brassicicola. J Chem Ecol 28(11): 2121-2140.

Karolczak-Bayatti, M., M. Sweeney, et al. (2011). Acetylation of heat shock protein 20 (Hsp20) regulates human myometrial activity. J Biol Chem.

Kawai, M., D. H. Rich, et al. (1983). The structure and conformation of HC-toxin. Biochem Biophys Res Commun 111(2): 398-403.

Keeling, P. J. (2009). Functional and ecological impacts of horizontal gene transfer in eukaryotes. Curr Opin Genet Dev 19(6): 613-619.

Keller, N. P. and T. M. Hohn (1997). Metabolic pathway gene clusters in filamentous fungi. Fungal Genet Biol 21(1): 17-29.

Keller, N. P., G. Turner, et al. (2005). Fungal secondary metabolism - from biochemistry to genomics. Nat Rev Microbiol 3(12): 937-947.

Kelly, B. G., A. Vespermann, et al. (2009). Gene transfer events and their occurrence in selected environments. Food Chem Toxicol 47(5): 978-983.

Kelly, B. G., A. Vespermann, et al. (2009). Horizontal gene transfer of virulence determinants in selected bacterial foodborne pathogens. Food Chem Toxicol 47(5): 969-977.

Khaldi, N., J. Collemare, et al. (2008). Evidence for horizontal transfer of a secondary metabolite gene cluster between fungi. Genome Biol 9(1): R18.

Khaldi, N. and K. H. Wolfe (2011). Evolutionary Origins of the Fumonisin Secondary Metabolite Gene Cluster in Fusarium verticillioides and Aspergillus niger. Int J Evol Biol 2011: 423821.

Kim, K. C., Z. Lai, et al. (2008). Arabidopsis WRKY38 and WRKY62 transcription factors interact with histone deacetylase 19 in basal defense. Plant Cell 20(9): 2357-2371.

Kim, K. H., Y. Cho, et al. (2007). Functional analysis of the Alternaria brassicicola non-ribosomal peptide synthetase gene AbNPS2 reveals a role in conidial cell wall construction. Mol Plant Pathol 8(1): 23-39.

Kirk, P., P. Cannon, et al. (2008). Dictionary of the Fungi 10th ed(Wallingford: CABI): 22.

Koonin, E. V., K. S. Makarova, et al. (2001). Horizontal gene transfer in prokaryotes: quantification and classification. Annu Rev Microbiol 55: 709-742.

Kwon, H. J., J. H. Kim, et al. (2003). Anti-parasitic activity of depudecin on Neospora caninum via the inhibition of histone deacetylase. Vet Parasitol 112(4): 269-276.

Kwon, H. J., T. Owa, et al. (1998). Depudecin induces morphological reversion of transformed fibroblasts via the inhibition of histone deacetylase. Proc Natl Acad Sci U S A 95(7): 3356-3361.

Labuda, R., P. Elias, Jr., et al. (2008). Alternaria jesenskae sp. nov., a new species from Slovakia on Fumana procumbens (Cistaceae). Microbiol Res 163(2): 208-214.

Lai, Z., F. Wang, et al. (2011). A critical role of autophagy in plant resistance to necrotrophic fungal pathogens. Plant J 66(6): 953-968.

Lawrence, J. (1999). Selfish operons: the evolutionary impact of gene clustering in prokaryotes and eukaryotes. Curr Opin Genet Dev 9(6): 642-648.

Lawrence, J. G. (1997). Selfish operons and speciation by gene transfer. Trends Microbiol 5(9): 355-359.

Lawrence, J. G. and J. R. Roth (1996). Selfish operons: horizontal transfer may drive the evolution of gene clusters. Genetics 143(4): 1843-1860.

Leipe, D. D. and D. Landsman (1997). Histone deacetylases, acetoin utilization proteins and acetylpolyamine amidohydrolases are members of an ancient protein superfamily. Nucleic Acids Res 25(18): 3693-3697.

Lenz, H. D., E. Haller, et al. (2011). Autophagy differentially controls plant basal immunity to biotrophic and necrotrophic pathogens. Plant J 66(5): 818-830.

Malonek, S., M. C. Rojas, et al. (2004). The NADPH-cytochrome P450 reductase gene from Gibberella fujikuroi is essential for gibberellin biosynthesis. J Biol Chem 279(24): 25075-25084.

Marcoux, D., F. Jafarian, et al. (2009). Deep cutaneous fungal infections in immunocompromised children. J Am Acad Dermatol 61(5): 857-864.

Matsumoto, M., S. Matsutani, et al. (1992). Depudecin: a novel compound inducing the flat phenotype of NIH3T3 cells doubly transformed by ras- and src-oncogene, produced by Alternaria brassicicola. J Antibiot (Tokyo) 45(6): 879-885.

Meeley, R. B., G. S. Johal, et al. (1992). A Biochemical Phenotype for a Disease Resistance Gene of Maize. Plant Cell 4(1): 71-77.

Meeley, R. B. and J. D. Walton (1991). Enzymatic Detoxification of HC-toxin, the Host-Selective Cyclic Peptide from Cochliobolus carbonum. Plant Physiol 97(3): 1080-1086.

Mehrabi, R., A. H. Bahkali, et al. (2011). Horizontal gene and chromosome transfer in plant pathogenic fungi affecting host range. FEMS Microbiol Rev 35(3): 542-554.

Mengiste, T., X. Chen, et al. (2003). The BOTRYTIS SUSCEPTIBLE1 gene encodes an R2R3MYB transcription factor protein that is required for biotic and abiotic stress responses in Arabidopsis. Plant Cell 15(11): 2551-2565.

Monneret, C. (2005). Histone deacetylase inhibitors. Eur J Med Chem 40(1): 1-13.

Moran, N. A. and T. Jarvik (2010). Lateral transfer of genes from fungi underlies carotenoid production in aphids. Science 328(5978): 624-627.

Mukherjee, A. K., S. Lev, et al. (2009). A compatible interaction of Alternaria brassicicola with Arabidopsis thaliana ecotype DiG: evidence for a specific transcriptional signature. BMC Plant Biol 9: 31.

Multani, D. S., R. B. Meeley, et al. (1998). Plant-pathogen microevolution: molecular basis for the origin of a fungal disease in maize. Proc Natl Acad Sci U S A 95(4): 1686-1691.

Navarro, L., R. Bari, et al. (2008). DELLAs control plant immune responses by modulating the balance of jasmonic acid and salicylic acid signaling. Curr Biol 18(9): 650-655.

Oh, I. S., A. R. Park, et al. (2005). Secretome analysis reveals an Arabidopsis lipase involved in defense against Alternaria brassicicola. Plant Cell 17(10): 2832-2847.

Oh, I. S., A. R. Park, et al. (2005). Secretome Analysis Reveals an Arabidopsis Lipase Involved in Defense against Alternaria brassicicolaW. Plant Cell 17(10): 2832-2847.

Oikawa, T., C. Onozawa, et al. (1995). Depudecin, a microbial metabolite containing two epoxide groups, exhibits anti-angiogenic activity in vivo. Biol Pharm Bull 18(9): 1305-1307.

Osbourn, A. (2010). Secondary metabolic gene clusters: evolutionary toolkits for chemical innovation. Trends Genet 26(10): 449-457.

Panaccione, D. G., J. W. Pitkin, et al. (1996). Transposon-like sequences at the TOX2 locus of the plant-pathogenic fungus Cochliobolus carbonum. Gene 176(1-2): 103-109.

Panaccione, D. G., J. S. Scott-Craig, et al. (1992). A cyclic peptide synthetase gene required for pathogenicity of the fungus Cochliobolus carbonum on maize. Proc Natl Acad Sci U S A 89(14): 6590-6594.

Pandey, R., A. Muller, et al. (2002). Analysis of histone acetyltransferase and histone deacetylase families of Arabidopsis thaliana suggests functional diversification of chromatin modification among multicellular eukaryotes. Nucleic Acids Res 30(23): 5036-5055.

Parfrey, L. W., D. J. Lahr, et al. (2011). Estimating the timing of early eukaryotic diversification with multigene molecular clocks. Proc Natl Acad Sci U S A 108(33): 13624-13629.

Park, J. S., K. R. Lee, et al. (1999). A hemorrhagic factor (Apicidin) produced by toxic Fusarium isolates from soybean seeds. Appl Environ Microbiol 65(1): 126-130.

Pastor, F. J. and J. Guarro (2008). Alternaria infections: laboratory diagnosis and relevant clinical features. Clin Microbiol Infect 14(8): 734-746.

Pedley, K. F. and J. D. Walton (2001). Regulation of cyclic peptide biosynthesis in a plant pathogenic fungus by a novel transcription factor. Proc Natl Acad Sci U S A 98(24): 14174-14179.

Pichersky, E. and E. Lewinsohn (2011). Convergent evolution in plant specialized metabolism. Annu Rev Plant Biol 62: 549-566.

Pitkin, J. W., D. G. Panaccione, et al. (1996). A putative cyclic peptide efflux pump encoded by the TOXA gene of the plant-pathogenic fungus Cochliobolus carbonum. Microbiology 142 (Pt 6): 1557-1565.

Pontiki, E. and D. Hadjipavlou-Litina (2010). Histone deacetylase inhibitors (HDACIs). Structure-activity relationships: history and new QSAR perspectives. Med Res Rev.

Postberg, J., S. Forcob, et al. (2010). The evolutionary history of histone H3 suggests a deep eukaryotic root of chromatin modifying mechanisms. BMC Evol Biol 10: 259.

Ransom, R. F. and J. D. Walton (1997). Histone Hyperacetylation in Maize in Response to Treatment with HC-Toxin or Infection by the Filamentous Fungus Cochliobolus carbonum. Plant Physiol 115(3): 1021-1027.

Richards, T. A., D. M. Soanes, et al. (2011). Horizontal gene transfer facilitated the evolution of plant parasitic mechanisms in the oomycetes. Proc Natl Acad Sci U S A 108(37): 15258-15263.

Robey, R. W., A. R. Chakraborty, et al. (2011). Histone deacetylase inhibitors: emerging mechanisms of resistance. Mol Pharm.

Rocha, E. P. (2008). The organization of the bacterial genome. Annu Rev Genet 42: 211-233.

Rosewich, U. L. and H. C. Kistler (2000). ROLE OF HORIZONTAL GENE TRANSFER IN THE EVOLUTION OF FUNGI. Annu Rev Phytopathol 38: 325-363.

Rossi, V., S. Locatelli, et al. (2007). Maize histone deacetylase hda101 is involved in plant development, gene transcription, and sequence-specific modulation of histone modification of genes and repeats. Plant Cell 19(4): 1145-1162.

Rushton, P. J., I. E. Somssich, et al. (2010). WRKY transcription factors. Trends Plant Sci 15(5): 247-258.

Scheffer, R. P. a. U., A.J. (1965). A host specific toxic metabolite from Helminthosporium carbonum. Phytopathology 55: 1037-1038.

Schenk, P. M., K. Kazan, et al. (2003). Systemic gene expression in Arabidopsis during an incompatible interaction with Alternaria brassicicola. Plant Physiol 132(2): 999-1010.

Scott-Craig, J. S., D. G. Panaccione, et al. (1992). The cyclic peptide synthetase catalyzing HC-toxin production in the filamentous fungus Cochliobolus carbonum is encoded by a 15.7-kilobase open reading frame. J Biol Chem 267(36): 26044-26049.

Shwab, E. K. and N. P. Keller (2008). Regulation of secondary metabolite production in filamentous ascomycetes. Mycol Res 112(Pt 2): 225-230.

Sindhu, A., S. Chintamanani, et al. (2008). A guardian of grasses: specific origin and conservation of a unique disease-resistance gene in the grass lineage. Proc Natl Acad Sci U S A 105(5): 1762-1767.

Slot, J. C. and D. S. Hibbett (2007). Horizontal transfer of a nitrate assimilation gene cluster and ecological transitions in fungi: a phylogenetic study. PLoS One 2(10): e1097.

Slot, J. C. and A. Rokas (2010). Multiple GAL pathway gene clusters evolved independently and by different mechanisms in fungi. Proc Natl Acad Sci U S A 107(22): 10136-10141.

Slot, J. C. and A. Rokas (2011). Horizontal transfer of a large and highly toxic secondary metabolic gene cluster between fungi. Curr Biol 21(2): 134-139.

Staal, J., M. Kaliff, et al. (2008). RLM3, a TIR domain encoding gene involved in broad-range immunity of Arabidopsis to necrotrophic fungal pathogens. Plant J 55(2): 188-200.

Su'udi, M., M. G. Kim, et al. (2011). Arabidopsis cell death in compatible and incompatible interactions with Alternaria brassicicola. Mol Cells 31(6): 593-601.

Sweat, T. A., J. M. Lorang, et al. (2008). Characterization of natural and induced variation in the LOV1 gene, a CC-NB-LRR gene conferring victorin sensitivity and disease susceptibility in Arabidopsis. Mol Plant Microbe Interact 21(1): 7-19.

Sweigard, J., Chumley, F., Carroll, A., Farrall, L., and Valent, B. (1997). A series of vectors for fungal transformation. Fung. Gen. Newsl.(44): 52-53.

Tanaka, M., T. Fujimori, et al. (2000). Biosynthesis of depudecin, a metabolite of Nimbya scirpicola. Biosci Biotechnol Biochem 64(2): 244-247.

Taunton, J., C. A. Hassig, et al. (1996). A Mammalian Histone Deacetylase Related to the Yeast Transcriptional Regulator Rpd3p. Science 272(5260): 408-411.

Thines, B., L. Katsir, et al. (2007). JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. Nature 448(7154): 661-665.

Thomma, B. P. (2003). Alternaria spp.: from general saprophyte to specific parasite. Mol Plant Pathol 4(4): 225-236.

Thomma, B. P., K. Eggermont, et al. (1998). Separate jasmonate-dependent and salicylate-dependent defense-response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens. Proc Natl Acad Sci U S A 95(25): 15107-15111.

Thomma, B. P., I. Nelissen, et al. (1999). Deficiency in phytoalexin production causes enhanced susceptibility of Arabidopsis thaliana to the fungus Alternaria brassicicola. Plant J 19(2): 163-171.

Tian, L. and Z. J. Chen (2001). Blocking histone deacetylation in Arabidopsis induces pleiotropic effects on plant gene regulation and development. Proc Natl Acad Sci U S A 98(1): 200-205.

Ton, J., J. A. Van Pelt, et al. (2002). Differential effectiveness of salicylate-dependent and jasmonate/ethylene-dependent induced resistance in Arabidopsis. Mol Plant Microbe Interact 15(1): 27-34.

Tweedy, B. G. and D. Powell (1963). The Taxonomy of Alternaria and Species of This Genus Reported on Apples. Botanical Review 29 (3): 405-412.

Ullstrup, A. J. (1941). Inheritance of susceptibility to infection by Helminthosporium maydis race 1 in maize. J. Agric. Res. 63: 331-334.

van Berkel, W. J., N. M. Kamerbeek, et al. (2006). Flavoprotein monooxygenases, a diverse class of oxidative biocatalysts. J Biotechnol 124(4): 670-689.

van Wees, S. C., H. S. Chang, et al. (2003). Characterization of the early response of Arabidopsis to Alternaria brassicicola infection using expression profiling. Plant Physiol 132(2): 606-617.

Veronese, P., X. Chen, et al. (2004). The BOS loci of Arabidopsis are required for resistance to Botrytis cinerea infection. Plant J 40(4): 558-574.

Vining, L. C. (1990). Functions of secondary metabolites. Annu Rev Microbiol 44: 395-427.

Vining, L. C. (1992). Secondary metabolism, inventive evolution and biochemical diversity--a review. Gene 115(1-2): 135-140.

Walton, J. D. (1996). Host-selective toxins: agents of compatibility. Plant Cell 8(10): 1723-1733.

Walton, J. D. (2000). Horizontal gene transfer and the evolution of secondary metabolite gene clusters in fungi: an hypothesis. Fungal Genet Biol 30(3): 167-171.

Walton, J. D. (2006). HC-toxin. Phytochemistry 67(14): 1406-1413.

Walton, J. D., E. D. Earle, et al. (1982). Purification and structure of the host-specific toxin from Helminthosporium carbonum race 1. Biochem Biophys Res Commun 107(3): 785-794.

Walton, J. D. and D. G. Panaccione (1993). Host-selective toxins and disease specificity: perspectives and progress. Annu Rev Phytopathol 31: 275-303.

Westerheide, S. D., J. Anckar, et al. (2009). Stress-inducible regulation of heat shock factor 1 by the deacetylase SIRT1. Science 323(5917): 1063-1066.

Wiest, P. K. W., Michael R. Jacobs, Anne B. Morrissey, Tom I. Abelson, William Witt and Michael M. Lederman (1987). Alternaria Infection in a Patient with Acquired Immunodeficiency Syndrome: Case Report and Review of Invasive Alternaria Infections. Reviews of Infectious Diseases 9(4): 799-803.

Wight, W. D., K. H. Kim, et al. (2009). Biosynthesis and role in virulence of the histone deacetylase inhibitor depudecin from Alternaria brassicicola. Mol Plant Microbe Interact 22(10): 1258-1267.

Winkler, C. J., A. Ponce, et al. (2010). Groucho-mediated repression may result from a histone deacetylase-dependent increase in nucleosome density. PLoS One 5(4): e10166.

Wolpert, T. J., L. D. Dunkle, et al. (2002). Host-selective toxins and avirulence determinants: what's in a name? Annu Rev Phytopathol 40: 251-285.

Wong, S. and K. H. Wolfe (2005). Birth of a metabolic gene cluster in yeast by adaptive gene relocation. Nat Genet 37(7): 777-782.

Wu, K., K. Malik, et al. (2000). Functional analysis of a RPD3 histone deacetylase homologue in Arabidopsis thaliana. Plant Mol Biol 44(2): 167-176.

Wu, K., L. Tian, et al. (2003). Repression of gene expression by Arabidopsis HD2 histone deacetylases. Plant J 34(2): 241-247.

Yoshida, M., M. Kijima, et al. (1990). Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. J Biol Chem 265(28): 17174-17179.

Yoshida, S., S. Maruyama, et al. (2010). Horizontal gene transfer by the parasitic plant Striga hermonthica. Science 328(5982): 1128.

Young, C., L. McMillan, et al. (2001). Molecular cloning and genetic analysis of an indole-diterpene gene cluster from Penicillium paxilli. Mol Microbiol 39(3): 754-764.

Zhang, S., B. J. Monahan, et al. (2004). Indole-diterpene gene cluster from Aspergillus flavus. Appl Environ Microbiol 70(11): 6875-6883.

Zheng, Y., J. D. Szustakowski, et al. (2002). Computational identification of operons in microbial genomes. Genome Res 12(8): 1221-1230.

Zhou, C., L. Zhang, et al. (2005). HISTONE DEACETYLASE19 is involved in jasmonic acid and ethylene signaling of pathogen response in Arabidopsis. Plant Cell 17(4): 1196-1204.