DETECTION OF QoI FUNGICIDE RESISTANCE AND GENOMIC ANALYSIS FOR IDENTIFICATION OF POLYKETIDE SYNTHASES AND METHYLTRANSFERASES IN *ASCOCHYTA RABIEI*

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Javier Andres Delgado

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Detection of QoI fungicide resistance and genomic analysis for identification of polyketide synthases and methyltransferases in *Ascochyta rabiei*

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

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ABSTRACT

Delgado, Javier Andres; Ph.D.; Department of Plant Pathology; College of Agriculture, Food Systems, and Natural Resources; North Dakota State University; September 2011. Detection of QoI Fungicide Resistance and Genomic Analysis for Identification of Polyketide Synthases and Methyltransferases in *Ascochyta rabiei*. Major Professors: Dr. Rubella S. Goswami and Dr. Samuel G. Markell.

Ascochyta blight is the most important disease of chickpeas in North America since the fungal pathogen *Ascochyta rabiei* was introduced to this region in 1986. Ascochyta blight epidemics can cause yield and economic losses of up to 100%. Currently available chickpea cultivars are partially resistant to *A. rabiei,* and the disease is primarily managed with the aid of seed and foliar fungicide treatment throughout the growing season. Development of resistance to highly effective fungicides such as the quinone outside inhibitor (QoI) fungicides has therefore significantly threatened chickpea production in the US and Canada. In this dissertation, the mutation associated with resistance to QoI fungicides was identified in *A. rabiei* in North Dakota from which a quick and accurate diagnostic assay was developed for the detection of QoI resistant isolates of *A. rabiei*. The detection of QoI resistant isolates is important for designing management strategies aimed to controlling *A. rabiei* in the field. Polyketide synthases (PKSs) and methyltransferases have been associated with virulence in several fungi but these genes have not been identified or characterized previously in *A. rabiei*. Therefore, a bioinformatic resource was developed for the identification of PKS proteins from the genome of *A. rabiei*. This was developed using the β-ketoacyl synthase and acyltransferase domains from PKS proteins exclusively belonging to the fungal species and was used to identify PKS genes from the *A. rabiei* genome. Several methyltransferase genes were also identified using a similar strategy. The characterization of the identified *A. rabiei* PKSs and methyltransferases was conducted to study the effect of the divalent cations present in chickpea

seed extracts on the accumulation of phytotoxic compounds, vegetative growth, and conidial production. This study provides an insight into the effect of these divalent cations on the expression of genes that modulate biological processes that may be directly associated with infection and colonization of the host.

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LITERATURE REVIEW

Production of Chickpeas

Chickpea (*Cicer arietinum* L.) is an important self-pollinated, annual edible legume that belongs to the family Leguminosae (Cubero, 1987). This crop was domesticated approximately 9,500 to 10,500 years ago in the Levant, which is comprised of today's geographical region along the eastern shore of the Mediterranean Sea, including Syria, Lebanon, Israel, and western Jordan (Abbo *et al.*, 2003). Chickpea and chickpea-based products are used for human consumption and animal feed because they are an excellent source of protein and carbohydrate (Jodha and Subba Rao, 1987; Saxena, 1987). Chickpea plants as well as other legume crops have the ability to fixate atmosphetic nitrogen that increases soil fertility, causing legumes to be highly recommended in rotations with crops such as cereals (Jodha and Subba Rao, 1987). In 2009, chickpeas were grown in 52 countries throughout Africa, America, Asia, Europe and Oceania. In the same year, 78,300 hectares of chickpeas were harvested in the United States and Canada with a yield of 3.6 tons per hectare, which corresponded to 0.68% and 5.6% of the world's harvested area and yield, respectively (FAOSTAT, 2011). Chickpea production in the United States during this period was led by the state of Washington, followed by Idaho, California, North Dakota, and Montana with 227.3, 188.6, 129.1, 88.2, and 8.2 tons, respectively (USDA NASS, 2011).

There are two types of chickpea plants known as Kabuli and Desi. These two types have been separated according to plant and seed characteristics. Kabuli-type chickpea most commonly have large, uni-foliate leaves while Desi-type chickpea have small, fern-like leaves. Seeds of Kabuli type are larger than those of Desi type. Kabuli seeds are also commonly rounded and pale with a thin coat, while Desi seeds are colored with thick or shriveled coats. Kabuli chickpeas are the most

commonly sold type of chickpeas in the United States and Canada. These are sold as either dried or canned chickpeas, and also as hummus (van Der Maesen, 1987).

Chickpea plants thrive in arid or semi-arid conditions. High humidity, rainfall and sprinkler irrigation have detrimental effects to the plant causing growth inhibition, health problems, and even delaying maturity (Abbo *et al.*, 2003; Saxena, 1987; van Der Maesen, 1987). Worldwide, chickpea fields have been reported to be affected by over 50 different pathogens (Nene and Reddy, 1987). However, Ascochyta blight, a disease that has been reported in over 35 countries, is the most important disease in the United States and Canada (Chen *et al.*, 2004a; Gan *et al.*, 2006; Khan *et al.*, 1999; Trapero-Casas and Kaiser, 1992a; 1996).

Ascochyta Blight of Chickpeas and its Causal Agent

Ascochyta blight is the most important disease of chickpeas (*Cicer arietinum*) worldwide, causing severe yield losses and significantly reducing seed quality (Chen *et al.*, 2004a; Gan *et al.*, 2006; Nene and Reddy, 1987; Shtienberg *et al.*, 2000). Chickpea production and acreage have decreased in United States and Canada since the introduction of *A. rabiei*-infected chickpea seeds into the production areas (Chen *et al.*, 2004a; Chongo *et al.*, 2004; Trapero-Casas and Kaiser, 1992). Ascochyta blight of chickpea is caused by the necrotrophic ascomycete *Ascochyta rabiei* (Pass.) Labr. (teleomorph *Didymella rabiei* (Kovacheski) v. Arx), which belongs to the phylum Ascomycota, class Dothideomycetes, and order Pleosporales (Alexopoulos *et al.*, 1996; Tree of Life Project, 2007).

Symptoms of Ascochyta blight often develop under cool, wet weather producing lesions in all above ground plant parts, including leaves, stems, and pods. Infections may occur at any growth stage of chickpea plants (Kaiser, 1997). The symptoms are visualized as concentric rings of black

pycnidia within round brown to gray lesions (Akem, 1999; Gan *et al.*, 2006; Jayakumar *et al.*, 2005). Infected chickpea seeds may appear to be shrunken or shriveled with brown to gray discoloration on the seed coat, while more severe infections may cause deep brown lesion or cankers with concentric rings of pycnidia on the seeds (Dey and Singh, 1994). Sowing of infected chickpea seeds have been found to lead to lower germination rates, lower seedling emergence, damping off after emergence, stem lesions, plant stunting and yellowing. Also, the sowing of infected chickpea seeds into new geographical areas may caused severe epidemics due to introduction of new genetic material of the pathogen (Chongo *et al.*, 2004; Jayakumar *et al.*, 2005; Kaiser *et al.*, 1973; Rhaiem *et al.*, 2007; Taylor and Ford, 2007; Udupa *et al.*, 1998). Ascochyta blight epidemics have been commonly reported in the production areas of the United States and Canada, since the introduction of *A. rabiei* in North America in 1986 (Kaiser and Hannan, 1987; Kaiser, 1997).

The teleomorph *Didymella rabiei* is heterothallic for which mating type idiomorphs MAT1-1 and MAT1-2 have been described (Barve *et al.*, 2003). Both mating types have been found to occur at similar frequencies in chickpea fields from the Pacific Northwest and North Dakota in the United States, and in chickpea fields from Saskatchewan, Canada (Armstrong-Cho *et al.*, 2001; Barve *et al.*, 2003; personal communication Dr. Steven Meinhardt). *D. rabiei* overwinters as a saprophyte on chickpea debris developing the melanized sexual reproductive structures called pseudothecia in which hyaline, two-celled ascospores are formed only at temperatures between 5 and 10 °C. Moisture has been reported to be critical for pseudothecia formation and ascospore discharge (Gamliel-Atinsky *et al.*, 2005; Trapero-Casas and Kaiser, 1992b). The anamorph *A. rabiei* produces hyaline, oval-shaped asexual spores called pycnidiospores (Kovachevski, 1936; Luthra and Sattar, 1935; Singh *et al.*, 1997). Young pycnidiospores are single-celled and as they mature, some of the pycnidiospores may become two-celled (Kovachevski, 1936; Luthra and Sattar, 1935; Singh *et al.*,

1997). Pycnidiospores ooze from melanized, asexual reproductive structures called pycnidia in the presence of moisture (Akamatsu *et al.*, 2010; Nene and Reddy, 1987). The optimum temperature for pycnidiospore germination is 20 °C. However, pycnidiospores are able to germinate from 5 to 32 °C. Ascospores and pycnidiospores can germinate at temperatures lower and higher than 20 °C but significant decrease of the overall disease severity has been reported when both ascospores and pycnidiospores were exposed to temperatures higher than 20 °C (Trapero-Casas and Kaiser, 1992a). Both these spores are important in disease development, but ascospores have been proven to be more resistant to adverse conditions than pycnidiospores. Moreover, ascospores are able to germinate faster and in a wider range of relative humidity than pycnidiospores. Both ascospores and pycnidiospores form germ tubers after 2 hours of favorable conditions (Armstrong-Cho *et al.*, 2004; Trapero-Casas and Kaiser, 1992a, 2007). Ascochyta blight of chickpeas has been found to be favored by periods of leaf wetness of at least 4 hours. Longer periods of leaf wetness have been correlated with an increase in disease severity (Trapero-Casas and Kaiser, 1992a).

The infection process of *A. rabiei* on chickpea starts with spore landing onto the plant surface. This spore germinates under favorable conditions producing a germ tube, which secretes a mucilagenous substance that aids the adhesion of the spore to the plant cuticle (Jayakumar *et al.*, 2005; Tivoli and Banniza, 2007). An appressoria-like structure is developed at the end of the germ tube, which is able to penetrate leaves and stems (Tivoli and Banniza, 2007). Histological studies with *A. rabiei* transformed with GUS (*E. coli* β-glucuronidase) showed direct penetration of the cuticle and hydathodes. This fungus spreads mainly intercellularly within the first 24 hours after inoculation in the mesophyll tissue (Köhler *et al.*, 1995). Hyphae can move from infected leaves to the stem colonizing non-lignified and the cortical parenchyma tissues (Jayakumar *et al.*, 2005). The fungus can be often found in the phloem, but rarely in the xylem. Moreover, pycnidia have been

found near the vascular tissue in chickpea stems (Köhler *et al.*, 1995). Fungal colonization of the host involves diffusion of toxins and cell wall degrading enzymes that aids the fungus to overcome plant physical barriers. The appressoria-like structure of *A. rabiei* has been found to be non-melanized and to secrete cell wall-degrading enzymes, such as cutinases, pectinases, and xylanases, to aid the penetration of this fungal pathogen into the plant (Jayakumar *et al.*, 2005; Tivoli and Banniza, 2007).

Most fungi in the order of the Pleosporales including *A. rabiei* are considered to be a necrotrophic plant pathogens due to the fact that during plant infection these fungi secrete cell wall-degrading enzymes and phytotoxins that contribute to the destruction of the plant tissue surrounding the infection point (Oliver and Solomon, 2010). The most studied and important phytotoxins produced by *A. rabiei* are the polyketide solanapyrones A, B, and C, which are produced during the conidiation (Hölh *et al.*, 1991; Kaur, 1995; Jayakumar *et al.*, 2005; Latif and Strange, 1993). This fungus can also produce solanapyrones on synthetic liquid media amended with chickpea seed extracts or certain divalent cations. *A. rabiei* has been reported to be unable to synthesize solanapyrone A, B, and C in the absence of zinc. The same research also showed that solanapyrone production is greatly reduced in the absence of calcium, cobalt, and manganese (Chen and Strange, 1991). Chickpea seedlings treated with these phytotoxins have been shown to develop Ascochyta-like symptoms (Hölh *et al.*, 1991; Jayakumar *et al.*, 2005; Latif and Strange, 1993). However, to date only solanapyrone C has been detected from chickpea tissues infected with *A. rabiei* (Shahid and Riazuddin, 1998).

Field isolates of *A. rabiei* have been shown to have great variability in terms of pathogenicity in most countries where chickpeas are grown (Chen *et al.*, 2004a; Chongo *et al.*, 2004; Gowen *et al.*, 1989; Jamil *et al.*, 1993; Khan *et al.*, 1999; Rhaiem *et al.*, 2007; Udupa *et al.*

1998; Vail and Banniza, 2004). Pathogenic variability of *A. rabiei* has been thought to be attributed to different pathotypes of its field populations. A pathotype has been defined as pathogenicity of an isolate on a set of differential cultivars with the purpose of classifying pathogenic variations of a given pathogen (Agrios, 2005). Isolates of *A. rabiei* have been classified into different pathotypes using different pathotype systems since 1974 (Akem, 1999; Chen *et al.*, 2004a; Chongo *et al.*, 2004; Toker and Çanci, 2003; Udupa *et al.*, 1998). The current three-pathotype system has been developed and validated to explain the pathogenic variability of *A. rabiei* using a proposed set of differential chickpea accessions (Chen *et al.*, 2004a; Toker and Canci, 2003; Udupa *et al.*, 1998). This three-pathotype system can be used to classify isolates from different parts of the world for better comparison (Chen *et al.*, 2004a; Udupa *et al.*, 1998). In this system, isolates classified as pathotype I are the least aggressive, while isolates classified as pathotype III correspond to the most aggressive isolates of *A. rabiei* (Chen *et al.*, 2004a). Since 2002, all isolates of *A. rabiei* in the United States have been found to belong to pathotype II (Wise, 2008).

The genetic variability of *A. rabiei* has been characterized in field populations from countries such as Canada, Syria, and Tunisia. Molecular studies using RAPD and SSR markers have demonstrated the high genetic diversity of this pathogen within a certain geographical area, and also among isolates from different countries (Chongo *et al.*, 2004; Geistlinger *et al.*, 2000; Rhaien *et al.*, 2007; Udupa *et al.*, 1998). Several researchers have suggested that the development of more aggressive isolates of *A. rabiei* within field populations can be attributed to three important factors: 1) the movement of chickpea seeds between distant geographical areas, 2) the introduction of more tolerant chickpea cultivars, and 3) the presence of both mating types of *A. rabiei* in most chickpea growing areas around the world (Chongo *et al.*, 2004; Rhaiem *et al.*, 2007; Taylor and Ford, 2007; Udupa *et al.*, 1998). Recently, Imtiaz *et al.*, (2011) reported a new pathotype IV within

field populations of *A. rabiei* in Syria. Isolates belonging to this newly described pathotype were reported to be highly virulent because they are able to kill chickpea differential cultivars that the other pathotypes are not able to kill. Moreover, the locus-specific microsatellite markers developed by Geistlinger *et al.*, (2000) found polymorphism among all four pathotypes of *A. rabiei* (Imtiaz *et al.*, 2011).

Ascospores are commonly the primary inoculum of Ascochyta blight of chickpeas in countries where both mating types are present such as the United States and Canada (Armstrong-Cho *et al.*, 2001; Gan *et al.*, 2006; personal communication Dr. Steven Meinhardt). In countries where only one mating type has been found, such as Australia, the primary inoculum is pycnidiospores (Taylor and Ford, 2007). Also, infected seeds may serve as primary inoculum when these are introduced in *Ascochyta*-free geographical areas. Infected seeds may cause early infections leading to severe epidemics, which can cause 50 to 100% in yield losses (Gan *et al.*, 2006; Haware *et al.*, 1995; Kaiser, 1997; Shahid *et al.*, 2008; Tivoli and Banniza, 2007). Once the fungus has penetrated the plant tissue, symptoms can develop quickly under cool and wet weather (Tivoli and Banniza, 2007; Trapero-Casas and Kaiser, 1992a). Ascochyta blight is a polycyclic disease. Pycnidiospores oozed out of mature pycnidia are dispersed for a short distance by rain-splash or sprinkler irrigation (Tivoli and Banniza, 2007).

A. rabiei can overwinter as either pseudothecia or pycnidia on chickpea residues (Akem, 1999). These overwintering structures have been reported to survive as long as four years on chickpea debris in the United States and Canada. However, it has been shown that viability of *A. rabiei* field inoculum decreases after three years (Gossen and Miller, 2004). This fungus can also survive on or in chickpea seeds for as long as 13 years under storage conditions (Kaiser, 1997).

Disease Management of Ascochyta Blight of Chickpeas

The management of Ascochyta blight in the United States and Canada has been of great concern since the introduction of this disease to the chickpea growing areas. This disease has been managed using an integrated approach that combines cultural practices, seed treatment, host resistance, and foliar chemical treatment.

Standard cultural practices to manage Ascochyta blight in the field include three-year crop rotations with non-host crops, and removal of chickpea residue. These cultural practices have been shown to decrease the incidence, severity, and onset of the disease (Davidson and Kimber, 2007; Gan *et al.*, 2006). Chickpea growers are advised to sow clean or certified disease-free seeds. In order to have access to crop insurance, chickpea growers of the United States and Canada are required to sow chickpea seeds with a maximum threshold for Ascochyta seed infection of 0.3% as determined by laboratory testing (Gan *et al.*, 2006; Wise, 2008). There are several fungicide seed treatments available for use in Ascochyta blight and seedling diseases of chickpeas. Chemistries labeled in North Dakota to be used on chickpea seeds as treatment for seed-borne and soil-borne fungi include azoxystrobin, fludioxonil, pyraclostrobin and trifloxystrobin. Thiabendazole is registered for use on seed-borne Ascochyta (McMullen and Markell, 2010).

To date, there are no commercially available chickpea cultivars that exhibit complete resistance to *A. rabiei*. However, pulse breeding programs have developed cultivars with partial resistance against this pathogen. In this regard, it is recommended that an integrated approach be used to manage Ascochyta blight of chickpea, i.e. the use of cultivars with partial host resistance in combination with chemical seed and foliar fungicide treatments (Davidson and Kimber, 2007).

Host resistance has been introduced into chickpea cultivars from wild *Cicer* species as an effort of breeding programs around the world (Akem, 1999; Iruela *et al.*, 2007; Millan *et al.*, 2006;

Singh *et al.*, 2005). However, host resistance is often overcome quickly in the field by the pathogen. Many researchers attribute this situation to the development of new pathotypes within *A. rabiei* field populations (Udupa *et al.*, 1998). Moreover, partial resistance against to Ascochyta blight has not been found to be effective in fields with high inoculum pressure and during flowering (Basandri *et al.*, 2007; Chongo and Gossen, 2001).

Methods for assessment of resistance levels of chickpea lines against Ascochyta blight has not been found to be consistent among pulse breeding programs (Wise, 2008). Some programs rely on seedling inoculation or detached leaf assays, while others relay on phytotoxicity assays using culture filtrates containing solanapyrones (Ahmed *et al.*, 2006; Dolar *et al.*, 1994;, Tar'an *et al.*, 2007). Resistance against *A. rabiei* pathotype I is thought to be conditioned by a single major gene, while resistance to pathotype II may be conditioned by at least two quantitative trait loci, also known as QTLs (Flandez-Galvez *et al.*, 2003; Iruela *et al.*, 2007; Millan *et al.*, 2006; Tekeoğlu *et al.*, 1994; van Rheenen and Haware, 1994).

Foliar fungicide applications are important in the integrated management of Ascochyta blight of chickpeas. Currently, there are several labeled fungicides for use against Ascochyta blight of chickpeas, including chemistries with protective, preventative and post-infection properties grouped according to their mode of action by the Fungicide Resistance Action Committee (FRAC). Chlorothalonil and mancozeb, two major protectants, are labeled for Ascochyta blight of chickpeas in North Dakota. They belong to the FRAC groups M5 and M3, respectively, and have a multi-site mode of action. Being protective in action, they need to be applied on the plant before *A. rabiei* infections occur. The recommended application time is between two to four weeks before flowering (McMullen and Markell, 2010).

Fungicides with preventative and post-infection/curative properties have also been labeled for their use against Ascochyta blight of chickpeas. These include azoxystrobin and pyraclostrobin (FRAC group 11), boscalid (FRAC group 7) and prothioconazole (FRAC group 3) (FRAC, 2010a; McMullen and Markell, 2010). The fungicides azoxystrobin and pyraclostrobin are quinone outside inhibitors (QoIs) that halt spore germination by obstructing the quinone outside binding site of the cytochrome bc_1 complex (complex III), which results in energy deficiency because of ATP depletion (Bartlett *et al.*, 2002; Grasso *et al.*, 2006). These QoI fungicides have also been shown to decrease disease and increase yields on *A. rabiei* infected plots when used as part of a fungicide spray schedule (Chang *et al.*, 2007; Wise *et al.*, 2006). QoI fungicides have been widely used in disease management programs of many fungi from the phyla Ascomycetes, Basidiomycetes and Oomycetes (Bartlett *et al.*, 2002; Grasso *et al.*, 2006). However, they are considered to be at high risk for the development of fungicide resistance in field populations of fungi (Brent and Hollomon, 2007; Chin *et al.*, 2001; Gisi *et al.*, 2002; Grasso *et al.*, 2006). QoI fungicides are recommended to be used in alternation or in tank-mixes with foliar fungicides of a different FRAC groups to avoid or minimize the risk of developing fungicide resistance in *A. rabiei* in fields where resistance has not been reported yet (McMullen and Markel, 2010). QoI fungicides were released in the agricultural market place in 1996 and since then, many fungal pathogens have been reported to be less sensitive to these chemistries. Resistance to QoI fungicides occur because of single amino acid substitutions in the cytochrome b protein (Chin *et al.*, 2001; FRAC, 2010b; Gisi *et al.*, 2002; Grasso *et al.*, 2006; Kim *et al.*, 2003; Ma *et al.*, 2003; Pasche *et al.*, 2005; Sierotzki *et al.*, 2002). Different amino acid substitutions confer different levels of resistance to QoI fungicides (Brausser *et al.*, 1996; Grasso *et al.*, 2006). In this regard, a lower level of QoI resistance is conferred by the substitution of phenylalanine into leucine at position 129 (F129L). Also, the substitution of glycine into arginine at

position 137 (G137R) confers similar level of QoI resistance as F129L. The highest level of QoI resistance reported among fungal pathogens is conferred by a substitution from glycine to alanine at position 143 (G143A). Amino acid substitution G143A may result in complete loss of QoI sensitivity (FRAC, 2006, FRAC, 2007b; Gisi *et al.*, 2002).

In North Dakota, azoxystrobin and pyraclostrobin were the only locally systemic fungicides with preventative and post-infection/curative properties registered for their use on chickpeas before boscalid and prothioconazole were labeled for use in this crop in 2003 and 2007, respectively. However, boscalid and prothioconazole were allowed to be used on chickpeas in North Dakota as the Environmental Protection Agency (EPA) section 18 emergency exemptions (Wise, 2008). Resistance to QoI fungicides has been reported in major chickpea growing areas of the United States and Canada (Chang *et al.*, 2007; Gossen and Anderson, 2004; Wise, 2008). In the United States, QoI resistance has been reported in Montana and North Dakota (Wise, 2008; Wise *et al.*, 2008; 2009), and recently in Nebraska and South Dakota (unpublished data). Statewide surveys conducted in North Dakota have found that QoI resistant isolates of *A. rabiei* occur at high frequency. Wise *et al.*, (2009) reported a 300- and 600-fold decrease in sensitivity to azoxystrobin and pyraclostrobin, respectively, in field isolates of *A. rabiei*. The identification of QoI resistant isolates determines whether or not QoI fungicides can be used to manage Ascochyta blight of chickpeas. The use of QoI fungicides in fields affected by QoI-resistant isolates could cause severe economic losses due to the inability of the chemical to control disease. This makes it very important to monitor field populations of *A. rabiei* for shifts in sensitivity to QoI fungicides. Moreover, the monitoring of shifts in fungicide sensitivity in fungal plant pathogens is fundamental for the conservation of these antifungal chemistries (Wise, 2008). To date, this monitoring was conducted using *in vitro* conidial germination assay on solid media amended with QoI fungicides at different

rates (Wise *et al.*, 2008; 2009). This technique is very laborious and time-consuming. Therefore, there is a need for the development of a quick, effective and reliable technique for the monitoring of QoI resistance in the field isolates of *A. rabiei*. The particular mutation associated with QoI resistance has not been characterized in field isolates of *A. rabiei* collected in the United States, but the level of QoI sensitivities displayed by *A. rabiei* isolates suggests that amino acid substitution G143A may be present in field isolates of *A. rabiei* of North Dakota.

Virulence Factors Involved in Ascochyta Blight of Chickpeas

The molecular basis of the interaction between *A. rabiei* and chickpeas is still poorly understood (Geistlinger *et al.*, 2000). Coram and Pang (2005a; 2005b; 2006) studied the defense response of chickpea accessions against *A. rabiei* at a molecular level by constructing an expressed sequence tag (EST) library using a highly resistant, non-commercial chickpea accession. This library helped to study the defense response of susceptible, partially resistant and resistant chickpea accessions during the disease development using microarrays and real-time PCR. However, these studies were not focused on the expression profile of the pathogen. Geistlinger *et al.*, (2000) proposed that characterization of fungal pathogenicity and virulence factors is important in order to design adequate disease management strategies against Ascochyta blight of chickpeas. Molecular studies on this fungal pathogen, though in limited number, have led to the generation of *A. rabiei* strains transformed using *Agrobacterium tumefaciens* with reduced production of solanapyrone A; and also altered pathogenicity profile (Mogensen *et al.*, 2006; White and Chen, 2007). The construction of a genomic phage library led to the identification of acyl-CoA ligase and a melanin polyketide synthase, which have been characterized as pathogenicity factors in other fungal species (White and Chen, 2007). However, the only *A. rabiei* pathogenicity factor that has

been fully characterized is the polyketide synthase (PKS) melanin synthase (Akamatsu *et al.*, 2010). Moreover, we identified a PKS likely to code for melanin synthase and another reducing PKS in *A. rabiei* using a genome-walking approach with four digested-genomic DNA libraries (Delgado *et al.*, 2009). These last two PKSs are yet to be characterized regarding their effect on disease development on chickpea seedlings.

Virulence factors have been defined as gene products that contribute towards induction and development of disease without being essential for pathogenicity (Agrios, 2005). Trichothecene mycotoxins are examples of fungal virulence factors. Disruption of the trichodiene synthase (*Tri5*) gene abolishes the production of 4,15-diacetoxyscirpenol in *Gibberella pulicaris* reducing the virulence levels of the *Tri5*-mutants in parsnips compared to the wild-type strains. However, the disruption of the *Tri5* gene in *G. pulicaris* did not show differences in the virulence levels between the *Tri5*-mutants and the wild-type strain when infecting potato tubers (Desjardins *et al.* 1992). Thus, a gene product that acts as a virulence factor for a specific pathosystem may not be a virulence factor for another. In *A. rabiei*, virulence factors have not been extensively characterized. Melanin and solanapyrones are the only two studied virulence factors in this pathosystem, both of which are synthesized by PKS pathways (Akamatsu *et al.*, 2010; Chen, *et al.*, 2004b; Hölh *et al.*, 1991, Hopwood and Sherman, 1990; White and Chen, 2007). Another, secondary metabolite produced by *A. rabiei* is cytochalasin D, which like solanapyrones, has also been identified in the culture filtrates of isolates of *A. rabiei*. This secondary metabolite is an alkaloid that inhibits actin polymerization (Latif and Strange, 1993).

Solanapyrones. Solanapyrones A, B and C are polyketide metabolites produced by both *A. rabiei* and *Alternaria solani*. These polyketides are non-host specific. In *A. rabiei*, their maximum production has been found to occur on the onset of conidiation. They have also been found in

conidial germination fluids, which suggests a possible role in early disease development (Jayakumar *et al.*, 2005). Solanapyrones have been found to aid in plant tissue penetration by *A. rabiei*. (Jayakumar *et al.*, 2005; Kaur, 1995) and have been correlated with seedling growth inhibition. However, solanapyrone production was found to correlate weakly with disease rating in nine chickpea cultivars (Hamid and Strange, 2000). Culture filtrates of *A. rabiei* containing solanapyrones have been shown to produce blight-like symptoms on chickpea leaves. Solanapyrones are thought to attack the plant cell membranes causing loss of turgor of parenchyma cells nearby the stele (Hamid and Strange, 2000; Hölh *et al.*, 1991; Kaur, 1995). They are highly reactive and very difficult to detect *in vivo*. In fact, only solanapyrone C has been detected in plant tissue (Shahid and Riazuddin, 1998). The inability to detect solanapyrone A and B in plant tissue maybe due to the fact that they are present in low concentrations in plant tissue and/or that solanapyrone A has been found to be rapidly metabolized by the glutathione/glutathione-S-transferase system (Hamid and Strange, 2000). Solanapyrone A has been reported to be more toxic than solanapyrone B and C in bioassays using chickpea protoplasts both independently and in combination. Chickpea shoots treated with solanapyrone A reported loss of turgor, shriveling and breakage of stems, accompanied by flame-shaped, chlorotic lesions in leaflets. Likewise, solanapyrone B was found to cause twisted, chlorotic leaflets; some of which could be abscised but unlike solanapyrone A it did not cause loss of turgor (Hamid and Strange, 2000).

Recently, the solanapyrone gene cluster was identified and characterized in the genome of *A. solani* (Kasahara *et al.*, 2010). In this cluster, six genes were identified to be involved in the synthesis of solanapyrones including a gene that encodes for a fungal-specific transcription factor (*Sol4*). Briefly outlining the pathway, solanapyrone A and B are synthesized from the precursor acyl coenzyme A (acyl CoA), which is converted into desmethylprosolanapyrone by the PKS enzyme

named Sol1. This intermediate product is converted into solanapyrone A by subsequential enzymatic reactions with a *O*-methyltransferase (Sol2), a cytochrome P450 monooxygenase (Sol6), and an flavin-dependent oxidase which also catalizes Diels/Alder cyclization (Sol3). Solanapyrone B is synthesized from solanapyrone A by a dehydrogenase enzyme (Sol3) (Kasahara *et al.*, 2010). The solanapyrone cluster has not been identified in the genome of *A. rabiei*.

Melanin. Although the appressoria-like structure of *A. rabiei* is not melanized as in other fungal pathogens such *Magnaporthe grisea* (Howard and Ferrari, 1989; Jayakumar *et al.*, 2005), the role of melanin in Ascochyta blight of chickpeas as fungal pathogenicity factor was first suggested when spontaneous non-melanized mutants of *A. rabiei* were found to be non-pathogenic on chickpea seedlings in greenhouse trials (Chen *et al.*, 2004b). After an extensive examination of these spontaneous albino mutants, we found that most of these spontaneous albino mutants do not produce pycnidia. Some albino mutants seemed to produce non functional pycnidia since they were misshapen and did not produce pycnidiospores. These pycnidia appeared to be non functional. Disruption of the *A. rabiei* melanin polyketide synthase gene showed that melanin is required for the protection of pycnidiospores from UV irradiation. Pycnidiospores formed in non-melanized pycnidia were less likely to germinate *in vitro* after treatment with UV irradiation (Akamatsu *et al.*, 2010).

Polyketide synthases (PKSs). Polyketide compounds are synthesized by multidomain enzymes called PKSs. Polyketide compounds are synthesized from acyl coenzyme A (acyl CoA) through Claisen condensations, which are sequential decarboxylation condensations (Crawford *et al.*, 2008; Gokhale *et al.*, 2007; Schümann and Hertweck, 2006; Shen, 2003; Weissman, 2008). Three types of PKSs have been identified in eukaryotic and prokaryotic organisms, of which the most commonly found are ones classified as type I PKSs. Bacterial type I PKSs are mainly modular

proteins, while all identified fungal type I PKSs are iterative. Modular PKSs are arranged in more than one module which contains a set of domains. The synthesized polyketide is passed from module to module until the final product is fully synthezised. On the other hand, iterative PKSs have only one module containing all PKS-specific domains necessary for the synthesis of a given polyketide compound. Iterative PKSs synthesize polyketide compounds using these protein domains repetitively until the final products are formed (Gaitatzis *et al.,* 2002; Gokhale *et al.,* 2007; Hoffmeister and Keller, 2007; Shen, 2003; Shen and Hutchinson, 1996; Wiesmann *et al.,* 1995). Type I iterative PKSs may have up to eight PKS-specific protein domains. However, functional type iterative PKSs must have the following minimum domain architecture: β-ketoacyl synthase (KS), an acyltransferase (AT), and an acyl carrier protein (ACP) domain (Hopwood and Sherman, 1990).

Polyketides compounds have been categorized into reduced and non-reduced polyketides based on the linear or aromatic structure of the keto group, respectively (Gónzalez-Lergier *et al.,* 2005). Likewise, PKSs can be categorized into non-reducing and reducing enzymes by phylogenetic analysis of the KS domain, which is the most conserved PKS domain and is important for predicting the potential function of each PKSs (Bingle *et al.,* 1999; Kroken *et al.,* 2003; Langfelder *et al.,* 2003; Nicholson *et al.,* 2001). Identification of PKSs has been conducted using different strategies from PCR cloning to genome mining (Bingle *et al.,* 1999; Bok *et al.,* 2006; Fujii *et al.,* 2005; Gaffoor *et al.,* 2005; Kellner and Zak, 2009; Torto-Alalibo *et al.,* 2005; White and Chen, 2007; Yang *et al.,* 1996). PKS genes have been identified from the genome of *Fusarium graminearum* and *Aspergillus* species using bioinformatic tools such as profile hidden markov models abbreviated as pHMMs (Bok *et al.,* 2006; Gaffoor *et al.,* 2005). pHMMs are statistical models that are built using related protein sequences for the identification of protein sequences that belong to the protein family being studied (Birney, 2001; De Fonzo *et al.,* 2007; Eddy, 1998, 2008; Schuster-Böckler *et al.,* 2004).

Methyltransferases. Methyltransferases such as histone and arginine methyltrasferases, are important regulators of gene expression in eukaryotic organisms at the chromatin level (Hamahata *et al.*, 1996; Mowen *et al.*, 2001; Tamaru and Selker, 2001). Recently, a methyltransferase named LaeA was identified to be a master transcription factor for gene clusters associated with secondary metabolism in *Aspergillus* species (Bok and Keller, 2004). LaeA methyltransferase as well as histone and arginine methyltransferases have a conserved S-adenosylmethionone (SAM) binding site protein domain, which is involved in chromatin modification by demethylation of heterochromatin into euchromatin. (Bok and Keller, 2004; Hamahata *et al.*, 1996; Mowen *et al.*, 2001; Tamaru and Selker, 2001). Functional characterization of LaeA has shown that disruption of this gene abolished the production of several natural products in *Aspergillus* species; and that its overexpression increases the synthesis of compounds such lovastatin and penicillin (Bok and Keller, 2004). LaeA-homolog proteins have been identified in several ascomycetes through bioinformatic analysis (Barreau *et al.*, 2011; Bok *et al.*, 2005; Butchko *et al.*, 2011; Howlett, 2006; Karimi *et al.*, 2011; Wiemann *et al.*, 2010; Wu *et al.*, 2011; Xing *et al.*, 2010). Therefore, it is believed that this LaeA may play a role in the global expression of secondary metabolism gene clusters (Gacek *et al.*, 2011; Keller *et al.*, 2005).

Ascochyta blight is the most severe disease affecting all chickpea growing areas. There has been research on the biology of its causal agent, ecology and epidemiology (Geistlinger *et al.*, 2000; White and Chen, 2007) primarily aimed at designing better disease management. However, very limited information regarding the pathogenicity and virulence factors involved in the disease development of the *A. rabiei*-chickpea pathosystem is available. Moreover, there is a need for developing efficient methods for avoiding development and spread of fungicide resistance. Therefore, the research presented in this dissertation focuses on the development of a diagnostic

assay for the detection of QoI resistance and on the characterization of PKS and methyltransferase genes associated to the production of phytotoxic compounds.

Specifically, the objectives of this dissertation were, first, the characterization of the cytochrome b gene fragment responsible for the shift in QoI sensitivities, to identify the most prevalent mutation associated with resistance, and development of a molecular diagnostic assay for the detection of QoI resistance in isolates of *A. rabiei*. Second, the development of an identification and categorization method for iterative type I fungal PKSs using a bioinformatics approach. And third, the identification of *Ascochyta rabiei* PKS and methyltransferase proteins associated with phytotoxicity using a genome mining approach.

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PAPER ONE. IDENTIFICATION OF THE CYTOCHROME B GENE MUTATION RESPONSIBLE FOR RESISTANCE TO QOI FUNGICIDES AND ITS DETECTION IN *ASCOCHYTA RABIEI* (TELEOMORPH *DIDYMELLA RABIEI*)

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Introduction

Ascochyta blight is the most important disease of chickpeas (*Cicer arietinum*) due to its yield-limiting and devastating effects on chickpea producing areas (Chen *et al.*, 2004a; Gan *et al.*, 2006; Nene and Reddy, 1987; Shtienberg *et al.*, 2000). This disease is caused by the ascomycete pathogen *Ascochyta rabiei* (Pass.) Labrousse (teleomorph: *Didymella rabiei* (Kovachevski) Arx). Ascochyta blight epidemics have been commonly reported in the production areas of United States and Canada. Foliar and stem infections may occur at any growth stage of chickpea plants (Kaiser, 1997). Chickpea production and acreage have decreased in the United States and Canada since the introduction of the Ascochyta blight to the production areas (Chen *et al.*, 2004a; Chongo *et al.*, 2004; Trapero-Casas and Kaiser, 1992).

In the United States and Canada, the management of this disease relies primarily on fungicide seed treatments and multiple foliar applications throughout the growing season (Chang *et al.*, 2007; Shteinberg *et al.*, 2000; Tivoli and Banniza, 2007). Quinone-outside inhibitor (QoI) fungicides have been used as a foliar fungicide treatment to manage Ascochyta blight in chickpeas. QoI fungicides block the cytochrome bc_1 complex (complex III) in the mitochondrial respiration

chain. This causes depletion of ATP that ultimately halts spore germination due to energy deficiency (Bartlett *et al.*, 2002; Grasso *et al.*, 2006a). Azoxystrobin and pyraclostrobin, two QoI fungicides belonging to FRAC group 11, are registered for use on chickpea in North Dakota (McMullen and Markell, 2010). These two QoI fungicides have both preventative and post-infection/curative activity (Bartlett *et al.*, 2002; FRAC 2010a). QoI fungicides are considered to be at high risk for developing resistance due to their site-specific mode of action (Brent and Hollomon, 2007a; Chin *et al.*, 2001; Gisi *et al.*, 2002; Grasso *et al.*, 2006a). Since the introduction of QoI fungicides into the market in 1996, several fungal pathogens have been reported to have developed reduced levels of sensitivity to QoI fungicides due to single amino acid substitutions in the cytochrome b protein, which is part of the cytochrome bc_1 complex (Chin *et al.*, 2001; FRAC, 2010b; Gisi *et al.*, 2002; Grasso *et al.*, 2006a; Kim *et al.*, 2003; Ma *et al.*, 2003; Pasche *et al.*, 2005). There are three amino acid substitutions conferring QoI resistance that are often found in the cytochrome b protein of fungal plant pathogens. These substitutions confer different levels of resistance to QoI fungicides (Brausser *et al.*, 1996; Grasso *et al.*, 2006a). A low level resistance is conferred by a change from phenylalanine to leucine at position 129 (F129L) and a change from glycine to arginine at position 137 (G137R). F129L and G137R substitutions may yield resistance factors that range between 5- to 15-fold (FRAC, 2006). Among fungal species the amino acid substitution from glycine to alanine at position 143 (G143A) is more commonly found and confers a much higher level of resistance, commonly a resistance factor of over 100 fold (Brent and Hollomon, 2007b; FRAC, 2006; Gisi *et al.*, 2002), that may result in complete loss of sensitivity to QoI fungicides. To date, there are 36 fungal species that have been reported to exhibit resistance to QoI fungicides. Of these, field populations of *Plasmopara viticola* and *Pyricularia grisea* have been found to harbor both the F129L and G143A mutations, while *Pyrenophora tritici-repentis* has been found to harbor F129L, G137R and G143A

mutations. However, these mutations have not been found to occur in the same isolate (Förster *et al.*, 2009; FRAC, 2010b; Heaney *et al.*, 2000; Kim *et al.*, 2003; Sierotzki *et al.*, 2005, 2007; Stammler *et al.*, 2006; Vincelli and Dixon, 2002).

QoI resistant isolates of *A. rabiei* were identified in Canadian chickpea production areas in 2004 (Chang *et al.*, 2007; Gossen and Anderson, 2004). In the United States, resistance to QoI fungicides was first detected in *A. rabiei* isolates from North Dakota in 2005 and from Montana a year later (Wise, 2008; Wise *et al.*, 2008, 2009). Since then, QoI resistant isolates of *A. rabiei* have been found at high frequency during statewide disease surveys of chickpea fields in North Dakota. Field isolates of *A. rabiei* have shown more than 300- and 600-fold decrease in sensitivity to azoxystrobin and pyraclostrobin, respectively (Wise, 2008; Wise *et al.*, 2008, 2009). The high level of resistance to QoI fungicides exhibited by *A. rabiei* isolates in this area suggests the presence of the G143A amino acid substitution. However, the mutation associated with resistance in North Dakota has not been identified. Currently, monitoring of resistance to azoxystrobin and pyraclostrobin in *A. rabiei* field isolates is conducted using an *in vitro* spore germination assay on fungicide-amended media (Wise 2008, Wise *et al.*, 2008, 2009). This assay can be very laborious and time-consuming. Thus, the first objective of this study was to determine the mutation(s) causing resistance to QoI fungicides in this region by cloning and characterizing the fragment of the cytochrome b gene that has been associated with the shift in QoI sensitivities in fungal plant pathogens. The second objective was to develop a PCR-based method for screening and monitoring for the appearance of QoI resistant isolates of *A. rabiei*.

Materials and Methods

Isolates of *Ascochyta rabiei* and inoculations of chickpea plants. This study was conducted with a total of 70 *A. rabiei* isolates showing different levels of QoI sensitivity (Table 1.1). Initially, eight isolates collected between the 2005 and 2006 growing seasons were used for cloning and sequencing of the cytochrome b gene fragment. Subsequently, 48 additional isolates, collected between 2005 and 2007, were scrutinized for the presence of the QoI resistance allele using a PCR-based assay. The QoI sensitivities of these 56 isolates had been previously determined using *in vitro* assays (Wise *et al.*, 2009). Fourteen additional isolates collected during the 2010 chickpea growing season were screened for detection of QoI resistant allele using the PCR-based assay. Spore germination assays were performed on selected isolates using azoxystrobin at a discriminatory dosage of 1µg/ml (Wise *et al.*, 2009). Chickpea plants (cv. Burpee) were inoculated according to the protocol described in Wise *et al.*, (2009). Infected leaves were collected 10 days after inoculation for PCR-based detection of wild-type and QoI mutant cytochrome b alleles.

Genomic DNA extraction. Isolate of *A. rabiei* were grown on potato dextrose broth (PDB) at 20 °C for 4 days in the dark. The extraction of genomic DNA (gDNA) was carried out with a rapid mini-preparation protocol according to Liu *et al.*, (2000) with modifications. Briefly, mycelium from each isolate was transferred to a 2-ml lysing matrix A tube (MP Biomedicals, Solon, OH) containing 500 µl of lysis buffer (400 mM Tris-HCl, pH 8.0; 60 mM EDTA, pH 8.0; 150 mM NaCl; 1% sodium dodecyl sulfate; 5 µl/ml β-mercaptoethanol) and disrupted using the FastPrep® homogenizer (MP Biomedicals, Solon, OH) set at speed 5.0 for 40 s. The lysate was incubated at 37 °C for 20 min with 2 µl/ml RNAse A (700 U/ml) (Qiagen, Valencia, CA). Subsequently, 150 µl of 3 M potassium acetate buffer (3 M potassium acetate, 2 M acetic acid, pH 4.8) was added and mixed by inversion. The mixture was centrifuged at 12,000 × *g* for 2 min. Then, 600 µl of the supernatant was transferred to

a new, clean 1.5-ml tube and centrifuged again at 12,000 × *g* for 2 min to eliminate the remaining cell debris, 400 µl of the supernatant was transferred to a new, clean 1.5-ml tube containing 800 µl of ice cold isopropanol and mixed by inversion. The mixture was incubated at -20 °C for 20-30 min and centrifuged at 16,000 × *g* for 5 min. The supernatant was discarded and the DNA pellet was washed with 300 µl of 80% ethanol at room temperature and centrifuged at 16,000 × *g* for 1 min. The supernatant was discarded and the pellet was air dried for 15-30 min. The DNA pellet was resuspended by incubation at 4 °C overnight in 50 µl of nuclease-free water.

DNA extraction from infected chickpea plants was conducted using DNeasy Plant Mini kit (Qiagen, Valencia, CA) with modifications. Briefly, an infected leaf was placed in a 2-ml lysing matrix A tube (MP Biomedicals, Solon, OH) containing 600 µl of Buffer AP1 and disrupted using the FastPrep® homogenizer (MP Biomedicals, Solon, OH) set at speed 6.5 for 45 s. The lysate was centrifuge at 4,000 × *g* for 30 s and incubated at 65 °C for 10 min with 2 µl/ml RNAse A (700 U/ml). The genomic DNA extraction proceeded as indicated by manufacturer's instructions.

Total RNA extraction. Isolates Ar01, Ar02, Ar03, Ar04, Ar05, Ar06, Ar07, and Ar08 were grown in 4 ml potato dextrose broth (PDB) in the absence of light at 26 °C for 2 days with continuous shaking (200 rpm). Total RNA was isolated using the RNeasy Plant Mini kit (Qiagen, Valencia, CA) with modifications. Fresh fungal mycelia from each isolate (100 mg) was transferred to 2-ml lysing matrix C tubes (MP Biomedicals, Solon, OH) containing 450 µl of buffer RLC and disrupted using the FastPrep® homogenizer (MP Biomedicals, Solon, OH) set at a speed of 6.5 for 45 s, and centrifuged at 16,000 × *g* for 5 min, 400 µl of the lysate was transferred to a QIAshredder spin columns placed in 2-ml collection tubes. From this point onwards, the protocol was continued according to manufacturer's instructions. Total RNA was quantified using Nanodrop 1000 at 260 nm (Thermo Scientific, Wilmington, DE) and brought to a final concentration of 100 ηg/µl. First strand

of complementary DNA (cDNA) was synthesized using QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA).

Table 1.1. Isolates of *Ascochyta rabiei* used for cytochrome b gene sequencing and MAMA-PCR

Isolate Code	Isolate ID	Origin	Year collected	Azoxystrobin ED50 (µg/ml)[1]	QoI sensitivity[2]	G143A screening[3]
Ar01	05 105M9	Ward Co, ND	2005	0.026	Sensitive	GGT allele
Ar02	05 SuCo1	Sully Co, SD	2005	0.029	Sensitive	GGT allele
Ar03	05 APSN4	Cass Co, ND	2005	0.034	Sensitive	GGT allele
Ar04	05 412CA9	Foster Co, ND	2005	0.039	Sensitive	GGT allele
Ar05	06 KND1A-2	Ward Co, ND	2006	3.480	Resistant	GCT allele
Ar06	06 212M1	Ward Co, ND	2006	8.080	Resistant	GCT allele
Ar07	06 STC2-5	Foster Co, ND	2006	16.230	Resistant	GCT allele
Ar08	06 KND2A-9	Ward Co, ND	2006	37.680	Resistant	GCT allele
Ar09	05 BND1	Ward Co, ND	2005	0.033	Sensitive	GGT allele
Ar10	05 BND2	Ward Co, ND	2005	0.030	Sensitive	GGT allele
Ar11	05 101M2	Ward Co, ND	2005	0.033	Sensitive	GGT allele
Ar12	05 207C2	Foster Co, ND	2005	0.031	Sensitive	GGT allele
Ar13	05 202STM2	Ward Co, ND	2005	0.030	Sensitive	GGT allele
Ar14	05 210C6	Foster Co, ND	2005	0.030	Sensitive	GGT allele
Ar15	05 101STM8	Ward Co, ND	2005	14.550	Resistant	GCT allele
Ar16	06 TLND14	McLean Co, ND	2006	22.150	Resistant	GCT allele
Ar17	06 BWEF2-8	Mountrail Co, ND	2006	16.130	Resistant	GCT allele
Ar18	06 FRL1	Cass Co, ND	2006	4.880	Resistant	GCT allele
Ar19	07 JB9-5	Hettinger Co, ND	2007	na[4]	Sensitive	GGT allele
Ar20	07 DF8	Ward Co, ND	2007	na	Resistant	GCT allele
Ar21	06 BND11	Ward Co, ND	2006	na	Resistant	GCT allele
Ar22	10 CK01B	Stuntsman Co, ND	2010	nd[5]	nd	GCT allele
Ar23	10 CK01	Stuntsman Co, ND	2010	nd	nd	GCT allele
Ar24	10 CK01E	Stuntsman Co, ND	2010	nd	nd	GCT allele
Ar25	10 CK01N	Stuntsman Co, ND	2010	nd	nd	GCT allele
Ar26	10 CK01O	Stuntsman Co, ND	2010	nd	nd	GCT allele
Ar27	10 CK01P	Stuntsman Co, ND	2010	nd	nd	GCT allele
Ar28	10 CK03A	Stuntsman Co, ND	2010	nd	nd	GCT allele
Ar29	10 CK04A	Stark Co, ND	2010	nd	nd	GCT allele
Ar30	10 CK06A	Dunn Co, ND	2010	nd	nd	GGT allele
Ar31	10 CK07A	Stark Co, ND	2010	nd	nd	GCT allele
Ar32	10 CK12A	Ward Co, ND	2010	nd	nd	GCT allele
Ar33	10 CK12B	Ward Co, ND	2010	nd	nd	GCT allele
Ar34	10 CK13A	Ward Co, ND	2010	nd	nd	GCT allele
Ar35	10 CK13B	Ward Co, ND	2010	nd	nd	GCT allele

Table 1.1. (continued)

Isolate Code	Isolate ID	Origin[1] (County, State)	Year collected	Azoxystrobin ED50 (µg/ml)[2]	QoI sensitivity[3]	G143A screening[4]
Ar36	06 108C11	Foster, ND	2006	0.030	Sensitive	GGT allele
Ar37	06 109M6	Ward Co, ND	2006	0.032	Sensitive	GGT allele
Ar38	06 MT20	Unknown Co, MT	2006	0.032	Sensitive	GGT allele
Ar39	07 GMT6	Valley Co, MT	2007	na	Sensitive	GGT allele
Ar40	07 HRF-11	Williams Co, ND	2007	na	Sensitive	GGT allele
Ar41	07 HRF-67	Williams Co, ND	2007	na	Sensitive	GGT allele
Ar42	07 HRF-8	Williams Co, ND	2007	na	Sensitive	GGT allele
Ar43	07 KMB9F1-20	Mountrail Co, ND	2007	na	Sensitive	GGT allele
Ar44	07 KMB9F1-40	Mountrail Co, ND	2007	na	Sensitive	GGT allele
Ar45	07 KRF-F3-32	Williams Co, ND	2007	na	Sensitive	GGT allele
Ar46	07 KRF-F3-37	Williams Co, ND	2007	na	Sensitive	GGT allele
Ar47	07 SHRF-8	Williams Co, ND	2007	na	Sensitive	GGT allele
Ar48	07 TF-F1-10	Williams Co, ND	2007	na	Sensitive	GGT allele
Ar49	07 TSF2-13	Williams Co, ND	2007	na	Sensitive	GGT allele
Ar50	07 W201-16	Williams Co, ND	2007	na	Sensitive	GGT allele
Ar51	07 W201-18	Williams Co, ND	2007	na	Sensitive	GGT allele
Ar52	07 WD103-3	Williams Co, ND	2007	na	Sensitive	GGT allele
Ar53	07 WDY101-10	Williams Co, ND	2007	na	Sensitive	GGT allele
Ar54	05 101STM8	Ward Co, ND	2005	14.55	Resistant	GCT allele
Ar55	05 202STM4	Ward Co, ND	2005	na	Sensitive	GGT allele
Ar56	06 210M4	Ward Co, ND	2006	na	Sensitive	GGT allele
Ar57	06 BND11	Ward Co, ND	2006	na	Resistant	GCT allele
Ar58	06 TLND61	McLean Co, ND	2006	na	Resistant	GCT allele
Ar59	07 BMXPQ-24	Ward Co, ND	2007	na	Resistant	GCT allele
Ar60	07 CWF3-2	Williams Co, ND	2007	na	Resistant	GCT allele
Ar61	07 DX21	Ward Co, ND	2007	na	Resistant	GCT allele
Ar62	07 GF-F1-7	Williams Co, ND	2007	na	Resistant	GCT allele
Ar63	07 GF-F2-4	Williams Co, ND	2007	na	Resistant	GCT allele
Ar64	07 HRF-39	Williams Co, ND	2007	na	Resistant	GCT allele
Ar65	07 KRF-F1-5	Williams Co, ND	2007	na	Resistant	GCT allele
Ar66	07 M108-6	Ward Co, ND	2007	na	Resistant	GCT allele
Ar67	07 M116-9	Ward Co, ND	2007	na	Resistant	GCT allele
Ar68	07 M215-5	Ward Co, ND	2007	na	Resistant	GCT allele
Ar69	07 M409-6	Ward Co, ND	2007	na	Resistant	GCT allele
Ar70	07 MLMT13	Sheridan Co, MT	2007	na	Resistant	GCT allele

[1] ED_{50} value for azoxystrobin as previously determined by Wise, (2008).

[2] QoI sensitivity as previously determined *in vitro* by Wise (2008) using ED_{50} or discriminatory dosage assays.

[3] QoI sensitivity detected by MAMA-PCR using gDNA.

[4] na indicates not applicable. These isolates were tested for azoxystrobin resistance using a discriminatory dosage assay (Wise, 2008). The ED_{50} values of these isolates are not available.

[5] nd indicates not determined. These isolates were collected during statewide survey for Ascochyta blight of chickpeas. These isolates have not been characterized using *in vitro* assays.

Cloning and sequencing of the cytochrome b gene fragment. PCR assays for cloning the wild-type cytochrome b gene fragment were performed using gDNA of the QoI sensitive *A. rabiei* isolate Ar01 with the Top Taq DNA polymerase kit (Qiagen, Valencia, CA). The reaction volume was 30 µl and it contained 25 ηg of fungal DNA, 1X PCR buffer pH 8.7, 1.5 mM $MgCl_2$, 160 µM dNTPs, 0.5 units of Top Taq DNA polymerase and 160 ηM of each oligonucleotide primer from each primer pair, namely CytB01, CytB02, CytB03, CytB04, and CytB05 (Table 1.2). The PCR thermal cycling was initiated with denaturation at 94 °C for 10 min; followed by 35 cycles of 94 °C for 25 s, annealing for 25 s, and 72 °C for 25 s; and a final extension step at 72 °C for 10 min. The PCR products were separated on a 1% agarose gel and stained with the nucleic acid gel stain GelRed™ (Biotium, Inc., Hayward, CA) for 45 min at 100 V. PCR products were ligated to pGEM-T easy vector (Promega, San Luis Obispo, CA) and cloned in One Shot® Mach1™ competent cells (Invitrogen, Carlsbad, CA). Plasmid purification was carried out using the Wizard® Plus SV Miniprep DNA Purification Systems (Promega, San Luis Obispo, CA). The cloned PCR product was sequenced using M13 primers (McLab, San Francisco, CA).

PCR assays were conducted using gDNA and cDNA from the *A. rabiei* isolates Ar01, Ar02, Ar03, Ar04, Ar05, Ar06, Ar07, and Ar08. The reactions were performed using either 25 ηg of gDNA or cDNA, respectively. PCR assays with gDNA were conducted using primer pair CytB03. And, PCR assays with cDNA were performed using primer pairs CytB03 and CytB04. Each primer was used at a final concentration of 200 µM (Table 1.2). The PCR reactions were conducted using Advantage 2 PCR kit (Clontech, Mountain View, CA). Thermal cycling was initiated with denaturation at 95 °C for 10 min; followed by 40 cycles of 95 °C for 25 s, annealing for 25 s, and 68 °C for 30 s; and a final extension step at 70 °C for 10 min. The PCR fragments were separated on a 1.5% agarose gel with

GelRed™ nucleic acid gel stain (Biotium, Inc., Hayward, CA) for 70 min at 80 V. Selected PCR and RT-PCR products were cloned and sequenced as described above.

Mismatch amplification mutation assay (MAMA) for the detection of G143A mutants.

This PCR-based assay was developed to detect wild-type isolates of *A. rabiei* bearing G143-allele of the cytochrome b gene, and QoI mutant isolates bearing the A143-allele (Cha *et al.*, 1992; Siah *et al.*, 2010). The forward primer was designed using the Primer-BLAST program at the NCBI website (Table 1.2) and it was coupled with both the QoI sensitive reverse primer (ArCytBSen) and the QoI resistant reverse primer (ArCytBRes). Both reverse primers were designed to detect a single nucleotide polymorphism (SNP) at the second position of the codon 143 of the cytochrome b gene using gDNA. The last base at the 3'-end of the reverse primers corresponded to the targeted SNP. Additionally, a second mutation was introduced at the penultimate base at the 3'-end in the reverse primers. This creates a second nucleotide mismatch increasing annealing specificity with the DNA template (Cha *et al.*, 1992). Both QoI sensitive and QoI resistant PCR assays amplified a 202 bp DNA fragment with corresponding QoI sensitive and QoI resistant isolates. Sensitive and resistant MAMA-PCR reactions were optimized to a final volume of 25 µl containing 25 ηg of fungal DNA, 1X PCR buffer pH 8.7, 1.5 mM of $MgCl_2$, 160 µM of dNTPs, 160 ηM of each oligonucleotide primers, and 0.65 units of Top Taq DNA polymerase. The PCR thermal cycling was initiated with denaturation at 94 °C for 3 min; followed by 30 cycles of 94 °C for 10 s, 58 °C for 10 s, and 72 °C for 15 s; and a final extension step at 72 °C for 3 min. The PCR products were separated on a 1.0% agarose gel with GelRed™ nucleic acid gel stain (Biotium, Inc., Hayward, CA) for 30 min at 90 V, and the DNA fragments were visualized under UV light. The same protocol was used for detecting the presence of QoI resistant and QoI sensitive isolates in artificially infected chickpea plants grown under greenhouse conditions.

Table 1.2. Primers used for amplification of the cytochrome b gene fragment of *Ascochyta rabiei* and for screening of the G143A mutation

Primer pairs	Primers	Primer sequence (5' to 3')	Annealing temperature	Reference	Primer pair purpose
CytB01	cytb1	GAGCAYATYATGCGYGAYGT	56 °C	Sierotzki *et al.*, 2000	Cloning of cytochrome b gene (PCR)
	cytb2rev	GTRATRACDATRGCDCCCCA			
CytB02	As1F	CAGAGCACCTAGAACTCTAGTATGAA	55 °C	Pasche *et al.*, 2005	Cloning of cytochrome b gene (PCR)
	As1R	CCTCCTCAAATGAACTCAACAA			
CytB03	CBF1	TATTATGAGAGATGTAAATAATGG	48 °C	Ma *et al.*, 2003	Cloning of cytochrome b gene (PCR and RT-PCR)
	CBR2	AACAATATCTTGTCCAATTCATGG			
CytB04	CBF1	TATTATGAGAGATGTAAATAATGG	46 °C	Fraaije *et al.*, 2002; 2005	Cloning of cytochrome b gene (PCR and RT-PCR)
	CBR3	CCTAATAATTTATTAGGTATAGATCTTA			
CytB05	Pter23F	ACATAGTAATACTGCTTCAGC	48 °C	Sierotzki *et al.*, 2007; Kianianmomeni *et al.*, 2007	Cloning of cytochrome b gene (PCR)
	Pter25R	TACATTTGAGGCAAATATTTC			
ArCytB Sensitive	ArCytBF1	GGTCACTGATGGGTGTCTGAAATG	56 °C	This study	Determination of QoI-sensitive isolates (G143 allele)
	ArCytBSen	CACTCATAAGGTTAGTAATAACTGTAGCCC			
ArCytB Resistant	ArCytBF1	GGTCACTGATGGGTGTCTGAAATG	56 °C	This study	Determination of QoI-resistant isolates (A143 allele)
	ArCytBRes	CACTCATAAGGTTAGTAATAACTGTAGCCG			

Results

Cloning of cytochrome b gene fragment from *A. rabiei*. The initial PCR reaction was conducted using Top Taq DNA polymerase (Qiagen, Valencia, CA) with five sets of primers (Table 1.2), which showed that only the primer pair CytB03 yielded a PCR fragment of approx. 1.5 to 2.0 kb in length (Figure 1.1). This PCR assay was repeated with the CytB03 primer pair and with eight *A. rabiei* isolates of varying QoI sensitivities (Ar01 to Ar08). The PCR products of all eight isolates were amplified with primer pair CytB03 and the PCR products of three QoI sensitive isolates and one QoI resistant isolate were successfully cloned into the plasmids. The plasmids of these four clones were sequenced. The obtained sequences were analyzed using BLAST programs at the NCBI website. The consensus sequences created by aligning forward and reverse sequences of each sequenced clone were found to range between 1648 and 1651 bp in length and they all matched the nucleotide sequence of the cytochrome b gene of the ascomycete *Monilinia fructigena* (Table 1.3). These gDNA fragments encoded amino acid residues 68 to 162 and was found to have an intron of 1365 bp located between amino acid residue 131 and 132. This intron was predicted to belong to group I introns and encoded for GIY-YIG endonucleases (Table 1.3).

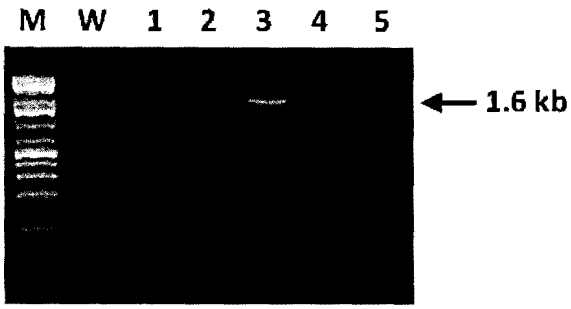

Figure 1.1. Partial PCR amplification of the cytochrome b gene using all five primer pairs with gDNA of the QoI sensitive *Ascochyta rabiei* isolate Ar01. (1) primer pair CytB01, (2) primer pair CytB02, (3) primer pair CytB03, (4) primer pair CytB04, and (5) primer pair CytB05. W represents the PCR where water was used as template. M represents 1 kb Plus DNA ladder from Fermentas (Glen Burnie, MD).

Table 1.3. Homologs to gDNA and cDNA sequence of the fragment of the cytochrome b gene of *Ascochyta rabiei* present in GenBank

Isolate	Sequence Length	BLAST program	Description of BLAST hit in GenBank	Accession Number	Organism	Query coverage	Maximum identities	Total score	E-value
gDNA fragment amplified with primer pair CytB03									
Ar01	1648	MegaBLAST	Cytochrome b gene	GU933644.1	*Monilinia fructigena*	90%	93%	2162	0.0
Ar02	1650	MegaBLAST	Cytochrome b gene	GU933644.1	*Monilinia fructigena*	90%	94%	2190	0.0
Ar03	1651	MegaBLAST	Cytochrome b gene	GU933644.1	*Monilinia fructigena*	90%	93%	2188	0.0
Ar05	1650	MegaBLAST	Cytochrome b gene	GU933644.1	*Monilinia fructigena*	90%	94%	2190	0.0
Ar01	1648	BLASTX	GIY-YIG endonuclease	ABU50155.1	*Cordyceps bassiana*	70%	56%	300	8.0 E -96
Ar02	1650	BLASTX	Putative endonuclease	CBM41828.1	*Millerozyma farinosa*	66%	38%	238	6.0 E -49
Ar03	1651	BLASTX	GIY endonuclease	YP_001249318.1	*Gibberella zeae*	45%	67%	327	2.0 E -66
Ar05	1650	BLASTX	Putative endonuclease	CBM41828.1	*Millerozyma farinosa*	66%	38%	237	6.0 E -49
Ar01	1648	BLASTX	Cytochrome b protein	AAP22126.1	*Alternaria alternata*	16%	96%	172	6.0 E -26
Ar02	1650	BLASTX	Cytochrome b protein	AAP22126.1	*Alternaria alternata*	16%	96%	172	6.0 E -26
Ar03	1651	BLASTX	Cytochrome b protein	AAP22126.1	*Alternaria alternata*	16%	96%	172	6.0 E -26
Ar05	1650	BLASTX	Cytochrome b protein	AAP22126.1	*Alternaria alternata*	16%	96%	174	6.0 E -26
cDNA fragment amplified with primer pair CytB04									
Ar01	665	MegaBLAST	Cytochrome b gene	DQ209285.1	*Alternaria solani*	85%	92%	826	0.0
Ar03	675	MegaBLAST	Cytochrome b gene	DQ209285.1	*Alternaria solani*	85%	93%	854	0.0
Ar04	675	MegaBLAST	Cytochrome b gene	DQ209285.1	*Alternaria solani*	85%	93%	854	0.0
Ar05	674	MegaBLAST	Cytochrome b gene	DQ209285.1	*Alternaria solani*	85%	92%	841	0.0
Ar06	675	MegaBLAST	Cytochrome b gene	DQ209285.1	*Alternaria solani*	85%	93%	848	0.0
Ar08	675	MegaBLAST	Cytochrome b gene	DQ209285.1	*Alternaria solani*	85%	93%	848	0.0
Ar01	665	BLASTX	Cytochrome b protein	YP_001427390.1	*Phaeosphaeria nodorum*	99%	88%	385	3.0 E-105
Ar03	675	BLASTX	Cytochrome b protein	YP_001427390.1	*Phaeosphaeria nodorum*	99%	89%	391	4.0 E -107
Ar04	675	BLASTX	Cytochrome b protein	YP_001427390.1	*Phaeosphaeria nodorum*	99%	89%	391	4.0 E -107
Ar05	674	BLASTX	Cytochrome b protein	YP_001427390.1	*Phaeosphaeria nodorum*	99%	88%	389	2.0 E -106
Ar06	675	BLASTX	Cytochrome b protein	YP_001427390.1	*Phaeosphaeria nodorum*	99%	88%	389	2.0 E -106
Ar08	675	BLASTX	Cytochrome b protein	YP_001427390.1	*Phaeosphaeria nodorum*	99%	88%	389	2.0 E -106

The primer pair CytB03 amplified a cytochrome b gene fragment that bears the most common QoI mutations in fungal plant pathogens, which are located between amino acid residues 120-160. The primer pair CytB04 amplified the cytochrome b gene region that contains all QoI related mutations, i.e. amplifying a DNA fragment that corresponds to amino acid residues 66 to 291. A second PCR assay was performed using primers CBF2 and CBR3. Primer CBF2 is the reverse complement of primer CBR2 and it was used as a forward primer coupled with the reverse primer CBR3 (Table 1.2). This PCR reaction was conducted to determine the length of this gDNA fragment using two *A. rabiei* isolates (Ar01 and Ar08). The amplified PCR product was between 3 to 4 kb in length in both QoI sensitive and resistant isolates (Figure 1.2). Therefore, the cytochrome b gene fragment located between primer CBF1 and CBR3 is at least 4.6 kb in length. Considering the large size of the amplicon, further characterization of this cytochrome b gene fragment pathogens was performed through a two-step RT-PCR using cDNA from the eight isolates aforementioned (Figure 1.3, Figure 1.4).

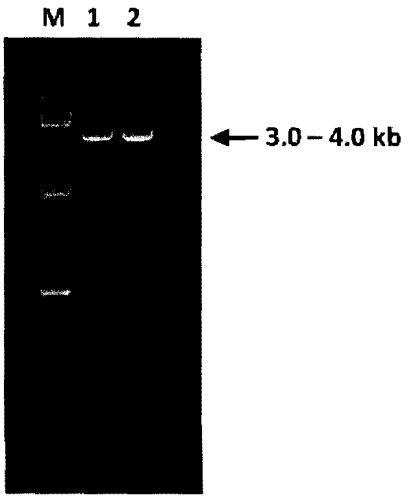

Figure 1.2. Partial PCR amplification of the cytochrome b gene using gDNA of two *Ascochyta rabiei* isolates with the reverse complement of CBR2 and CBR3. The reverse complement of primer CBR2 was used as forward primer in this PCR assay. (1) QoI sensitive *A. rabiei* isolate Ar01, (2) QoI resistant isolate Ar08. M represents 1 kb Plus DNA ladder from Fermentas (Glen Burnie, MD).

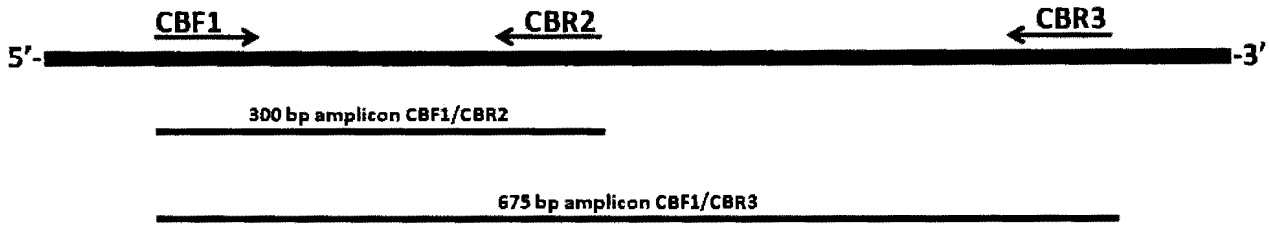

Figure 1.3. Fragment of the mRNA of the cytochrome b gene. This picture shows the positions of the primers CBF1, CBR2 and CBR3 used for the two-step RT-PCR amplifications.

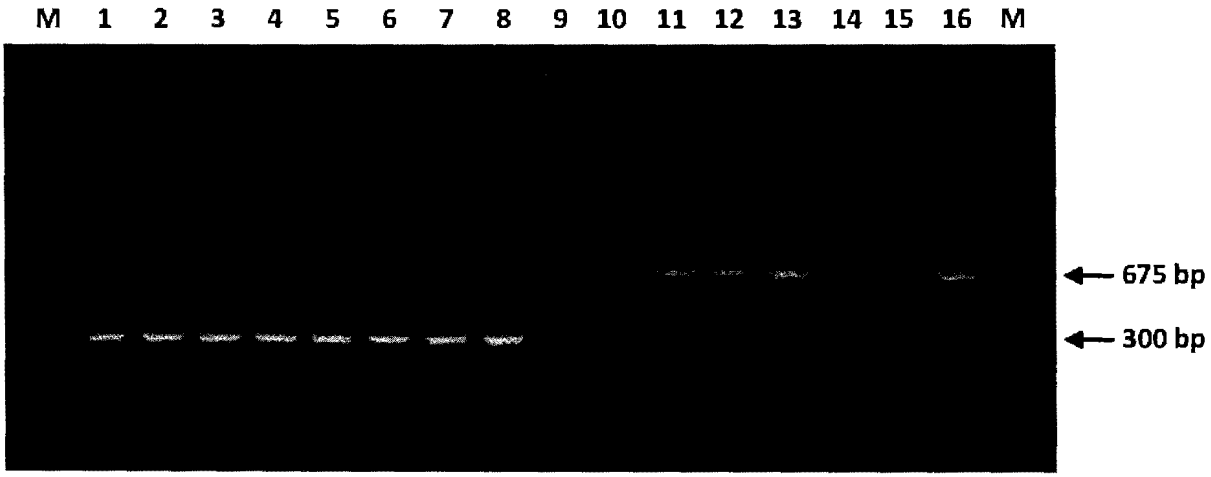

Figure 1.4. RT-PCR fragments of the cytochrome b gene obtained using primer pairs CytB03 and CytB04 with eight isolates of *Ascochyta rabiei* of different sensitivities to QoI fungicides. RT-PCR products obtained using CytB03 primers: (1) isolate Ar01, (2) Ar02, (3) Ar03, (4) Ar04, (5) Ar05, (6) Ar06, (7) Ar07, and (8) Ar08. RT-PCR products using CytB04 primer: (9) isolate Ar01, (10) Ar02, (11) Ar03, (12) Ar04, (13) Ar05, (14) Ar06, (15) Ar07, and (16) Ar08. M represents 1 kb Plus DNA ladder from Fermentas (Glen Burnie, MD).

CytB03 primer pair amplified a fragment of approximately 300 bp in length while the CytB04 primer pair (CBF1/CBR3) amplified a fragment 675 bp in length from cDNA obtained from *A. rabiei* isolates Ar01 to Ar08. The CytB04 PCR fragments were cloned; however, the PCR fragments of only six isolates showed positive transformed clones (Ar01, Ar03, Ar04, Ar05, Ar06, and Ar08). These were sent for sequencing. Sequence analyses of the gDNA and cDNA clones using ClustalW (www.clustal.org) revealed a SNP at position 228 of the PCR fragment. The characterized SNP

corresponded to the second nucleotide of the codon 143 of the cytochrome b gene shifting the codon from GGT to GCT. Analysis of the derived protein sequence obtained from PCR products amplified with primer pair CytB04 showed that these products corresponded to the amino acid residues 61 to 224. This characterized SNP causes the G143A amino acid substitution (Figure 1.5). F129L and G137R mutations were not found within the PCR fragments of the six *A. rabiei* isolates. G143A mutation was then associated with decreased QoI sensitivity in *A. rabiei*. Three other amino acid mutations were observed but they were not associated with decreased QoI sensitivity. Of the three, only two SNPs resulted in amino acid substitutions. Analysis of the cDNA sequences using MegaBLAST with NCBI database showed that the best match was the cytochrome b gene of the ascomycete *Alternaria solani*, while analyses using BLASTX showed that the best match was the cytochrome b protein of *Phaeosphaeria nodorum* (Table 1.3).

Mismatch amplification mutation assay (MAMA) for the detection of G143A mutants. This assay was developed to screen QoI sensitive and QoI resistant isolates of *A. rabiei* based on G143A mutation using gDNA sequence. Two reverse primers of 30 bp in length were designed to amplify either the wild-type G143-allele or the mutated A143-allele (Figure 1.6, Figure 1.7). Both MAMA-PCRs were conducted using the same forward primer and they amplified a fragment 202 bp in length. Differentiation between the G143- and A143-allele was achieved after PCR optimization using isolates with known QoI sensitivity levels previously determined through spore germination assays (Wise, 2008). QoI sensitive and the QoI resistant PCRs were able to differentiate between cytochrome b allele for 56 isolates of known sensitivities (Figure 1.7, Table 1.1). Of these, 29 isolates were previously characterized as QoI sensitive and 27 isolates as QoI resistant. The only

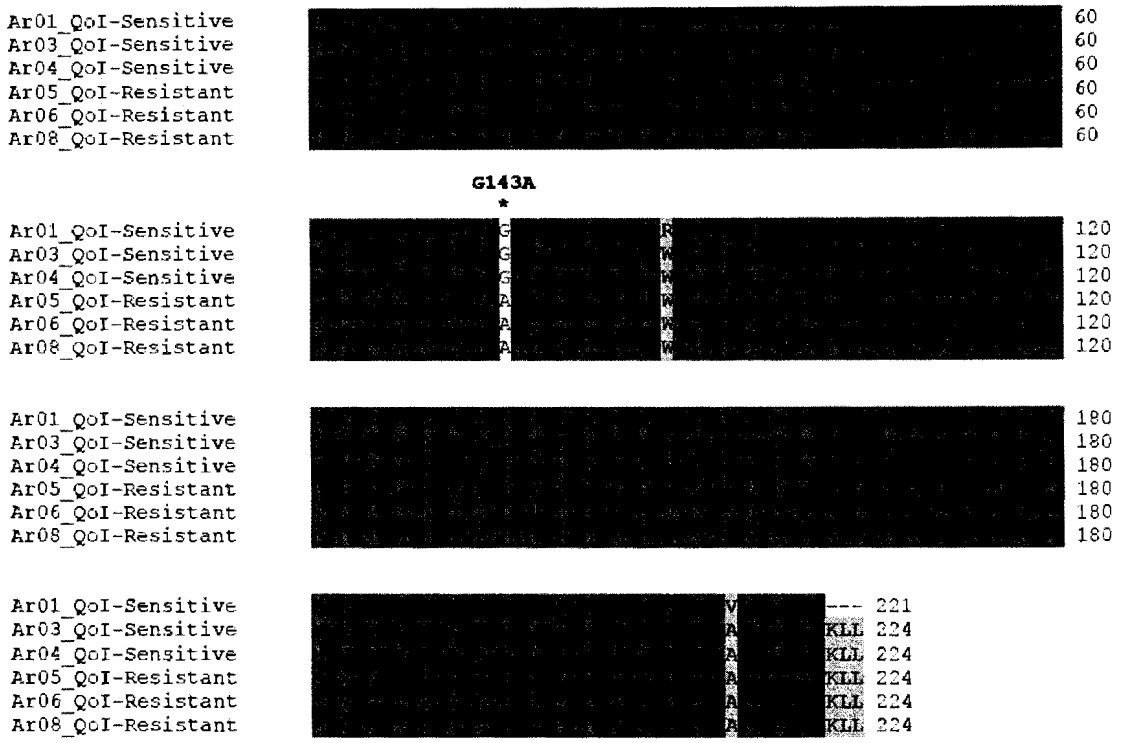

Figure 1.5. Partial protein sequence of the cytochrome b of six isolates of *Ascochyta rabiei* with different QoI sensitivities. The star indicates the G143A amino acid substitution that correlates with decreased sensitivity to QoI-fungicides. Dark gray highlighted areas represent 100% identities, light gray areas represent amino acid substitutions that are not associated with decreased QoI-sensitivity.

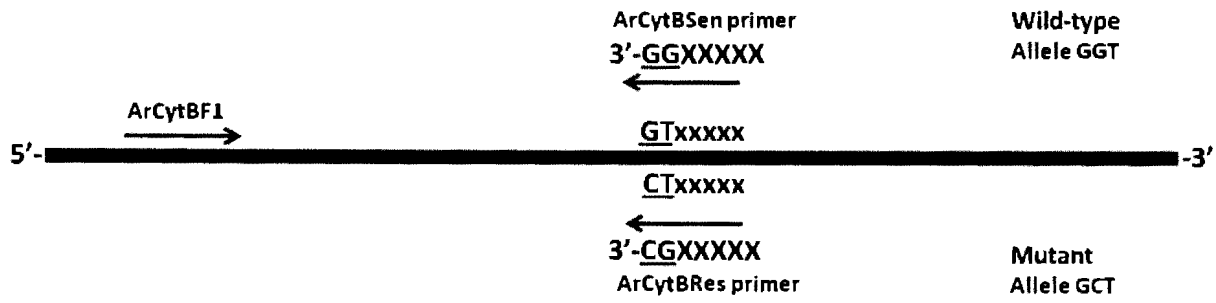

Figure 1.6. Fragment of the cytochrome b gene. This picture shows the positions of the primers used for the MAMA-PCR. The reverse primer ArCytBSen was used to amplify the GGT-allele and primer ArCytBRes was used to amplify the GCT-allele.

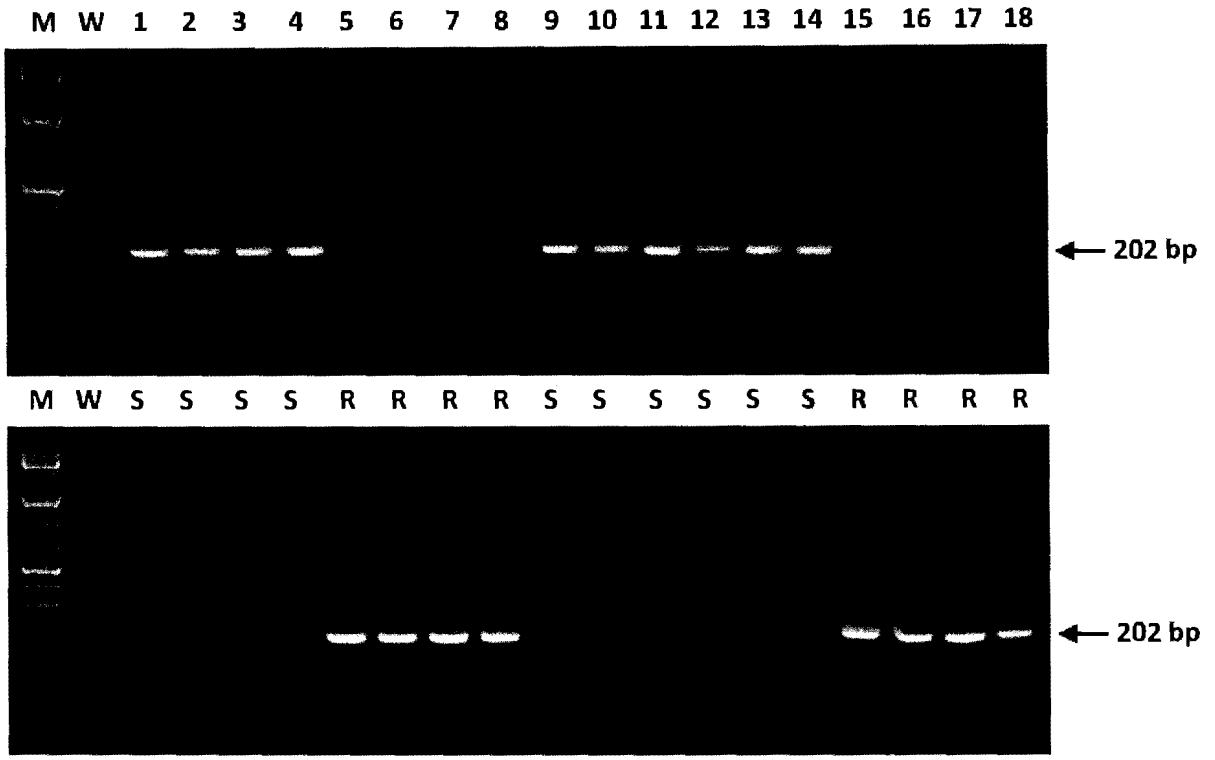

Figure 1.7. Mismatch amplification mutation assay (MAMA) for screening of the G143A mutation on the cytochrome b gene of *Ascochyta rabiei*. Top; ArCytB Sensitive MAMA-PCR. Bottom: ArCytB Resistant MAMA-PCR. Isolates: (1) Ar01, (2) Ar02, (3) Ar03, (4) Ar04, (5) Ar05, (6) Ar06, (7) Ar07, (8) Ar08, (9) Ar09, (10) Ar10, (11) Ar11, (12) Ar12, (13) Ar13, (14) Ar14, (15) Ar15, (16) Ar16, (17) Ar17, and (18) Ar18. W represents the PCR with water as template. M represents 1 kb Plus DNA ladder from Fermentas (Glen Burnie, MD). S indicates QoI sensitive isolates. R indicates QoI resistant isolates.

difference was that the MAMA-PCR showed that 31 isolates instead of 29 were QoI sensitive (GGT-allele) and 25 isolates were QoI resistant (GCT-allele). Isolates Ar55 and Ar56 had been previously characterized as QoI resistant in spore germination assays (Wise, 2008). But, the MAMA-PCR showed that these isolates harbored the GGT allele. To explain this discrepancy, the isolates Ar55 and Ar56 were retrieved from the original long-term culture stocks and grown on PDA plates to confirm the QoI sensitivity status of these isolates through spore germination assay as well as repeating the MAMA-PCR from newly extracted gDNA. The MAMA-PCR again showed that these isolates had the GGT allele. However, the discriminatory dosage assays using azoxystrobin

according to Wise *et al.*, (2009) demonstrated that isolates Ar55 and Ar56 were in fact sensitive to azoxystrobin with conidial germination of 0.0% and 1.3%, respectively.

Fourteen isolates collected during 2010 growing season were subsequently screened using this PCR-assay. It was determined that 13 of these isolates had the A143-allele, which correlates with resistance to QoI fungicides (Figure 1.8). The presence or absence of the A143-allele was confirmed by sequencing the cDNA fragments amplified with primer pair CytB03 using isolates Ar22, Ar28, Ar30 and Ar35. In this case, MAMA-PCR was found to be able to amplify PCR products

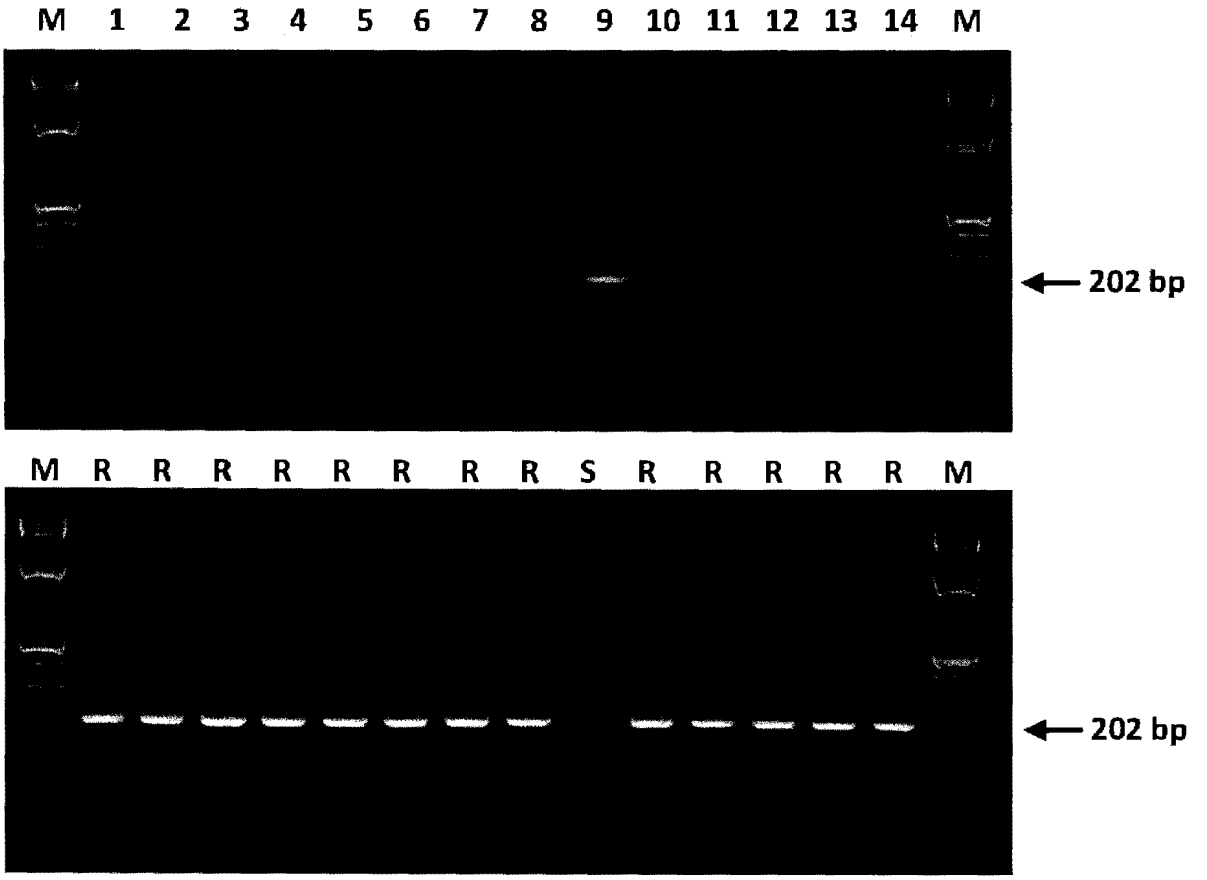

Figure 1.8. MAMA-PCR for screening of the G143A mutation on the cytochrome b gene of *Ascochyta rabiei* isolates collected during 2010 growing season from chickpea fields in North Dakota. Top: ArCytB Sensitive MAMA-PCR. Bottom: ArCytB Resistant MAMA-PCR. Isolates: (1) Ar22, (2) Ar23, (3) Ar24, (4) Ar25, (5) Ar26, (6) Ar27, (7) Ar28, (8) Ar29, (9) Ar30, (10) Ar31, (11) Ar32, (12) Ar33, (13) Ar34, and (14) Ar35. M represents 1 kb Plus DNA ladder from Fermentas (Glen Burnie, MD). S indicates QoI sensitive isolates. R indicates QoI resistant isolates.

with template concentration as low as 0.1 pg/µl when using gDNA from fungal mycelia (data not shown). This method was also used to test for the presence of resistant isolates directly from chickpea leaves inoculated with *A. rabiei*. The amplification of the G143 and A143-alleles was also possible using infected-plant material. These results correlated with the amplification performed on the same isolates grown on PDA (Figure 1.9). The MAMA-PCR primers were also tested against gDNA of fungal species that often grow on PDA along with *A. rabiei* during the isolation process such as *Fusarium graminearum* and *Alternaria solani*, as well as against *Ascochyta pinodes* that is believed to potentially infect chickpea cultivars (Bretag *et al.*, 2006). None of these showed amplification with either the G143-allele or the A143-allele while using MAMA-PCR.

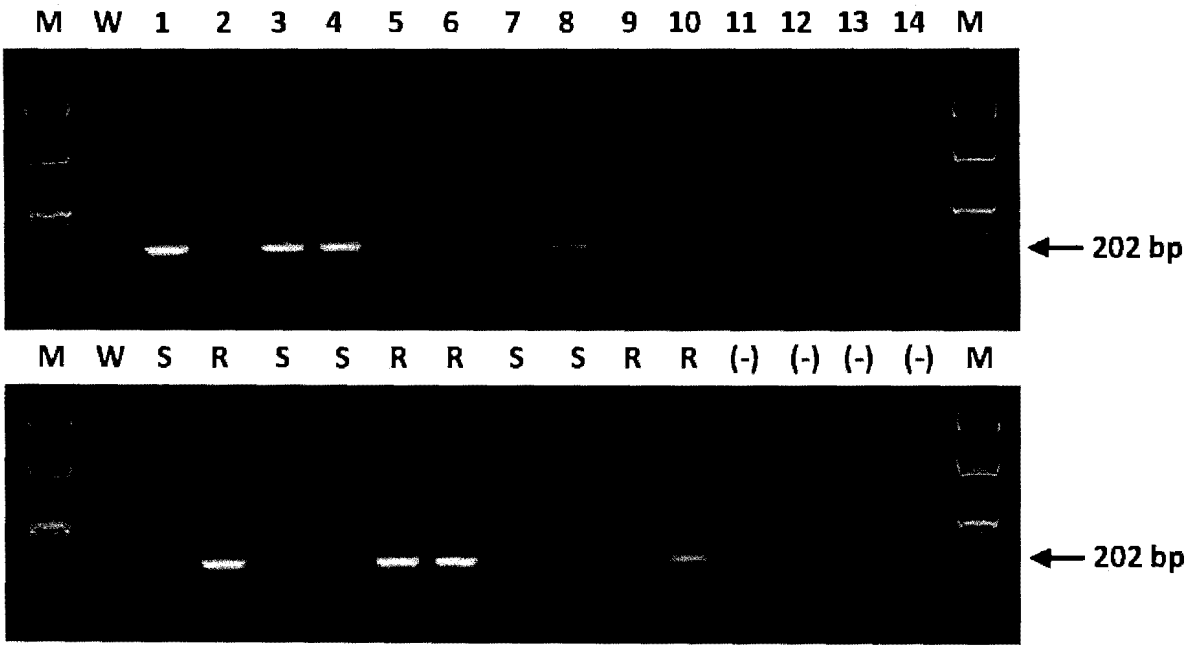

Figure 1.9. MAMA-PCR for screening of the G143A mutation in isolates of *A. rabiei* grown on PDA and inoculated chickpeas grown under greenhouse conditions. Top: ArCytB Sensitive MAMA-PCR. Bottom: ArCytB Resistant MAMA-PCR. Fungal mycelia: (1) Ar01 as QoI-sensitive reference, (2) Ar08 as QoI-resistant reference, (3) Ar13, (4) Ar19, (5) Ar20, (6) Ar21. Inoculated chickpea leaves: (7) Ar13, (8) Ar19, (9) Ar20, (10) Ar21, (11) non-infected leaves. Negative controls: (12) *Fusarium graminearum*, (13) *Alternaria solani*, and (14) *Ascochyta pinodes*. W represents the PCR where water was used as template. M represents 1 kb Plus DNA ladder from Fermentas (Glen Burnie, MD). S indicates QoI sensitive isolates. R indicates QoI resistant isolates.

Discussion

QoI resistant isolates of *A. rabiei* have been reported in Canada since 2004 and the United States since 2005 (Chang *et al.*, 2004; Wise *et al.*, 2009). Previous studies have shown that QoI resistant isolates are found at a high frequency throughout North Dakota and that the disease caused by these resistant isolates is poorly controlled by QoI fungicides (Wise *et al.*, 2009). Moreover, there are no commercially available chickpea cultivars with durable resistance to Ascochyta blight at the moment. The purpose of this study was to determine whether the decreased sensitivity of *A. rabiei* isolates to QoI fungicides was due to amino acid substitutions located in the cytochrome b protein of the cytochrome bc_1 complex and to develop a PCR-based diagnostic tool to monitor QoI resistance in *A. rabiei*. Most amino acid substitutions related to QoI resistance in fungal plant pathogens are situated in two major hot spots. These hot spots are located between amino acid residues 120-160 and 250-300, respectively, with the three most commonly reported mutations (F129L, G137R, and G143A) being located within the first hot spot (Brausser *et al.*, 1996; Grasso *et al.*, 2006a).

In this study, we cloned the cytochrome b gene fragment of *A. rabiei* that contains the amino acid residues from 61 to 224 using the primer pair CytB04 with cDNA synthesized from six *A. rabiei* isolates of varying QoI sensitivities. Prior to that, sequence analysis of the gDNA fragment amplified using the primer pair CytB03, which corresponded to the cytochrome b gene of *A. rabiei,* showed similarity to the cytochrome b gene of *Monilinia fructigena*. We found that the cytochrome b gene of *A. rabiei* is over 4.6 kb in length because PCR amplifications using gDNA with primers CBF1/CBR2 and CBF2/CBR3 yielded fragments of 1.6 Kb and 3.0 - 4.0 Kb in length, respectively. Primer pair CytB04, when used for RT-PCR, amplified a cDNA fragment 675 bp in length from the same isolates. Multiple alignments of the cDNA and protein sequences revealed a point mutation

on the second nucleotide of the codon 143 from GGT to GCT causing G143A mutation in the cytochrome b gene. G143A mutation has been previously described in the cytochrome b gene of other fungal plant pathogens and has been associated with higher levels of QoI resistance and even complete loss of disease control (Brasseur *et al.*, 1996; Brent and Hollomon, 2007b; Fraaije *et al.*, 2002; FRAC, 2006; Gisi *et al.*, 2002; Kianianmomeni *et al.*, 2007; Kim *et al.*, 2003). This supports the over 100-fold reduction in sensitivity to the QoI fungicides azoxystrobin and pyraclostrobin reported in North Dakota (Wise, 2008; Wise *et al.*, 2009).

The gDNA fragment of the cytochrome b gene amplified from *A. rabiei* was shown to contain a 1365 bp group I intron (Burke, 1988) between codons 131 and 132. Group I introns are characterized by the presence of intronic endonucleases and maturases, which are encoded within these introns. These intron encoded proteins seem to be associated with mitochondrial splicing and mobility (Saguez *et al.*, 2000). A BLASTX search of this region matched endonucleases from ascomycete species, which suggested the likelihood of the presence of these endonuclease encoding regions in the mitochondrial genome of *A. rabiei*. Grasso *et al.*, (2006a) found a maturase in the sequence of the group I intron located between codon 143 and 144 in *Puccinia* species. Interestingly, the G143A mutation has not been found in fungal species that have an intron immediately after the codon 143 (Banno, *et al.*, 2009; Grasso *et al.*, 2006b; Sierotzki *et al.*, 2007). Thus, this cytochrome b gene structure has not been associated with the occurrence of QoI mutation, i.e., fungal species such as rusts and *Alternaria solani* do not exhibit G143A mutation because the 5'-splice site is lethally affected by the nearby exonic flanking sequences (Banno *et al.*, 2009; Bolduc *et al.*, 2003; Burke, 1988; Cech, 1988; Grasso *et al.*, 2006b; Lambowiz and Belfort, 2003; Pasche *et al.*, 2005; Sierotzki *et al.*, 2007). However, the gene structure of the cytochrome b

gene of *A. rabiei* appears to be favorable for development of a point mutation related to QoI resistance at the codon 143.

Isolates of *A. rabiei* bearing the G143A mutations have higher probabilities of survival in a chickpea field in comparison to F129L and G137R mutants (Brasseur *et al.*, 1996; Brent and Hollomon, 2007b; Fraaije *et al.*, 2002; FRAC, 2006; Gisi *et al.*, 2002; Kianianmomeni *et al.*, 2007; Kim *et al.*, 2003). We only sequenced the cytochrome b cDNA fragment of six isolates with known QoI sensitivities; however, these isolates were chosen to represent both extremes of the QoI sensitivity values (EC_{50}) reported by Wise *et al.*, (2008; 2009). Ar01 isolate represented the most sensitive isolate to azoxystrobin while Ar04 represented the least sensitive among the QoI sensitive isolates. Likewise, Ar05 isolate represented the least resistant isolate to azoxystrobin while Ar08 represented the most resistant among the QoI resistant isolates (Table 1.1). The Ar05 isolate was found to be a G143A mutant and it was previously shown to have a decreased sensitivity to azoxystrobin and pyraclostrobin of 129 and 322-fold, respectively (Wise, 2008; Wise *et al.*, 2008, 2009). These findings and previously discussed evidence suggest that the presence of F129L and G137R mutants of *A. rabiei* populations in North Dakota is unlikely. However, a larger size sample is needed to completely support this hypothesis since different gene structures may affect a fungal population. For example, two different gene structures have been found in *Botrytis cinerea* population harboring G143A mutation (Banno *et al.*, 2009). Also, field populations of *Plasmopara viticola* and *Pyrenophora tritici-repentis* have been found to harbor more than one mutation associated to resistance to QoI fungicides (Förster *et al.*, 2009; FRAC, 2010b; Heaney *et al.*, 2000; Kim *et al.*, 2003; Sierotzki *et al.*, 2005, 2007; Stammler *et al.*, 2006; Vincelli and Dixon, 2002).

Monitoring shifts of fungicide sensitivity is one of the most efficient strategies to avoid plant disease epidemics, to ensure effective plant disease management and to prolong the effective

use of registered fungicides (Siah *et al.*, 2010; Wise *et al.*, 2008; 2009). However, diagnosis of QoI resistance using *in vitro* bioassays is time-consuming and laborious due to the large number of samples that need to be processed before, during and after each chickpea growing season. In our monitoring program, we have seen that *A. rabiei* is difficult pathogen to work with, because it is slow growing and is often overgrown during the isolation process by secondary fungal species that are present in the plated chickpea leaves. The conidial production is variable since this pathogen is very sensitive to light regimen, media composition and relative humidity. Moreover, *A. rabiei* has been reported to produce spontaneous albino mutants *in vitro* (Chen *et al.*, 2004b).

We were successful in developing a molecular diagnostic assay for QoI resistance on *A. rabiei* that will allow fast and efficient screening of G143A mutants. This has been suggested by other researchers (Wise *et al.*, 2009; Russell, 2004) and several others have successfully used molecular techniques to detect G143A, F129L and G137R mutants in other pathosystems (Banno *et al.*, 2008; Jiang *et al.*, 2009; Luo *et al.*, 2007; Ma *et al.*, 2005; Pasche *et al.*, 2005; Siah *et al.*, 2010). Most of these reports use real time PCR based strategies; however the advantage of the approach adopted in this study is that does not require expensive instrumentation. The allele-specific PCR using the mismatch amplification mutation assay (MAMA) approach allows the identification of GGT- and GCT-alleles in *A. rabiei* isolates using two simple endpoint PCRs (Cha *et al.*, 1992; Siah *et al.*, 2010).

During this study, we screened a total of 56 *A. rabiei* isolates that had been previously characterized for QoI resistance (Wise, 2008; Wise *et al.*, 2008; Wise *et al.*, 2009). Among these, we found two isolates bearig the wild-type allele (GGT) that had been previously characterized as azoxystrobin resistant using media amended with QoI fungicides (Wise, 2008; Wise *et al.*, 2009). This situation was re-assessed and the QoI sensitivity status of these isolates was re-confirmed

using the discriminatory dosage assay (Wise *et al.*, 2009) and MAMA-PCR repeated with newly extracted DNA. This re-assessment showed that these two isolates were in fact QoI sensitive. The MAMA-PCR strategy is highly reliable; however, we recommend conducting the discriminatory dosage assay in cases where all or most isolates have the GGT-allele if the field data shows loss of disease control after fungicide applications. Fungal pathogens may also develop QoI resistance due to mutations elsewhere in the cytochrome bc_1 complex (Fernández-Ortuño *et al.*, 2008) and they may also develop more than one mutation associated to QoI resistance within field populations (Förster *et al.*, 2009; FRAC, 2010b; Heaney *et al.*, 2000; Kim *et al.*, 2003; Sierotzki *et al.*, 2005, 2007; Stammler *et al.*, 2006; Vincelli and Dixon, 2002). Therefore, it is also judicious to monitor isolates with GGT-alleles due to the potential development of other mutations associated to QoI resistance.

We believe that this MAMA-PCR using allele-specific primers for G143A mutation in *A. rabiei* is a useful tool that can be implemented for QoI resistance screening. This newly developed tool will allow the screening of a large number of samples in a shorter period of time. Moreover, this method could potentially be modified in the future to detect other mutations. This newly developed MAMA-PCR will be used in our monitoring program for routine detection of QoI resistance in field isolates of *A. rabiei* along with the discriminatory dosage assay.

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PAPER TWO. A RESOURCE FOR *IN SILICO* IDENTIFICATION OF FUNGAL POLYKETIDE SYNTHASES FROM PREDICTED FUNGAL PROTEOMES

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Authour Note: My role in this manuscript was to design, conduct and analyze all the experiments that were carried out for this manuscript. I also brought forth and developed with the concept of this project. Furthermore, I wrote this manuscript and performed all corrections suggested by the co-authors and journal's reviewers.

Introduction

Natural products such as polyketides are of great pharmaceutical and agrochemical value due to their antibacterial, antifungal, immunosuppressant and other biological activities. Polyketide compounds are a large group of naturally occurring active compounds that are synthesized by a family of proteins known as polyketide synthases (PKSs). PKSs are considered to be the most successful means of discovering and creating new natural and engineered active compounds for the manufacture of novel pharmaceutical drugs and pesticides (Shen, 2003). Fungi are prolific producers of active compounds under natural conditions. However, fungal PKSs have been studied to a much lesser extent than bacterial PKSs. Fungal PKSs are involved in the biosynthesis of secondary metabolites and pigments, which have been shown to be important in the survival, competition and pathogenicity of several fungal species (Feng *et al.,* 1995; Gaffoor and Trail, 2006; Loppnau *et al.,* 2004; Zhang *et al.,* 2003). Also, polyketide compounds can be either beneficial or detrimental to human health (Keller *et al.,* 2005)

PKSs are multidomain proteins that biosynthesize polyketide compounds from the precursor acyl coenzyme A (acyl CoA) by sequential decarboxylative condensations, also known as

Claisen condensations (Crawford *et al.*, 2008; Gokhale *et al.*, 2007; Schümann and Hertweck, 2006; Shen, 2003; Weissman, 2008). Three types of PKSs have been described, namely type I, type II, and type III PKSs, which differ from each other based on their protein structure (Gokhale *et al.*, 2007). Type I PKSs are commonly found in both bacteria and fungi but bacterial type I PKSs are mostly modular proteins while fungal type I PKSs are iterative. These two types of PKSs present different biosynthesis mechanisms and domain architectures. Modular PKSs are arranged in modules with each module consisting of a set of domains. Iterative PKSs are organized in one single module with one set of domains that act in a repetitive fashion until the final polyketide product has been synthesized (Gaitatzis *et al.*, 2002; Gokhale *et al.*, 2007; Hoffmeister and Keller, 2007; Shen, 2003; Shen and Hutchinson, 1996; Wiesmann *et al.*, 1995).

Functional type I iterative PKSs can contain up to eight domains but they must have a minimum domain architecture consisting of a β-ketoacyl synthase (KS), an acyltransferase (AT), and an acyl carrier protein (ACP) domain (Hopwood and Sherman, 1990). Type I iterative PKSs activate the acyl CoA substrates through the ACP domain, while the KS domain elongates the carbon chain yielding the final product (Gokhale *et al.*, 2007; Shen, 2003). Polyketide compounds are classified into non-reduced, reduced, and highly reduced categories. Reducing type I iterative PKSs produce either partially reduced or highly reduced products by having multiple AT domains and/or other reducing domains, such as ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) (Gokhale *et al.*, 2007). Reduced polyketide compounds include 6-methylsalycilic acid-related compounds (patulin, ochratoxin, etc) and other mycotoxins (alternapyrone, T-toxin, etc). On the other hand, non-reduced compounds include fungal pigments (conidial pigments and melanin) and non-reduced mycotoxins (aflatoxin, cercosporin, etc). The KS domain is the most conserved PKS domain and can be used to phylogenetically categorize PKS proteins into reducing and non-

reducing types (Bingle *et al.,* 1999; Kroken *et al.,* 2003; Langfelder *et al.,* 2003; Nicholson *et al.,* 2001). This domain is persistently located toward the N-terminus of the protein sequence followed by the AT domain in type I iterative PKSs (Bingle *et al.,* 1999; Langfelder *et al.,* 2003).

The overall diversity and complexity of PKS proteins have made the molecular analysis and heterologous expression of these proteins much more difficult than originally expected. Evolution has separated fungal iterative PKSs from bacterial modular PKSs in such manner, that initial attempts of cloning fungal PKSs failed due to the use of genomic probes from bacterial PKSs (Schümann and Hertweck, 2006; Weissman, 2008). Also, our preliminary studies showed that bacterial PKS prediction models are not suitable for genome mining of fungal iterative PKSs. Fungal iterative PKS genes and proteins have been cloned and characterized in several different ways, such as mapping the locus of T-toxin from *Cochliobolus heterostrophus* (Yang *et al.,* 1996), PCR cloning using degenerate primers for the KS and AT domains of highly-reducing, partially-reducing and non-reducing PKSs (Bingle *et al.,* 1999; Fujii *et al.,* 2005; Kellner and Zak, 2009), probe hybridization to localize a melanin PKS in *Ascochyta rabiei* (White and Chen, 2007), and genome-based PKS mining (Bok *et al.,* 2006; Gaffoor *et al.,* 2005). In one such study, 15 iterative PKSs were identified and disrupted in the proteome of *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schwein.) Petch) using a genome-based PKS mining approach (Gaffoor *et al.,* 2005). Fungal type I iterative PKSs have been shown to group separately from bacterial PKSs and related proteins such as fatty acid synthases (FAS), during phylogenetic analysis of the protein sequence of the KS domain. It has also been shown that the KS domain alone is enough to discriminate between reducing and non-reducing fungal iterative PKSs (Kroken *et al.,* 2003).

pHMMs (profile Hidden Markov Models) are statistical models that have been used with great success in bioinformatics (Birney, 2001; De Fonzo *et al.,* 2007; Eddy, 1998). The idea behind

pHMMs is to represent positions in a set of aligned sequences as hidden states. The actual amino acid instances are considered as emissions from the hidden match states. Insertions and deletions with respect to the match states are represented through insertion and deletion states, respectively. Emission probabilities as well as transition probabilities from and to the match states are determined based on a set of target sequences that show the protein domain of interest. The first step in building a pHMM is to assemble those sequences that are to be represented and construct a multiple alignment. The sequences that are to be included in a pHMM is a design choice that depends on the objectives for using the pHMM. A common use of pHMMs is to search databases for members of protein families (Schuster-Böckler *et al.,* 2004). pHMMs can, however, also be built to be more specific and distinguish subtypes of protein domains. A match in a pHMM search is characterized by a bit score (S), i.e. a statistical value based on the raw alignment score that has been normalized for comparison among different searches or databases. When the bit score of a match with a pHMM for one subtype is higher than that of the other subtype, this information can be used to differentiate such subtypes.

With the increasing number of fungi having whole genome sequences and predicted proteomes already available, a tool to identify PKS proteins for further studies would be very valuable, particularly where complete annotation of the genome is not available. Computational tools are already available on the web. However, they are limited in their ability to detect fungal PKSs, mainly because these models were constructed using bacterial modular PKSs (Li *et al.,* 2009; Starcevic *et al.,* 2008; Tae *et al.,* 2009). Foerstner *et al.* (2008) manually curated PKS domains retrieved from PKSDB, a modular bacterial PKSs database (Yadav *et al.* 2003) to build eight pHMMs for the following proteins domains: AT, ACP, KS, DH, ER, KR, C-methyltransferase (MeT) and thioesterase (TE). These domain pHMMs were shown to be a better predictor for PKS domains in

metagenomics samples than the ones available at PFAM and TIGRFAM. However, bit score values were not found to be sufficient to discriminate true PKS domains and non-PKS domains and reliable PKS and non-PKS discrimination was only achieved by phylogenetic analyses (Foerstner *et al.*, 2008). The ACP domain, though essential for creating a functional PKS (Hopwood and Sherman, 1990), was shown to be the least discriminative of all domains based on bit scores and phylogenetic analysis (Foerstner *et al.*, 2008). Khaldi *et al.* (2010) created a web-based software called SMURF (Secondary Metabolite Unique Regions Finder) for the prediction of secondary metabolite clusters, which included PKSs, nonribosomal peptide synthetases (NRPSs), hybrid PKS/NRPSs and prenyltransferases. SMURF predicts the backbone enzyme of each cluster and the decorating enzymes. The backbone enzymes are the main enzymes that catalyze the biosynthesis of the main product intermediate. The decorating enzymes modify the product intermediate catalyzing the final product. Khaldi *et al.* (2010) used the pHMMs for the PKS domains available in Pfam and TIGRFAM to search for backbone enzymes. The search for PKSs was performed using the AT domain, the KS C-terminal domain and the KS N-terminal domain. The predictions carried out in this work were found to be accurate. But, this model was found to yield false positives and false negatives; which could be due to the fact that they used pHMMs for PKS domains available at Pfam and TIGRFAM. As mentioned earlier, the PKS pHMMs from Pfam and TIGRFAM were found to be less accurate than the pHMMs developed by Foerstner *et al.*, (2008). We believe that the approaches suggested by Foerstner *et al.* (2008) and Khaldi *et al.* (2010) are valid and effective, however they do suffer from limitations posed by predictions yielded by mixed models. We believe that these limitations can be overcome by constructing protein sequence models based on the KS and AT domains of fungal PKSs. These could then be used to identify and study type I iterative PKSs within fungal genomes, predicted transcriptomes and proteomes. Therefore, the objectives of the present study were; 1)

assembly of the aforementioned databases to create two pHMMs corresponding to fungal KS and AT domains. 2) application of these two newly built fungi-based pHMMs for identification of putative type I iterative PKSs from fungal proteomes, and 3) comparison of these fungi-based pHMMs against mixed-kingdom KS and AT pHMMs that were built using the KS and AT domain sequences from different taxonomic groups.

Materials and Methods

Fungal genomes. Predicted proteomes of five plant pathogens with fully sequenced genomes were selected for this study. This included the cereal pathogen *Fusarium graminearum*, which has a fully sequenced and annotated genome, where all the PKSs have been identified and characterized. Based on the PKS identification status, *F. graminearum* was used for validation of the model. The predicted proteome of the *F. graminearum* isolate PH-1 (NRRL 31084) was obtained from the *F. graminearum* Genome Database (http://mips.helmholtz-muenchen.de/genre/proj/fusarium). This study also included the use of predicted proteomes of the pathogens *F. oxysporum* f. sp. *lycopersici*, *Verticillium albo-atrum* and *V. dahliae* where automated annotation of the genome is available. The proteomes of these three pathogens were retrieved from the Broad Institute (http://www.broadinstitute.org/). The genome sequence of the fungus *A. brassicicola* (ATCC 96836) was downloaded from Genome Sequencing Center at the Washington University School of Medicine (http://genome.wustl.edu). Gene identification was carried out using FGENESH, the gene finding algorithm from http://www.softberry.com with organism parameters specific to *Alternaria*. This algorithm yielded an output indicating the number of predicted genes, predicted exons, predicted mRNA, and predicted proteins.

Data compilation, model building, and PKS prediction. Fungal PKS protein sequences used to build the PKS identification model were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/), excluding proteins derived from the *A. brassicicola*, *F. graminearum*, *F. oxysporum* f. sp. *lycopersici*, *V. albo-atrum*, and *V. dahliae*. The KS and AT domains were manually curated from each PKS protein sequence using the Conserved Domain Search (CDS) at NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The protein sequences that had complete amino acid sequences for both KS and AT domains were trimmed and edited to create two fasta files containing the KS and AT domains, respectively. Each domain sequence was given the same accession number as the original protein sequence. In order to build pHMMs, multiple alignments were performed for the sequences of each domain. The pHMMs were named fungi-based KS model (FungalKS.hmm) and fungi-based AT model (FungalAT.hmm). The pHMMs were first tested against the predicted proteins from *F. graminearum* to determine the performance of the pHMMs; then against the predicted proteins of *V. albo-atrum* to further validate the pHMMs' performance. Later, the predicted proteins of *A. brassicicola*, *F. oxysporum* f. sp. *lycopersici*, and *V. dahliae* were searched against the pHMMs to identify potential PKS sequences by looking for KS and/or AT domain matches. The models were built using the HMMER (version 2.3.2) software and the search was performed using the default parameters (i.e. local alignment) of the software (http://hmmer.wustl.edu). New version of HMMER (HMMER3) can be found at http://hmmer.janelia.org. The fungi-based KS and AT sequences were further separated according to their predicted reducing nature to create four additional pHMMs: Reducing fungi-based KS model (RedFungalKS.hmm), Non-reducing fungi-based KS model (NrFungalKS.hmm), Reducing fungi-based AT model (RedFungalAT.hmm), and Non-reducing fungi-based AT model

(NrFungalAT.hmm). These pHMMs were used to predict the reducing nature of the putative PKS sequences by comparison of the bit scores (S).

The domain architectures of the predicted PKSs were analyzed using Conserved Domain Architecture Retrieval Tool (CDART) and CDS at NCBI. A predicted PKS was considered to be a functional PKS when the minimum domain architecture requirements were met, i.e. all KS, AT and ACP domains were present. The closest relative of each functional PKS was determined by BLASTP at NCBI with an E-value cutoff of 1.0×10^{-5} (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Stand-alone BLASTP was performed against the non-redundant (nr) protein sequence database using the default general and scoring parameters. Figure 2.1 describes the work flow carried out from the PKS protein collection to model validation and predictions. The reducing nature of the PKSs predicted in this study was determined by phylogenic analysis of the KS domain (Kroken *et al.*, 2003; Tamura *et al.*, 2007). The neighbor joining phylogeny algorithm was used with the Poisson correction model, 20 000 replications in bootstrap test, and the gaps were treated with pairwise deletion.

Comparison of the fungi-based PKS model to a mixed-kingdom model. The performance domain sequences were scored as 1, and non-PKS domains as 0. Thus, true-PKS domains predicted with a $S > 0$ were considered to be true positive (TP); non-PKS domains predicted with a $S < 0$ true negative (TN); true-PKS domains predicted with a $S < 0$ were considered to be false negative (FN); and non-PKS domains predicted with a $S > 0$ were considered to be false positive (FP). The performance of the model was evaluated as follows: accuracy= [TP+TN]/[TP+TN+FP+FN]; sensitivity= TP/[TP+FN]; specificity= TN/[TN+FP]; and precision= TP/[TP+FP] (Baldi *et al.*, 2000).

Validation of the reducing and non-reducing pHMMs was performed using a two-fold cross validation. Prior to validation, the PKS sequences were screened for duplicate sequences and even

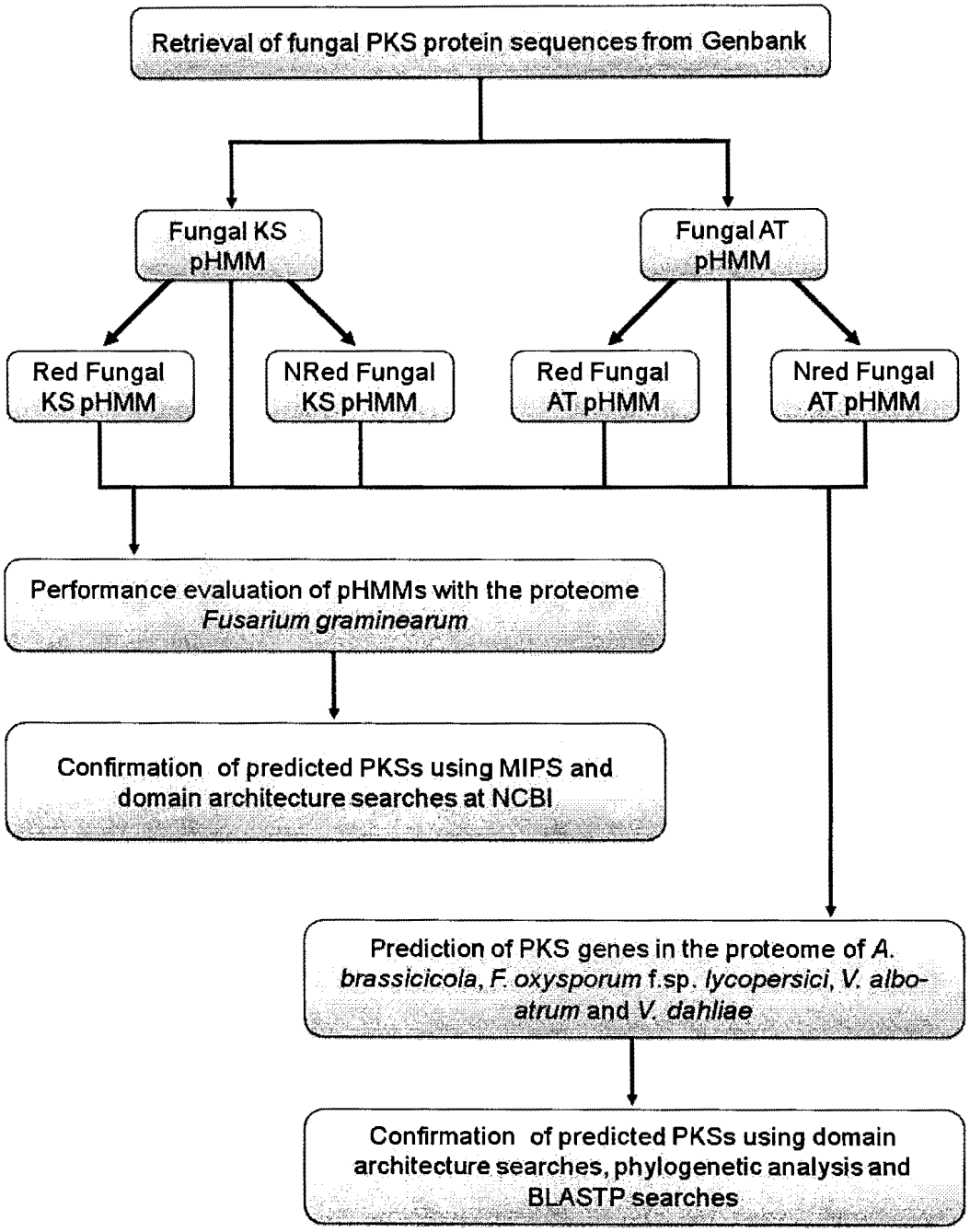

Figure 2.1. Flow chart of profile hidden markov model building, PKS validations and predictions in fungal proteomes. Abbreviations= PKS: polyketide synthase, KS: beta-ketoacyl synthase domain, AT: acyltransferase, Comp: compiled, Red: reducing, NRed: non-reducing, pHMM: profile hidden markov model, MIPS: Münich information center for protein sequences, BLASTP: protein basic local alignment search tool, NCBI: National center for biotechnology information.

multiple versions of the same sequence to assure the independency of the training set and test set to one another. The KS domain sequences were separated into two files, the first with 202 reducing KS sequences and 70 non-reducing KS sequences. The second file contained 202 reducing KS sequences and 69 non-reducing KS sequences. The AT domain sequences were also separated into two files as previously described. The class labels were as follows: reducing sequences were scored as 1, and non-reducing sequences as 0. Thus, reducing sequences predicted as reducing were considered to be true positive (TP); non-reducing sequences predicted as non-reducing were considered to be true negative (TN); reducing sequences predicted as non-reducing were considered to be false negative (FN); and non-reducing sequences predicted as reducing were considered to be false positive (FP). The performance of the models was also evaluated in comparison to the phylogenetic analysis. The performance of the models was evaluated using the parameters of accuracy, sensitivity, specificity, and precision calculated as mentioned above.

To further compare the fungi-based PKS model to the mixed-kingdom PKS model, statistical analyses were performed using the Statistical Analysis Software (SAS) version 9.1 (SAS Institute, Inc., Cary, NC). Comparisons of prediction of true PKSs between the fungi-based and mixed-kingdom PKS model for both the KS and AT domains were analyzed using paired T-test analysis (PROC TTEST) under the hypothesis that the mean difference between the models is equal to zero. Comparisons between the predictions of true-PKS versus non-PKS by model were analyzed using independent group analysis (PROC TTEST) under the hypothesis that the means of each group are equal. Box plot representations were obtained using PROC BOXPLOT. All analyses were performed using bit scores as the variable (α= 0.05).

DNA extraction, PCR assays and DNA sequencing of *Verticillium dahliae* PKSs. Four isolates of *V. dahliae* were provided by Dr. Neil Gudmestad from North Dakota State University.

Isolates H5, M5, U1 and U2 were obtained from potato plants affected by Verticillium wilt from Central Minnesota, USA. The isolates were grown in 100 ml of potato dextrose broth (EMD Chemicals, Darmstadt, Germany) for 10 days at room temperature. Fifty milligrams of freeze-dried mycelium was placed in a lysing matrix A (MP Biomedicals, Solon, OH) with 700 µl of nuclei lysis solution and homogenized using the FastPrep® homogenizer (MP Biomedicals, Solon, OH) set at a speed of 6.0 for 40 seconds. DNA extraction from fungal isolates was performed using Wizard® Genomic DNA Purification Kit (Promega, San Luis Obispo,CA) according to manufacturer's instructions. The predicted PKS genes from the *V. dahliae* isolates were amplified using specific PCR primers designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) (Table 2.1). PCR assays of *V. dahliae* PKSs were carried out using the Top Taq DNA polymerase kit (Qiagen, Valencia, CA) with reaction for the volume of 25 µl containing 25 ηg of fungal DNA, 1X PCR buffer pH 8.7, 2.0 µM of $MgCl_2$, 200 µM of dNTPs, 200 ηM of oligonucleotide primers, and 0.5 units of Top Taq DNA polymerase. The amplicons were separated on a 1% agarose gel with GelRed™ nucleic acid gel stain (Biotium, Inc., Hayward, CA) for 45 min at 90 mV. Selected amplicons were ligated to the pGEM-T easy vector (Promega, San Luis Obispo, CA) and cloned in One Shot® Mach1™ competent cells (Invitrogen, Carlsbad, CA). The plasmids were isolated using Wizard® Plus SV Miniprep DNA Purification Systems (Promega, San Luis Obispo, CA). The cloned insert was sequenced using M13 forward and reverse primers at McLab DNA sequencing services (San Francisco, CA).

Results

Data retrieval and model building. A total of 543 PKS protein sequences were compiled from GenBank. The presence of complete KS and AT domains in all the sequences was confirmed by

Table 2.1. Domain-specific primers for predicted PKS protein sequences from *Verticillium dahliae*

Gene Designation[1]	PKS Domain	Domain-specific PKS Primers		
		Forward Sequence (5' to 3')	Reverse Sequence (5' to 3')	Size of PCR Product[2]
VDAG_00190	KS	GAGCTTCTGTCGCAGGGCTT	AACCATTCTGCCGACGCCAT	752 bp
	AT	GCAAGGACCTCTACGCCCAC	CCCGTCAGCCTGTGGACTTC	716 bp
VDAG_01835	KS	GGTCACGGGCTCCAAGACTG	TACTCGTGTTGGAGCACGGC	772 bp
	AT	CTTTGTACGGCGCTCCAGGT	ATCAGCAGCAGCGGCCTAAG	708 bp
VDAG_01856	KS	TTGTCGATTCCCCGGCACAG	TCGCCCCCAAGACTCTCCAA	1334 bp
	AT	CCGCAGCCTATGCTTGTGGA	AGCCCTTGGGGAACTCTGGT	708 bp
VDAG_03466	KS	CCCGAACCCCGAGAAAAGGG	ACCGGCTTTGTTGAGGCACA	960 bp
	AT	GCTCGGAGCCACTTTCTCCC	TACGTCCCTTCGCTGAAGCG	822 bp
VDAG_07270	KS	TACGCGACGCACTATGACGG	CCTGTTGACCAGCCGCTCAT	1022 bp
	AT	GACTCGGCTCTGCAGGCATT	TGCCGGTCCTCTATCGCAGA	769 bp
VDAG_07928	KS	GTCGAGGCCCACAGCATTGA	ACACCTGCTGGAGACCCCAT	963 bp
	AT	GCTTCTCGTTGGAGCGGACA	CCGTATCCCGTACACTGGCG	806 bp
VDAG_08448	KS	CTGCCGAGTTCTGGGAGCTG	GACGGCGAAAGATCCGCTCT	1079 bp
	AT	TAGCCAGCCATCTTGCACGG	CCCGCAAAAAGGACGCCATC	766 bp
VDAG_09534	KS	AGCATTTCTGAGCGGTCGCA	ACTCCCCTAGCCAACGAGCA	778 bp
	AT	TATGCGTTGACGCGGCTCTT	TCGTACTCACCATCGCTGCG	828 bp

[1] Broad Institute protein designation
[2] PCR product from genomic DNA

conserved domain search (CDS) at NCBI. The Genbank accession numbers of the 543 fungal PKS proteins and their reducing nature can be accessed in Appendix A. PKS protein sequences with incomplete domains were not included in the pHMMs. A total of eight pHMMs were built, six fungi-based iterative PKS pHMMs containing the KS and AT domains from reducing and non-reducing iterative PKSs both separately and in combination (FungalKS.hmm, FungalAT.hmm, RedFungalKS.hmm, NrFungalKS.hmm, RedFungalAT.hmm, and NrFungalAT.hmm), and two mixed-kingdom PKS pHMMs (MixedKS.hmm and MixedAT.hmm). The mixed-kingdom KS and AT pHMMs were built using domain sequences obtained from Foerstner *et al.* (2008), which included PKS protein domains from the following taxonomic groups: Actinobacteria, Proteobacteria, Fungi, Cyanobacteria, Firmicutes, Animal, Mycetozoa, Alveolata, Viriplantae, Chloroflexi, Planctomycetes, Bacteroidetes/Chlorobi, among others. The six pHMMs based on the reducing, non-reducing and

combined KS and AT domain sequences of fungal origin were considered for the fungi-based iterative PKS model. The fungi-based and mixed-kingdom models were used to identify potential iterative PKSs from the predicted proteomes of all fungal species included in this study.

PKS predictions using fungi-based KS and AT pHMMs. The fungi-based PKS model predicted a total of 54 true KS domains and 56 true AT domains that represented a total of 53 putative iterative PKS proteins with both KS and AT domains (Table 2.2). Further evaluation of the entire protein sequence showed that 45 out of 53 predicted iterative PKSs were expected to be functional based on the presence of the ACP domain in their protein sequence (Table 2.3). In the predicted proteome of *F. graminearum*, 14 iterative PKSs out of 15 were found to contain the ACP domain and expected to be functional. Likewise, six out of 10 were expected to be functional iterative PKS proteins in the proteome of *V. albo-atrum* due to the presence of the ACP domain.

True PKSs and non-PKS proteins were determined based on their length, domain architecture, previous annotation as well as BLASTP matches using the non-redundant protein database at NCBI. Predicted domains were considered to be true PKS domains when yielded bit scores higher than zero (S >0). On the other hand, predicted domains that yielded bit scores lower than zero (S <0) when using the fungi-based PKS model were considered to be non-PKS domains. The fungi-based KS and AT pHMMs were able to separate true PKS proteins from non-PKS proteins in all studied fungal proteomes. Among all five proteomes, 26 non-PKS proteins were predicted by the fungi-based KS pHMM, and 39 by the fungi-based AT pHMM (Figure2.2).

Model predictions showed nine KS and nine AT domains in the proteome of *V. dahliae*. Among these, eight predicted iterative PKSs had both domains along with the ACP domain, and were therefore expected to be functional. The protein sequence VDAG_04539 (1114 aa) was

Table 2.2. Summary of PKS and non-PKS predictions by the fungi-based and mixed-kingdom PKS models. True PKS were separated from non-PKS proteins according to their protein length, previous annotation, BLASTP annotation using NCBI protein database, and protein domain architecture

Proteome	Score of the KS domain pHMM			Score of the AT domain pHMM		
	Predicted domains	Mean	Range	Predicted domains	Mean	Range
Prediction of true PKSs by the fungal-based models						
F. graminearum	15	791.0	603.9 - 935.4	15	412.9	204.7 - 497.4
A. brassicicola	7	795.9	449.7 - 910.7	8	353.3	46.3 - 530.0
F. oxysporum	13	767.0	579.6 - 958.4	14	354.3	27.0 - 516.1
V. albo-atrum	10	749.1	515.3 - 928.1	10	386.2	9.1 - 525.0
V. dahliae	9	751.4	506.5 - 928.8	9	340.3	73.7 - 527.1
Total	54	770.8	449.7 - 958.4	56	369.4	9.1 - 530.0
Prediction of non-PKSs by the fungal-based models						
F. graminearum	4	-242.6	-309.0 - -77.7	12	-149.8	-175.3 - -37.0
A. brassicicola	6	-276.9	-314.0 - -112.5	14	-156.8	-178.9 - -69.2
F. oxysporum	4	-180.2	-296.1 - -9.1	9	-143.1	-172.7 - -70.2
V. albo-atrum	6	-253.2	-312.1 - -84.5	9	-149.2	-177.3 - -8.7
V. dahliae	6	-272.2	-311.7 - -48.9	8	-149.1	-176.8 - -67.2
Total	26	-245.0	-314.0 - -9.1	52	-149.6	-178.9 - -8.7
Prediction of true PKSs by the mixed-kingdom models						
F. graminearum	15	178.4	123.8 – 216.0	15	278.8	197.7 – 347.1
A. brassicicola	7	171.1	65.9 – 212.9	8	238.1	29.5 – 354.6
F. oxysporum	13	148.2	78.5 – 242.8	14	241.8	27.7 – 497.7
V. albo-atrum	10	159.9	43.3 – 243.8	10	254.7	43.9 – 379.1
V. dahliae	9	151.7	60.5 – 243.9	9	221.6	52.1 – 380.8
Total	54	161.9	43.3 – 243.9	56	247.0	27.7 – 497.7
Prediction of non-PKSs by the mixed-kingdom models						
F. graminearum	2	12	-5.4 – 30	13	-59.7	-112.8 – 173.2
A. brassicicola	13	-7.2	-14.3 – 7	20	-91.9	-116.3 – 82.2
F. oxysporum	2	-1.8	-1.8	8	-9.9	-108.6 – 92.1
V. albo-atrum	8	-4.7	-11.8 – 29.1	29	-90.7	-114.5 – 198.9
V. dahliae	14	-5.5	-11.6 – 37.1	34	-98.4	-114.5 – 128.0
Total	39	-1.4	-14.3 – 37.1	104	-70.1	-116.3 – 198.9

Table 2.3. Protein domain architecture of predicted PKS protein sequences from all five fungal predicted proteomes

Protein	Protein size (aa)	Domain Architecture[1]	Functional status[2,3]
Alternaria brassicicola			
Ab001seq371	2376	KS-AT-DH-ER-KR-ACP	Functional PKS
Ab001seq648	1985	KS-AT-ACP-TE	Functional PKS
Ab003seq685	2905	KS-AT-DH-KR-TE	Nonfunctional PKS
Ab003seq693	460	[KS]-AT	Distorted PKS
Ab004seq368	2370	KS-AT-DH-MeT-ER-KR-ACP	Functional PKS
Ab004seq469	2998	MeT-KS-AT-DH-MeT-ER-KR	Nonfunctional PKS
Ab005seq505	2493	KS-AT-DH-MeT-ER-KR-ACP	Functional PKS
Ab008seq352	2200	KS-AT-DH-MeT-ER-KR-ACP	Functional PKS
Fusarium graminearum			
FG01790	2466	KS-AT-DH-MeT-ER-KR-ACP	Functional PKS
FG02395	1344	KS-AT	Nonfunctional PKS
FG03340	2566	KS-AT-DH-MeT-ER-KR-ACP	Functional PKS
FG03964	2030	KS-AT-DH-ACP-TE	Functional PKS
FG04588	2173	KS-AT-ACP-MeT	Functional PKS
FG05794	3178	KS-AT-DH-MeT-ER-KR-ACP	Functional PKS
FG08795	2351	KS-AT-DH-ER-KR-ACP	Functional PKS
FG10548	2464	KS-AT-DH-ER-KR-ACP	Functional PKS
FG12040	2068	KS-AT-ACP-TE	Functional PKS
FG12055	2346	KS-AT-DH-ER-KR-ACP	Functional PKS
FG12100	3921	KS-AT-DH-MeT-KR-ACP-CD-LuxE-ACP-KR	Functional PKS/NRPS
FG12109	2567	KS-AT-DH-MeT-ER-KR-ACP	Functional PKS
FG12121	2643	KS-AT-DH-MeT-KR-ACP	Functional PKS
FG12125	2288	AT-KS-AT-ACP-ACP-TE	Functional PKS
FG12977	2564	KS-AT-DH-MeT-ER-KR-ACP	Functional PKS
Fusarium oxysporum* f. sp. *lycopersici			
FOXT_02741	2553	KS-AT-DH-MeT-ER-KR-ACP	Functional PKS
FOXT_02884	1982	KS-AT-DH-ER-KR-ACP	Functional PKS
FOXT_03051	2358	KS-AT-DH-ER-KR-ACP	Functional PKS
FOXT_03945	3802	[KS]-AT-DH-MeT-KR-ACP-CD-LuxE-KR	Distorted PKS/NRPS
FOXT_04757	1241	KS-AT	Nonfunctional PKS
FOXT_05816	2084	KS-AT-ACP-ACP-TE	Functional PKS
FOXT_10805	2524	KS-AT-DH-MeT-ER-KR-ACP	Functional PKS
FOXT_11892	2439	KS-AT-DH-MeT-KR-ACP	Functional PKS
FOXT_11954	2454	KS-AT-DH-MeT-ER-KR-ACP	Functional PKS

Table 2.3. (continued)

Protein	Protein size (aa)	Domain Architecture[1]	Functional status[2,3]
FOXT_14587	3585	KS-AT-DH-MeT-KR-CD-LuxE-ACP-KR	Functional PKS/NRPS
FOXT_14850	2183	KS-AT-DH-MeT-ER-KR-ACP	Functional PKS
FOXT_15248	2377	KS-AT-DH-ER-KR-ACP	Functional PKS
FOXT_15296	3895	KS-AT-DH-MeT-KR-CD-LuxE-ACP-KR	Functional PKS/NRPS
FOXT_15886	2166	KS-AT-DH-MeT-ER-KR-ACP	Functional PKS
Verticillium dahliae			
VDAG_00190	2190	KS-AT-ACP-ACP-TE	Functional PKS
VDAG_01835	2624	KS-AT-DH-MeT-ER-KR-ACP	Functional PKS
VDAG_01856	2403	KS-AT-DH-ER-KR-ACP	Functional PKS
VDAG_03466	2161	KS-AT-DH-ER-KR-ACP	Functional PKS
VDAG_04539	1114	[KS]-AT-DH	Distorted PKS
VDAG_07270	2240	KS-AT-DH-MeT-KR-ACP	Functional PKS
VDAG_07928	4043	KS-AT-DH-MeT-KR-ACP-CD-LuxE-ACP-KR	Functional PKS/NRPS
VDAG_08448	2211	KS-AT-DH-ER-KR-ACP	Functional PKS
VDAG_09534	1857	KS-AT-DH-ACP-ACP-TE	Functional PKS
Verticillium albo-atrum			
VDBG_00162	2167	KS-AT-DH-ER-KR-ACP	Functional PKS
VDBG_00580	2006	KS-AT-ACP-ACP-TE	Functional PKS
VDBG_01329	1365	KS-AT-DH	Nonfunctional PKS
VDBG_01693	1971	KS-AT-DH-ER-KR	Nonfunctional PKS
VDBG_01714	2496	KS-AT-DH-MeT-ER-KR	Nonfunctional PKS
VDBG_04992	596	KS-AT	Nonfunctional PKS
VDBG_04998	3999	KS-AT-DH-MeT-KR-ACP-CD-LuxE-ACP-KR	Functional PKS/NRPS
VDBG_06312	1756	KS-AT-DH-ER-KR-ACP	Functional PKS
VDBG_09122	2207	KS-AT-DH-MeT-KR-ACP	Functional PKS
VDBG_09801	1912	KS-AT-DH-ACP-ACP-TE	Functional PKS

[1] Abbreviations= PKS: polyketide synthase, NRPS: nonribosomal peptide synthase, PKS/NRPS: hybrid proteins, KS: beta-ketoacyl synthase domain, AT: acyltransferase domain, DH: dehydratase domain, MeT: C-methyltransferase domain, ER: enoylreductase domain, KR: ketoreductase domain, ACP: acyl carrier protein domain, TE: thioesterase domain, CD: condensation domain of nonribosomal peptide synthases, LuxE: acyl-protein synthetase domain. Brackets means truncated domain.
[2] Functional protein status was predicted according to the presence or absence of the ACP domain in the protein architecture
[3] PKS/NRPS is a hybrid enzyme composed of PKS domains and nonribosomal peptide domains.

predicted only by AT pHMM with a bit score of 73.7. This sequence was also predicted by the fungi-based KS pHMM but the bit score was -239.7. Domain architecture analysis showed the presence of a truncated KS domain (91 aa). Also, the protein sequence VDAG_05633 (402 aa) was predicted

only by the KS pHMM with a bit score of 506.5, which was determined to be a true KS domain protein by CDS at NCBI.

In the proteome of *F. oxysporum* f.sp. *lycopersici*, 12 out of 13 predicted PKS were considered functional. Sequence FOXT_03945 (3802 aa) was predicted only by AT pHMM with a bit score of 467.3, but the KS pHMM predicted it with a bit score of -9.1. Further domain architecture analysis showed the presence of a truncated KS domain (166 aa). Its domain architecture resembled a PKS/NRPS protein. Due to the presence of a truncated KS domain, this sequence was considered to be a distorted PKS. Such distortion could be caused by several factors including among others the state of scaffold assembly, gene prediction errors, and mutations.

The publicly available genome sequence of *Alternaria brassicicola* had a total of 838 scaffolds that were analyzed using FGENESH to predict a total of 7840 genes. The translated proteins were subsequently analyzed by the KS and AT models to identify putative PKS sequences. Five out of seven predicted PKSs were expected to be functional based on the presence of all KS, AT and ACP domains. The sequence Ab003seq693 (460 aa) was predicted only by AT pHMMs with a bit score of 46.3. Its domain architecture showed the presence of a true AT domain and a truncated KS domain (71 aa). This sequence was also considered distorted.

Discrimination of reducing and non-reducing iterative PKSs. Two approaches were tested for the efficacy of these pHMMs in discriminating between reducing and non-reducing iterative PKSs. The first approach involved phylogenetic analysis of the KS domain based on Kroken *et al.* (2003) and the second approach involved the use of bit scores from predictions using the reducing and non-reducing models (RedFungalKS.hmm, NrFungalKS.hmm, RedFungalAT.hmm, and NrFungalAT.hmm).

The KS domains of all 53 putative PKS proteins were analyzed phylogenetically by neighbor joining analysis (Figure 2.2). This analysis showed that the predicted fungal iterative PKSs were grouped in clusters that were different from the modular bacterial PKSs. Moreover, within the main grouping the fungal iterative PKSs separated in two distinctive clusters. The separation of the fungal iterative PKSs occurred according to their reducing nature. The reducing fungal iterative PKS cluster contained 41 of the predicted sequences while the non-reducing fungal iterative PKS cluster grouped 12 sequences. According to this grouping, 10 iterative PKSs were reducing and five were non-reducing in *F. graminearum*; six iterative PKSs were determined to be reducing and one to be non-reducing in the proteome of *A. brassicicola*; 11 were reducing and two were non-reducing in *F. oxysporum* f. sp. *lycopersici*; eight were reducing and two were non-reducing in *V. albo-atrum*; and six were reducing and two were non-reducing in *V. dahliae*.

Comparison of the bit scores obtained from the searches performed using the reducing and non-reducing models showed that 42 of the predicted fungal iterative PKSs were reducing and 11 were non-reducing. Sequence of the predicted protein FG04588 of *F. graminearum* was predicted differently by the two methods. It was predicted as non-reducing by the phylogenetic analysis but as reducing by bit score comparison of the reducing and non-reducing model predictions. Previous phylogenetic studies had concluded that this protein is a non-reducing iterative PKS (Gaffoor *et al.*, 2005; Kroken *et al.*, 2003). The bit scores for this sequence were 569.9 and 520.9 when using RedFungalKS and NrFungalKS, respectively; as well as 180.4 and 69.6 when using RedFungalAT, and NrFungalAT pHMMs, respectively. Comparison of the reducing and nonreducing models for each domain suggested a reducing nature for this sequence because a higher bit scores for both domains while using the reducing pHMM. The sequence VDBG_00162 of *V. dahliae* had been annotated as

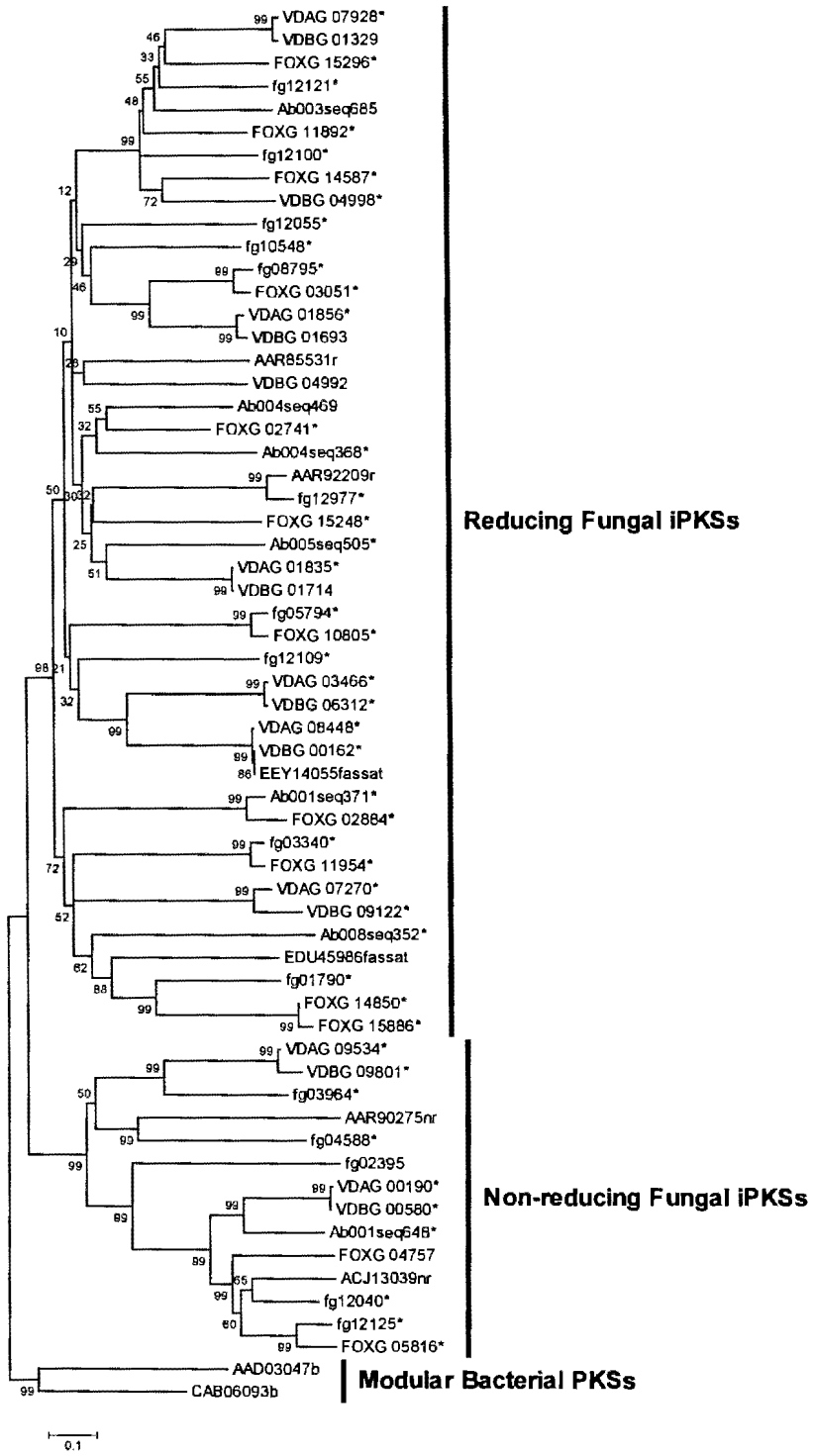

Figure 2.2. Phylogenetic analysis of the KS domain of type I PKS proteins identified from all five fungal proteomes. The star (*) next to the protein IDs indicates functional PKSs, b indicates bacterial PKS, nr is non-reducing, r is reducing, , and fassat is fatty acid synthase S-acetyltransferase.

fatty acid synthase S-acetyltransferase (FAS S-acetyltransferase) through automated annotation by the Broad Institute; however, our fungi-based model predicted this sequence as PKS. Domain architecture analysis showed that this sequence contained true PKS domains. Furthermore, phylogenetic analysis of the KS domain showed that this sequence grouped with reducing fungal iterative PKSs. BLASTP analysis of this sequence showed that one of its closest relative is in fact a polyketide synthase from the fungus *Botryotinia fuckeliana* (GenBank accession No. AAR90244) with an E-value of 0.0 and a bit score value of 5185, previously characterized by Kroken *et al.*, (2003). The best matches for all predicted iterative PKSs in GenBank were determined to be PKSs by BLASTP (Appendix B).

Comparison of the fungi-based PKS model to a mixed-kingdom model. The performance of the fungi-based PKS model was evaluated in comparison to the mixed-kingdom PKS model by calculation of the accuracy, sensitivity, specificity and precision parameters (Table 2.4). Both the fungi-based PKS model and the mixed-kingdom PKS model yielded same number of true PKS predictions in the proteome of *F. graminearum* and in all five fungal proteomes but they differed in their performance as measured by the aforementioned parameters and statistical analyses of predictions using bit scores. The fungi-based KS and AT models performed with higher accuracy, specificity and precision than the mixed-kingdom KS and AT models, respectively, when evaluated using the *F. graminearum* proteome alone and when evaluated using all five fungal proteomes.

Paired t-test analyses showed that the mean bit score of true KS domains was significantly different (P <0.0001) between the fungi-based KS model and the mixed-kingdom KS model. Overall, the bit score values for the fungi-based KS model ranged from 449.7 to 958.4 with a mean of 771.5, while for the mixed-kingdom KS model ranged from 43.3 to 243.9 with a mean of 162.5 (Figure 2.3A). Likewise, the paired t-test analyses determined that mean bit score of true AT domains was

significantly different (P <0.0001) between the fungi-based AT model and the mixed-kingdom AT model. Overall, the bit score values for the fungi-based AT model ranged from 9.1 to 530.0 with a mean of 372.5, while for the mixed-kingdom AT model ranged from 27.7 to 497.7 with a mean of 249.6 (Figure 2.3B).

Table 2.4. Performance of fungi-based and mixed-kingdom KS and AT pHMMs[1]

Parameters	Proteome of *Fusarium graminearum*				Proteome of all five fungal species[2]			
	Fungal-based pHMM		Mixed-kingdom pHMM		Fungal-based pHMM		Mixed-kingdom pHMM	
	KS domain	AT domain	KS domain	AT domain	KS domain	AT domain	KS domain	AT domain
True Positives	15	15	15	15	54	56	54	56
True Negatives	4	12	1	10	26	52	34	91
False Positives	0	0	1	3	0	0	5	13
False Negatives	0	0	0	0	0	0	0	0
Accuracy	100.0	100.0	94.1	89.3	100.0	100.0	94.6	91.9
Sensitivity	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Specificity	100.0	100.0	50.0	76.9	100.0	100.0	87.2	87.5
Precision	100.0	100.0	93.8	83.3	100.0	100.0	91.5	81.2

[1] PKS domains identified as PKS domains (S >0) were considered to be a true positive, non-PKS domains identified as non-PKS domains were considered a true negative (S <0), non-PKS domains identified as PKS domains (S >0) were considered to be a false positive, and PKS domains identified as non-PKS domains were considered a false negative (S <0)

[2] *Fusarium graminearum*, *Fusarium oxysporum* f. sp. *lycopersici*, *Alternaria brassicicola*, *Verticillium albo-atrum* and *Verticillium dahliae*.

Independent group analyses (t-test) showed that the mean bit score of true PKS domains was significantly different (P <0.0001) than the non-PKS domains for all fungi-based KS, fungi-based AT, mixed-kingdom KS, and mixed-kingdom AT models. There was no bit score overlap between bit score values of true PKS domains and non-PKS domains when the fungal proteomes were analyzed with both the fungi-based KS and AT models (Table 2.2, Figure 2.3). However, there was an overlap between bit scores of true PKS domains and non-PKS domains when the fungal proteomes were analyzed with both the mixed-kingdom KS and AT models (Table 2.2, Figure 2.3). Moreover, the non-PKS domains predicted by the both fungi-based models did not present positive bit score values (S <0), in contrast with the mixed-kingdom models where some non-PKS domains showed

positive bit score values (S >0) leading to the prediction of false positives (Figure 2.3C and Figure 2.3D). Overall, the fungi-based KS and AT models performed better than the mixed-kingdom KS and AT models respectively.

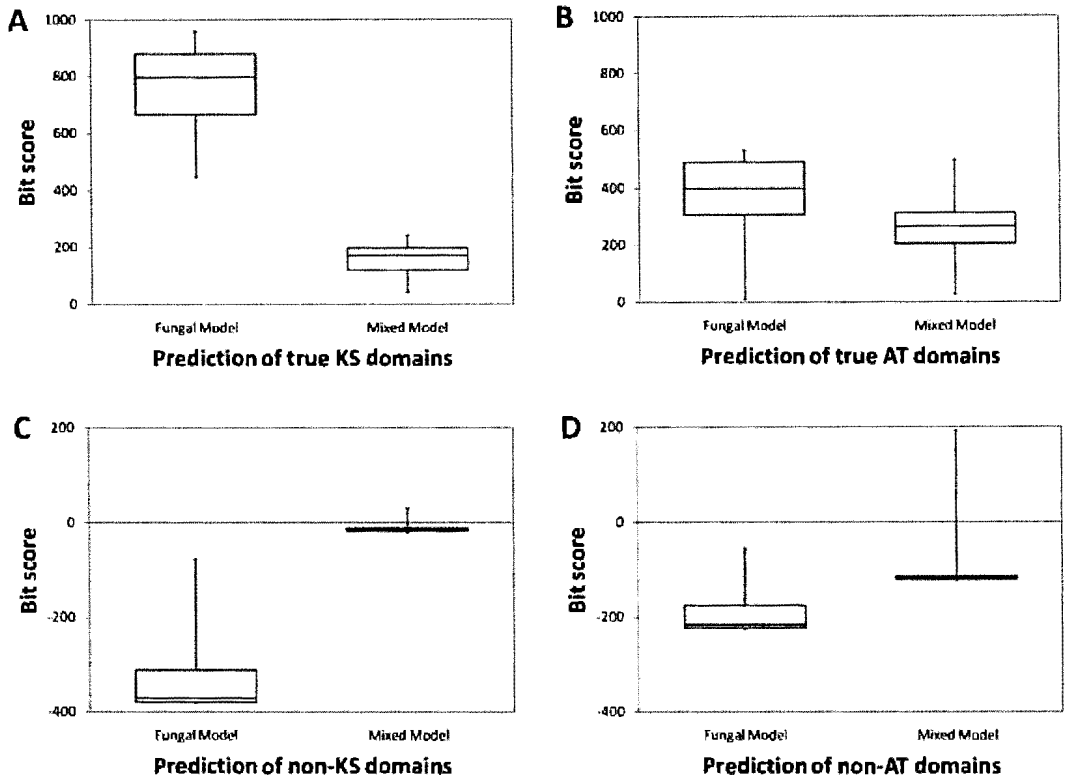

Figure 2.3. Box plots of the bit score distribution of pHMM searches. A) bit score distribution of true KS domains predicted by the fungi-based KS model and mixed-kingdom KS model, B) bit score distribution of true AT domains predicted by the fungi-based KS model and mixed-kingdom KS model, C) bit score distribution of non-KS domains predicted by the fungi-based KS model and mixed-kingdom KS model, D) bit score distribution of non-AT domains predicted by the fungi-based KS model and mixed-kingdom KS model. The vertical bars represent the range of bit scores for each prediction. The bottom horizontal bar of the boxplot represents the 25th percentile, the middle represents the median, and the top bar represents the 75th percentile.

Validation of the reducing/non-reducing fungi-based models. Both KS and AT models were cross-validated using a two-fold cross validation approach with the reducing and non-reducing

databases. In the KS model, 403 true positives, 139 true negatives, one false positive and no false negatives were determined, showing that the KS model had an accuracy of 99.8%, sensitivity of 100%, specificity of 99.3% and precision of 99.8%. Similarly, the AT model was found to have an accuracy of 99.8%, sensitivity of 99.8%, specificity of 100% and 100% of precision with 403 true positives, 139 true negatives, no false positives and one false negative.

PCR assays for confirmation of *V. dahliae* PKS sequences. Domain-specific primers (Table 2.1) were designed for the KS and AT domains of each *V. dahliae* PKS sequence in order to confirm the presence of the genes in four isolates obtained from potato plants affected by Verticillium wilt. Initially, sets of primers were tested to determine their ability to amplify the KS and AT domains of *VDAG_00190*, *VDAG_01856*, *VDAG_03466*, and *VDAG_07928* genes from the isolates M5 and U2 obtained from infected potato stems. The PCR products were found to match the predicted sizes for each gene and were subsequently sequenced. The sequences of these amplicons were analyzed using BLASTX, confirming either the KS or the AT identity of each PCR product (data not shown). All *V. dahliae* isolates, namely H5, M5, U1, and U2 showed the presence of seven of the eight predicted PKSs (Figure 2.4). The KS and AT domains of VDAG_01835 were not amplified in any of the isolates, suggesting it may be unique to strains most closely related to the sequenced isolate.

Discussion

The present study explored the possibility of identifying type I iterative PKSs in predicted fungal proteomes using fungal PKS-specific domain pHMMs to provide a simple method for identifying iterative PKSs from predicted proteomes of unannotated genome sequences or transcript data. In order to achieve this goal, we manually selected and curated intact, complete

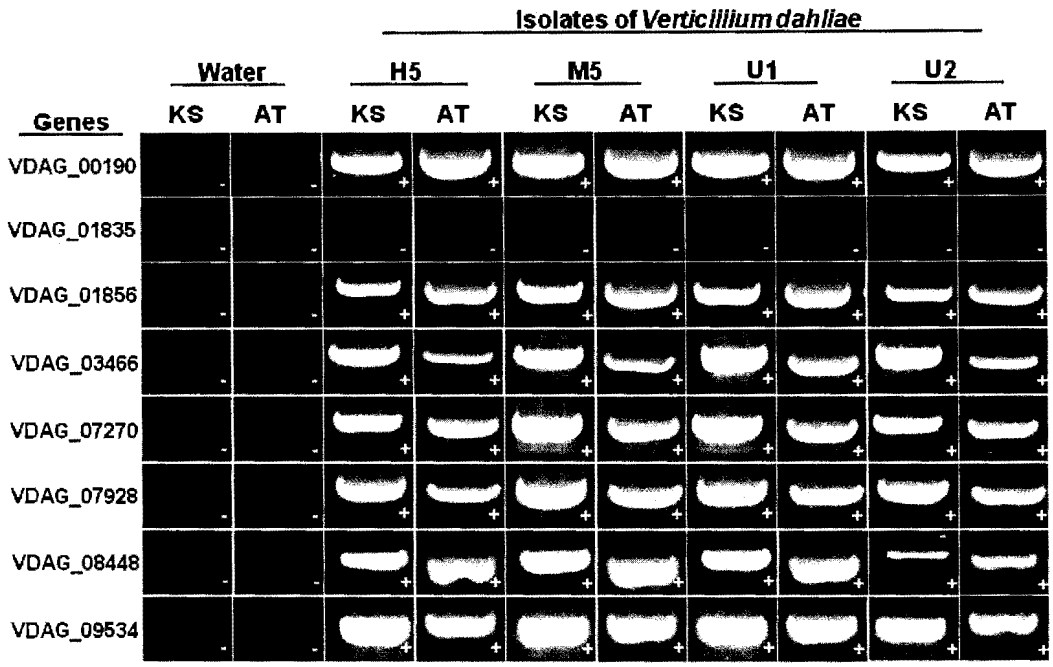

Figure 2.4. PCR assay for PKS genes of four *Verticillium dahliae* isolates using KS and AT domain-specific primers. Rows represent the different PKS genes. Isolates are arranged by columns.

protein sequences of KS and AT domains to create domain databases. Two compiled pHMMs (containing sequences of each domain from reducing and non-reducing fungal iterative PKSs) and four pHMMs (containing only reducing or non-reducing sequences for each domain) were built based on the two protein domains and used to identify fungal PKS proteins from the *F. graminearum*, *V. albo-atrum*, *F. oxysporum* f. sp. *lycopersici*, *V. dahliae* and *A. brassicicola* proteomes. Similar studies have demonstrated the advantages of identifying specific types of PKSs including type I iterative PKSs using a bioinformatic-based or genomic-based approach versus classical molecular cloning using degenerate primers (Bingle *et al.*, 1999; Kroken *et al.*, 2003; Gaffoor *et al.*, 2005). Among the available approaches, the most recently published resource was developed by Khaldi *et al.*, (2010). This approach called SMURF is a web-based resource for the identification of gene clusters of secondary metabolites, including PKSs. This software has been successfully used to identify fungal iterative PKSs. However, it has been shown to yield false

positives and more importantly false negatives. We believe that this might be due to the use of Pfam and TIGRFAM pHMMs to predict PKS proteins. The pHMMs available at Pfam and TIGRFAM are mixed-kingdom models and have been shown to perform less accurately than the mixed models used by Foerstner *et al.*, (2008). To our knowledge, there are no publicly available pHMMs solely based on protein domains of fungal PKSs.

The *F. graminearum* genome was sequenced in 2003 (Cuomo *et al.*, 2003) and extensive studies have since been conducted for gene prediction and characterization including functional evaluation of all 15 iterative PKSs found in this fungus through gene disruption (Gaffoor *et al.*, 2005). Therefore, the proteome of *F. graminearum* was used as control for validation and evaluation of our fungi-based PKS model. Our goal was to develop a method for identification of type I iterative PKSs from fungal plant pathogens for further analysis of their function, their classification into reducing and non-reducing types, and to study their regulation in association with disease initiation and development.

Three PKS domains are considered to be the minimal domain architecture for a PKS protein to be functional, namely KS, AT and ACP domains (Hopwood and Sherman, 1990). Our model was built using only the KS and AT domains, as it has been previously demonstrated that the ACP domain is the least suitable domain for discrimination between true PKS and non-PKS proteins through phylogenetic and pHMM analyses due to their short length (Foerstner *et al.*, 2008). Later, each protein sequence predicted by both the fungi-based KS and AT pHMMs was analyzed by protein domain architecture searches to determine if predicted proteins were functional or not, based on the presence of the ACP domain in their domain architecture. Our models predicted 53 iterative PKSs, of which 45 were predicted to be functional according to their domain architecture. Out of the 15 iterative PKSs identified by our model in the predicted proteome of *F. graminearum*,

14 were found to be functional. *F. graminearum* PKS protein FG02395 is believed to be non-functional due to the lack of the ACP domain in its domain architecture. The length of this protein, according to the *Fusarium graminearum* Genome Database at Munich Information Center for Protein Sequences, was of 1344 amino acids and its domain architecture was KS-AT. However, further evaluation showed that this sequence has been experimentally determined to be 1995 amino acids long and its domain architecture was KS-AT-ACP (Gaffoor *et al.*, 2005). This iterative PKS is functional and encodes for a PKS involved in the biosynthesis of zearalenone in *F. graminearum* isolates (Gaffoor *et al.*, 2005; Gaffoor and Trail, 2006). This is an example of an atypical protein for which an earlier gene prediction program did not predict the complete gene. These and other results support the conclusion that our model is well suited for identification of putative iterative PKSs in fungal genomes and that our strategy of including only the KS and AT domains in our fungi-based PKS model is well justified.

The fungi-based KS model also showed the ability to exclude truncated KS domains. Truncated PKS proteins were only predicted by the fungi-based AT model. It was reported by Wight *et al.*, (2009) that the genome of *A. brassicicola* had nine iterative PKSs but details of these iterative PKSs were not available. The only *A. brassicicola* PKS published as being associated with virulence (Genbank accession no. ACZ57548) was found to be similar to Ab001seq371 identified by the fungi-based PKS model. Our model only identified seven PKS sequences plus one truncated PKS sequence. We attribute this disagreement to differences in the gene prediction and to scaffold assembly. We found 13 PKS sequences in the proteome of *F. oxysporum* f. sp. *lycopersici* that had previously been annotated as conserved hypothetical proteins. However, the domain architecture as well as phylogenetic analysis of the KS domain clearly defines them as iterative PKS proteins.

Their closest relatives were also identified as PKSs by stand-alone BLASTP against NCBI nr protein database.

We compared our fungi-based PKS model against a mixed-kingdom PKS model derived from the mining of PKSs within metagenomic libraries (Foerstner *et al.,* 2008). This mixed-kingdom model contained PKS domains from different taxonomic groups, including bacteria and fungi, among others. Models developed by Foerstner *et al.* (2008) were proven to perform better than the models available at PFAM and TIGRFAM, which are also considered to be mixed-kingdom models. Similarly, our fungi-based KS and AT models were shown to perform even better than the mixed-kingdom KS and AT models in the prediction of iterative PKSs from fungal species. Though overall iterative PKS predictions between the two models were similar, our fungi-based PKS models were found to predict fewer false positives and false negatives. Moreover, the fungi-based model was significantly better in identifying iterative PKS proteins from closely related proteins. The performance was evaluated using the *F. graminearum* proteome and also all five fungal proteomes. We observed that the fungi-based PKS models predicted all true PKS domains with positive bit score values (S >0), while non-PKS domains were predicted with bit score values below zero (S <0). The mixed-kingdom KS and AT models failed to discriminate closely related PKS domains yielding false positives due to some non-PKS domains being predicted with bit score over zero (S >0). Also, the fungi-based KS model predicted true KS domains with higher bit scores than the mixed-kingdom model with no overlap of bit score values. The fungi-based AT model had a bit score mean significantly different from the mixed-kingdom AT model but there was overlap of bit score values. This can be justified by the fact that the KS domain is more conserved than the AT domain within the kingdoms (Bingle *et al.,* 1999; Langfelder *et al.,* 2003; Nicholson *et al.,* 2001). The only discrepancies observed in the predictions were in the case of the sequences VDBG_00162 and

VDAG_03466 from *V. albo-atrum* and *V. dahliae*, respectively, which have been annotated by the Broad Institute as FAS S-acetyltransferases, and the sequence VDAG_01835 that has been annotated as a FAS. All three sequences were not discriminated as non-PKSs by the fungi-based PKS models, the mixed-kingdom PKS models or phylogenetic analysis. Further analysis showed that these proteins were also indistinguishable from PKSs according to their domain architecture. Therefore, it is likely that their true identity can only be resolved by gene disruption experiments coupled with metabolite screening analyses, which have yet to be conducted. In fact, fungal FAS S-acetyltransferases have been only identified in the predicted proteome *V. albo-atrum*, *Paracoccidioides brasiliensis* and *Pyrenophora tritici-repentis*; all through automated annotation by the Broad Institute.

During our search for PKS prediction tools currently available, we came across a computational tool called MAPSI (Management and Analysis for polyketide synthase type I), which is an updated version of the previous tool named ASMPKS (Analysis system of modular PKSs) (Ansari *et al.*, 2004; Tae *et al.*, 2009). According to the information available, MAPSI is able to analyze not only modular type I PKSs but also iterative type I PKSs from bacterial and fungal genomes previously uploaded to the software or manually uploaded by the user. This software works under the assumption that several organisms may share the same PKS gene clusters. This software identifies PKS genes, describes their domain architectures and associates the PKS genes with polyketide products. In the database, each PKS protein sequence is associated to a GenBank accession number as well as a polyketide product. The database consists of a total of 215 PKS protein sequences, of which 193 sequences are bacterial PKSs and only 22 are fungal PKSs. The database MAPSI consists of 65 different polyketide products of which 45 are synthesized by modular PKSs and 20 by iterative PKS. There are only 17 polyketide products of fungal origin, all

from iterative PKSs. We have not been able to upload and evaluate the fungal genomes included in this study as the "user genome" option seems to be under construction. However, we feel that a more diverse fungal PKS protein database is needed in order to identify novel PKS proteins that have yet to be associated with a polyketide product. We also explored the possibility of using other bacterial-based PKS predictors before building our fungi-based PKS model. In this regard, we evaluated the softwares ClustScan (Starcevic *et al.,* 2008) and NP.searcher (Li *et al.,* 2009) with all five fungal genomes. The latter was initially developed for nonribosomal peptide synthases. ClustScan was not appropriate for fungal genomes as the software input requires for nucleotide sequences from which PKS genes are predicted using bacterial gene predictors. The NP.searcher on the other hand failed to predict any PKS from all five fungal genomes.

We observed that comparison of bit scores from reducing and non-reducing models of either the KS or AT domains could be used for determining the reducing nature of the predicted proteins before conducting a phylogenetic analysis of the KS domain. This observation was confirmed with phylogenetic analyses of the identified type I iterative PKSs from all five fungal proteomes using KS domain sequences as previously reported (Kroken *et al.,* 2003). For example, the PKS sequence FG04588 had been previously classified as a non-reducing PKS (Gaffoor *et al.,* 2005; Kroken *et al.,* 2003), but our model classified it as reducing. The bit score comparison was a good estimator of the reducing nature of PKSs; however, phylogenetic analysis had even more discriminatory power as concluded by other authors (Foerstner *et al.,* 2008, Kroken *et al.,* 2003). Previous authors have also concluded that phylogenetic analyses are more powerful in distinguishing true PKS and non-PKS domains (Foerstner *et al.,* 2008). The reducing and non-reducing pHMMs presented high accuracy, sensitivity, specificity and precision (99-100%).

Therefore, we believe that bit scores could be used effectively for initial categorization into reducing and non-reducing PKS immediately after screening through the predicted proteome.

We feel that the use of the two fungi-based KS and AT models would be helpful in the identification and annotation of iterative PKSs from predicted fungal proteomes; and, that fungal genomes and predicted transcriptomes could be screened to narrow down the number of sequences to study when dealing with whole genome BLAST searches. This fungi-based PKS model has been successfully used in the genome of the legume fungal pathogen *Ascochyta rabiei*, at a scaffold stage, from which we have predicted its iterative PKSs and confirmed them through PCR and RT-PCR analyses.

User instructions for the use of the HMMER3 software with the fungi-based KS and fungi-based AT alignments are accessible in the Appendix C. HMMER3 can be downloaded from http://hmmer.janelia.org (Eddy, 1998, 2008). The multiple alignments of fungal KS and AT domains have been made available at SMART, which is a user-friendly web-based protein domain database (http://smart.embl.de/help/additional_alignments.shtml). The submitted alignments contain the 543 KS and AT domains obtained from fungal PKSs from NCBI that were manually curated for this work; and the KS and AT domains obtained from the fungal proteomes included in this study, i.e. they included KS and AT domains from *Alternaria brassicicola*, *Fusarium graminearum*, *F. oxysporum* f. sp. *lycopersici*, *Verticillium albo-atrum* and *V. dahliae*.

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PAPER THREE. GENOME-WIDE IDENTIFICATION AND CHARACTERIZATION OF POLYKETIDE SYNTHASES AND METHYLTRANSFERASES FROM THE FUNGAL PLANT PATHOGEN *ASCOCHYTA RABIEI*

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Authour Note: My role in this manuscript was to design, conduct and analyze all the experiments that were carried out for this manuscript. I also brought forth and developed with the concept of this project. Furthermore, I wrote this manuscript and performed all corrections suggested by the co-authors.

Introduction

Fungal secondary metabolites include chemically diverse compounds of low molecular weight, which may have biological activities such as allelopathy, enzyme inhibition, immunosuppression, cytotoxicity, phytotoxicity, mutagenesis, carcinogenesis, teratogenesis; as well as antibacterial, antifungal, and antitumor activities among others (Keller *et al.*, 2005). However, these compounds are not considered to be essential for the normal growth and development of fungal species under laboratory conditions (Bennett, 1983; Gaffoor and Trail, 2006). Secondary metabolites are synthesized by a number of pathways from precursors derived from the primary metabolism. They are produced during certain stages of morphological development in response to certain media components and/or environmental conditions (Keller *et al.*, 2005). The biosynthetic pathway genes of secondary metabolites as well as many other biosynthetic pathway genes in filamentous fungi are organized in clusters (Keller and Hohn, 1997). The gene clusters of secondary metabolites are often located in the subtelomeric regions of fungal chromosomes, and are inherited as single loci (Keller *et al.*, 2005; McDonaugh *et al.*, 2008; Perri *et al.*, 2007). Some enzymes encoded by genes in these secondary metabolite gene clusters include synthases,

transcription factors, glutathione S-transferases, cytochrome P450s, and *O*-methyltransferases among others (Keller *et al.*, 2005; McDonaugh *et al.*, 2008; Perri *et al.*, 2007). Fungal secondary metabolites are classified into indole alkaloids, nonribosomal peptides, polyketides, and terpenes (Keller *et al.*, 2005).

Polyketides are the most abundant secondary metabolites amongst fungal species (Keller *et al.*, 2005), a number of which has been found to function as virulence factors, such as cercosporin, botcinic acid, elsinochrome, depudecin, and T-toxin, among many others (Choquer *et al.*, 2005; Dalmais *et al.*, 2011; Liao and Chung, 2008; Wight *et al.*, 2009; Yang *et al.*, 1996). These are synthesized through multidomain enzymes known as polyketide synthases (PKSs). PKSs synthesize polyketides compounds from acyl coenzyme A (acyl CoA) via Claisen condensations (Crawford *et al.*, 2008; Gokhale *et al.*, 2007; Shen, 2003; Weissman, 2008). There are three types of PKSs, among which fungal PKSs are classified as type I iterative PKSs (Gokhale *et al.*, 2007). Type I iterative PKSs may contain up to eight protein domains that are used in a repetitive manner until the final product is synthesized (Gokhale *et al.*, 2007; Hoffmeister and Keller, 2007; Shen, 2003). However, only three protein domains are considered necessary to create functional PKSs. These include one β-ketoacyl synthase (KS), one acyltransferase (AT), and one acyl carrier protein (ACP) domain (Hopwood and Sherman, 1990; Keller *et al.*, 2005). PKS proteins are often classified into non-reducing and reducing PKSs according to the aromatic or linear structure, respectively, of the keto group of the resulting polyketide (Gónzalez-Lergier *et al.*, 2005). The reducing nature of a given PKS can be distinguished using phylogenetic analysis of the KS domain (Kroken *et al.*, 2003) and/or by the domain architecture of the given PKS (Gaffoor *et al.*, 2005). Protein domains of reducing PKSs include β-ketoacyl reductase (KR), dehydratase (DH), and enoyl reductase (ER)

domains; as well as multiple AT domains (Fujii *et al.*, 2004; Gaffoor and Trail, 2006; Gokhale *et al.*, 2007; Kroken *et al.*, 2003).

The genes involved in secondary metabolite biosynthesis, including PKSs, are co-regulated by pathway-specific transcription factors and global regulators (Keller *et al.*, 2005). Both pathway-specific transcription factors and global regulators of secondary metabolism are responsible for detecting environmental and/or substrate cues that affect the expression of fungal genes associated with the synthesis of these natural products (Howlett, 2006; Martin, 2000; Tilburn *et al.*, 1995). Pathway-specific transcription factors are located within the corresponding biosynthetic gene cluster. They positively regulate the expression of all genes within a given cluster. The most commonly found transcription factor in filamentous fungi, which also happens to be unique to this kingdom is Zn $(II)_2$ Cys_6 zinc binuclear transcription factor (Fernandes *et al.*, 1998; Keller *et al.*, 2005; Proctor *et al.*, 1995; Woloshuk *et al.*, 1994). In the last decade, a novel global regulator of secondary metabolism that belongs to the methyltransferase protein family was identified in *Aspergillus* species. This regulatory protein is known as LaeA (Bok *et al.*, 2004). Deletion of the gene encoding for LaeA in *Aspergillus* species abolished the production of lovastatin, penicillin and sterigmatocystin. Conversely, the overexpression of *LaeA* increased the production of lovastatin and penicillin (Bok and Keller, 2004). Homologs of *LaeA* have been identified in fungal species such as *Coccidioides immitis*, *Cochliobolus heterostrophus*, *Fusarium graminearum*, *F. oxysporum*, *F. sporotrichioides*, *F. verticillioides*, *Gibberella fujikuroi*, *Magnaporthe grisea*, *Neurospora crassa*, *Penicillium citrinum*, *Trichoderma reesi* (Barreau *et al.*, 2011; Bok *et al.*, 2005; Butchko *et al.*, 2011; Howlett, 2006; Karimi *et al.*, 2011; Wiemann *et al.*, 2010; Wu *et al.*, 2011; Xing *et al.*, 2010), and *Verticillium albo-atrum* (www.broadinstitute.org). All the proteins encoded by these genes contain the S-adenosylmethionone (SAM) binding site domain, previously

described in nuclear methyltransferases (Bok and Keller, 2004; Hamahata *et al.*, 1996). Due to the presence of the SAM protein domain, these particular methyltransferases are thought be involved in chromatin modification by demethylation of heterochromatin into euchromatin, i.e. making the secondary metabolism gene clusters available for gene expression events (Gacek *et al.*, 2011; Keller *et al.*, 2005).

Ascochyta rabiei is a necrotrophic fungal pathogen that belongs to the order Pleosporales. This fungus is responsible for causing Ascochyta blight of chickpeas, which has been associated with severe economic losses in chickpeas worldwide (Chen *et al.*, 2004a; Gan *et al.*, 2006; Nene and Reddy, 1987; Shtienberg *et al.*, 2000). Ascochyta blight is the most important foliar disease of chickpeas, affecting all above ground parts of the plant. Although Ascochyta blight has been of great concern, most of the research conducted on this pathosystem has been focused on the host with limited information available regarding virulence factors of *A. rabiei*. In fact, melanin and solanapyrones, both polyketides, are the only virulence factors directly associated with the chickpea-*A. rabiei* pathosystem to date (Akamatsu *et al.*, 2010; Chen, *et al.*, 2004b; Hölh *et al.*, 1991, Hopwood and Sherman, 1990; White and Chen, 2007).

Fungal phytotoxins are often considered to be important for infection of plant hosts, where they may act as virulence factors. They are known to cause plant cell death at low concentrations (below 10 mM), causing symptoms such as wilting, growth inhibition, chlorosis, and necrosis (Berestetsky, 2008). In necrotrophic fungi, phytotoxins can elicit a hypersensitive response to obtain the nutrients released from the dead plant tissue (Möbius and Hertweck, 2009). Solanapyrones are non-host specific polyketide phytotoxins secreted mainly during conidiation, which have been shown to aid tissue penetration (Jayakumar *et al.*, 2005; Kaur, 1995). They have also been correlated with seedling growth inhibition, and fungal culture filtrates containing

solanapyrones have been shown to produce blight-like symptoms on chickpea leaves (Hölh *et al.*, 1991; Kaur, 1995). However, solanapyrones are highly reactive and very difficult to detect *in vivo*, and only solanapyrone C has been detected in plant tissue (Shahid and Riazuddin, 1998). Solanapyrone A, B, and C are known to be produced by two fungal plant pathogens from the order Pleosporales, *Alternaria solani* and *Ascochyta rabiei* (Alam *et al.*, 1989; Chen and Strange, 1991; Ichihara *et al.*, 1983). The solanapyrone gene cluster has been recently characterized from *A. solani* (Kasahara *et al.*, 2010), where it was found to consist of a PKS protein in association with five other proteins including a pathway-specific transcription factor (Kasahara *et al.*, 2010). The gene associated with the production of solanapyrone in *A. rabiei* are however yet to be identified and characterized.

The role of melanin in Ascochyta blight of chickpeas was demonstrated by Chen *et al.* (2004b), where they showed that spontaneous non-melanized mutants of *A. rabiei* were non-pathogenic on chickpea seedlings (Chen *et al.*, 2004b). These spontaneous albino mutants lacked functional pycnidia. Melanin has been previously demonstrated to be deposited in pycnidial walls protecting pycnidiospores from UV irradiation, and disruption of the melanin synthase gene lead to the development of albino mutants that when exposed to UV irradiation had significantly lower rates of germination compared to the wild-type parents (Akamatsu *et al.* 2010).

PKSs have recently been identified using genome-wide analysis as in the fungal pathogens *Fusarium graminearum* and *Aspergillus* species using profile hidden markov models (pHMMs), which are statistical models commonly used in the identification of protein families (Birney, 2001; Bok *et al.*, 2006; De Fonzo *et al.*, 2007; Eddy, 1998, 2008; Gaffoor *et al.*, 2005; Schuster-Böckler *et al.*, 2004). Recently, we developed pHMMs specifically for the identification of PKS proteins of fungal origin based only on the protein sequence of KS and AT domains. These

pHMMs were found to perform better in the identification of fungal PKS proteins than other previously built models (Delgado *et al.*, 2011).

Based on the potential role of PKSs and global regulators in virulence and disease development, the objectives of this study were to identify the PKS and methyltransferase (*LaeA*-like) genes from the recently sequenced genome of *A. rabiei*, and to characterize their response to light stimuli and divalent cations previously determined to be essential for solanapyrone biosynthesis. Additionally, the toxicological potential of the crude and organic extracts of *A. rabiei* cultures exposed to light and/or divalent cations stimuli was also evaluated on actively growing cultures of *Chlamydomonas reinhardtii*.

Materials and Methods

Identification of PKSs from the *A. rabiei* genome. Isolate ME14 of *A. rabiei* from Australia has been sequenced by Dr. Richard Oliver's team at Curtin University, Perth, Australia. To date, the genome has been assembled to the stage of scaffolds. These scaffolds were searched against a fungal PKS protein database to identify the ones that might bear PKS genes using the BLASTX program included in the publicly available software BioEdit (Hall, 1999). The selected scaffolds were then analyzed using fgenesh (www.softberry.com). The fgenesh-predicted proteins were analyzed using profile hidden markov models based on the previously built fungal KS and AT pHMMs (Delgado *et al.*, 2011). Predicted PKSs were subsequently compared to the non-redundant protein database at NCBI using BLASTP. The domain architecture of each putative PKS was determined using the Conserved Domain Search (CDS) at NCBI. The reducing nature of these PKSs was determined by phylogenetic analysis of the KS domain using neighbor joining phylogeny algorithm (Delgado et al., 2011; Kroken *et al.*, 2003; Tamura *et al.*, 2007)

Similarly, the identification of methyltransferases was performed by first creating a database of 28 fungal methyltransferases (Appendix D) to search the *A. rabiei* scaffolds and identify ones that might bear methyltransferase orthologs using TBLASTN. These were then analyzed by fgenesh as previously described. The fgenesh-predicted proteins were further analyzed with a pHMM built using the protein domain characteristic to arginine methyltransferase, histone methyltransferase, and LaeA methyltransferases known as SAM binding site domain. Further analysis of the predicted methyltransferases was conducted as previously described for PKS proteins.

Fungal culture conditions and plant inoculations. Conidial suspensions of the *A. rabiei* isolate Ar666 from Washington state at a concentration of 1×10^5 conidia mL^{-1} were prepared from 7 to 10 days old cultures grown on PDA. For the axenic cDNA library, 260 µL of the conidial suspension was inoculated into 200-mL of liquid media. The cultures were grown under different conditions of divalent cations and light regimen. Four conditions were evaluated: (1) Czapek Dox media (CDM) incubated under dark conditions, (2) CDM incubated under a 12 h light regimen, (3) Czapek Dox liquid media amended with divalent cations (CDCLM) incubated under dark conditions, and (4) CDCLM incubated under a 12 h light regimen. CDCLM was prepared according to Mogensen *et al.*, (2006) where CDM liquid media was amended with 100 mg L^{-1} $CaCl_2 \times 2H_2O$, 20 mg L^{-1} $CoCl_2 \times 6H_2O$, 20 mg L^{-1} $CuCl_2 \times 2H_2O$, 20 mg L^{-1} $MnCl_2 \times 4H_2O$, and 50 mg L^{-1} $ZnSO_4 \times 7H_2O$. All cultures were incubated at 22 °C for 14 days. At the end of the incubation period, fungal mycelia were collected for total RNA extraction and for fungal biomass quantification. The fungal mycelia were dried at 60 °C for 48 hours before estimation the fungal biomass. The culture filtrates were collected and pooled together for toxicity assays. Isolate Ar666 was also grown on CDM and CDCLM agar to determine the effect of light and divalent cation stimuli on the conidial production of *A.*

rabiei according to Rivera-Varas *et al.*, (2007). Later, *A. rabiei* grown in CDM amended with each individual divalent cation was also tested under 12-hour light regimen. Fungal biomass was also assessed in these cultures.

Chickpea seedlings (cv. Burpee) were inoculated 10-14 days after germination with 1×10^5 conidia mL^{-1} until runoff. Control plants were inoculated with sterile water. The inoculated plants were incubated according to the mini-dome technique, and they were evaluated after 10 days of inoculation when characteristic Ascochyta blight lesions were visible (Chen and Muehlbauer, 2003; Chen *et al*, 2004a). All collected tissue was flash frozen and kept at -80 °C until total RNA isolation.

Phytotoxicity of *A. rabiei* culture filtrates and organic extraction. *Chlamydomonas reinhardtii* strain CC-124 or CC-1690 were obtained from the Chlamydomonas Center at University of Minnesota, Saint Paul, MN and grown on high salt high acetate (HSHA) plates at 20 °C for 7 days with a 12-hour light regimen (Sueoka, 1960). The *C. reinhardtii* cells were subsequently collected and grown in 1000-mL HSHA liquid media for 7 days with continuous shaking (150 rpm) until the optical density (O.D.) was equal to or greater than 0.4 at 550 nm. The cultures were then transferred to sterile 50-ml tubes and centrifuged at 5000 *xg* for 5 minutes at 12 °C. The cell pellets were resuspended in fresh HSHA and transferred to a 1000-ml flask. The final volume was then adjusted to 1000 ml with fresh HSHA and incubated overnight to obtain a final O.D. equal to or greater than 0.5. Later, 20 mL of *C. reinhardtii* culture was transferred to 100-ml flasks and 4-mL of *A. rabiei* crude extract or 400-µL of organic extract resuspended in DMSO was added to each flask and incubated at 20 °C for 24 hours with 12-hour light regimen. The phytotoxicity assay was performed in two independent trials. The culture filtrates used in the phytotoxicity assay were sterilized using a 0.2 µm filter. The organic extractions were conducted as described by Hamid and Strange (1997). The phytotoxicity levels of both the crude and organic extracts were estimated by

measuring the chlorophyll content of each treated *C. reinhardtii* culture (Arnon, 1949). Chlorophyll extraction was conducted by transferring 1.5 mL of treated *C. reinhardtii* cells to a 2.0-mL centrifuge tubes, followed by centrifugation at 16,000 x *g* for 10 min at room temperature. Subsequently, 1.35 mL of the supernatant was discarded from each tube and replaced with same volume of acetone. The tubes were incubated overnight at -20 °C with occasional inversion. Total chlorophyll absorbance was measured at wavelength of 652 nm and total chlorophyll content was calculated according to Arnon, (1949). The organic extracts resuspended in DMSO were tested on shoots of chickpea for phytotoxicity as described by Hamid and Strange (2000).

Nucleic acids extraction, PCR, RT-PCR of putative PKS and methyltransferase genes. *A. rabiei* isolate Ar666 was grown on potato dextrose broth at room temperature for four days in darkness for extraction of genomic DNA. The DNA extraction was conducted according to Liu *et al.*, (2000) with modifications described in Delgado *et al.*, (201X). Extractions of total RNA from axenic cultures (CDM dark, CDM light, CDCLM dark, and CDCLM light) and infected-chickpea seedlings were carried out using the RNeasy Plant Mini kit (Qiagen) with on-column DNA digestion with modifications. Briefly, freeze-dried mycelia or infected leaves were placed in a 2-ml lysing matrix C tube (MP Biomedicals, Solon, OH) containing 600 µl of Buffer RLC. The tissue was disrupted using the FastPrep® homogenizer (MP Biomedicals, Solon, OH) set at speed 6.5 for 45 s, followed by an incubation step at 56 °C for 3 min. Subsequently, total RNA extraction was conducted according to manufacturer's instructions. The first-strand cDNA synthesis was performed using the QuantiTect Reverse Transcription kit (Qiagen). PKS and methyltransferase gene-specific primers were designed to be used with genomic DNA and cDNA templates (Table 3.1).

PCR and RT-PCR assays were conducted in a final reaction volume of 20 µl, containing 15 ηg of fungal DNA or cDNA, 1X PCR buffer pH 8.7, 1X CoralLoad concentrate, 2.5 mM $MgCl_2$, 150 µM

dNTPs, 0.5 units of Top Taq DNA polymerase and 125 ηM of each oligonucleotide primer. The PCR thermal cycling started with an initial denaturation at 94 °C for 5 min; followed by 40 cycles of denaturation at 94 °C for 10 s, annealing at 66 °C for 10 s, and 72 °C for 15 s; and a final extension step at 72 °C for 3 min. The PCR products were separated on a 1.2% agarose gel with GelRed™ nucleic acid gel stain (Biotium, Inc., Hayward, CA) for 80 min at 100 V. All PCR and RT-PCR assays were carried out using the housekeeping gene glyceraldehide-3-phosphate (*G3P*) gene as control.

Table 3.1. Primers used for amplification of predicted PKS and methyltransferase genes and cDNAs from *Ascochyta rabiei* genome and transcriptome, respectively

Gene Name	Sequence of forward primer (5' to 3')	Sequence of reverse primer (5' to 3')	Size of PCR product (bp)	Size of RT-PCR product (bp)
G3P	CCGAGGGCAAGCTCAAGGGC	ACACGGCGGGAGTAACCCCA	175	175
ArPKS01	TCGAGCGGCTTCCCGTCGCT	TGCCCTCGGGCCAAGCTTGC	200	153
ArPKS02	TGGACCCTCAGCAGCGGTTGCT	CCGGGTCTCGGAACAGGTCTGCC	207	146
ArPKS03	AAGCGCACGGTACCGGCACAC	TGGCTTGCGTGACCTTCAACAGTGC	248	176
ArPKS04[1]	AGCGCGCCCTCGCCGTAGTA	GTGCGCACGTGGTGGGCATGA	186	na[2]
ArPKS04	TGCGGTGCTTGAGCGCGAGG	ATGGCACAGGCACGCAGGCG	na[2]	197
ArPKS05	TGGCGCCGTGTTCAGCAAGCC	ACACGCAATCGAGCGGATCGCC	231	179
ArPKS06	AGCAGGAGGCACACAAGCTGAGCG	ATCGGCGAGCGTCGTGGGAGA	220	169
ArPKS07	ACCAGGGCGATCTGCAACGCC	TGTAGTGACGCGCTGCAACGCAT	192	130
ArPKS08	TCGCTCGCCCGCGCAACAAA	TCGAGGTGGCCCGCTGACAGT	223	166
ArPKS09	TGGCACTTCTGCAGACCCAGAGCC	TTGCGAGAGAAGCGGTGGTCACCG	202	146
ArPKS10	CGCGGTATCGACGATCTTCGCCG	TCAGTTGCTGACTACGGCGTCGACA	223	149
ArPKS11	AGCCAGCGGTGGTGGGTGCT	AGTCATCCGCGAGGCCGGCA	203	203
ArPKS12	AACTCCGACGGCCGCACGAAC	TGGCCGATCCATGGCGCAGCAT	167	113
ArPKS13	AGCTGGTCAAACGCGCTTCGACTG	TCCGTAGCGTCTCCAGGAAGGGTGT	169	103
ArMet01	ACGCTGAAGCAGCGCATGCT	TCGCGCTGATAGCGGCCGATTT	246	120
ArMet02	GAGATGGGCGACCGCTTCGG	GCAGTTGGTTCCACCGGCCA	166	166
ArMet03	AACAAGCTCCACCTGGCGCC	TGGGGGAGAGGTCTACGCCG	180	128
ArMet04	TGGAGTCTTGGCTACTTCCGCGA	ACAGGGTATGGACCGCAGAGCT	150	90

[1]designed for amplification of genomic DNA
[2]not applicable

Identification of the solanapyrone biosynthesis cluster. The scaffolds of the *A. rabiei* genome were searched with TBLASTN using the BioEdit software (Hall, 1999). TBLASTN was conducted with the solanapyrone biosynthesis proteins from *Alternaria solani* (Kasahara *et al.*, 2010). The identified scaffolds were first aligned to the nucleotide sequence of the solanapyrone cluster from *A. solani* using the TBLASTX-alignment option available at NCBI. Subsequently, these scaffolds were analyzed using fgenesh at softberry website. The resulting proteins were analyzed using BLASTP with the non-redundant protein database at NCBI. The orientation of the corresponding open reading frames was assessed using the results from the above TBLASTN output.

Statistical analysis. All experiments were conducted in a complete random design with three replications, and at least two independent trials. Independent trials that showed no significant difference between or among each other according to Levene's test (PROC TTEST) were combined for further analysis. ANOVA analyses were conducted using PROC ANOVA with Statistical Analysis Software (SAS) version 9.2 (SAS Institute, Inc., Cary, NC).

Results

Identification of PKSs and methyltransferase genes/proteins using *A. rabiei* genome. Twenty nine scaffolds of the *A. rabiei* genome bearing nucleotide sequences similar to known PKS genes were identified using BLASTX with a database containing 543 fungal PKS proteins. This BLASTX search was performed with an E-value cut off $\geq 1 \times 10^{-40}$. These scaffolds were analyzed using the fgenesh algorithm yielding 179 genes. The corresponding 179 proteins were subsequently analyzed with the fungal KS and AT domain pHMMs from PKSs with a bit score cut off equal to 0.0 as described in Delgado *et al.* (2011). This pHMM analysis predicted 14 putative PKS proteins, of

which 13 proteins had the KS domain and 12 had the AT domain. Only 11 proteins were predicted by both the KS and AT pHMMs (Table 3.2). Two of the proteins predicted by the KS domain (ArPKS01 and ArPKS04) were found to have truncated AT domains by analysis of their domain structure. Likewise, domain analysis of the protein sequence Ar120226.01, predicted only by the fungal AT pHMM, showed that this sequence also contained a DH domain. Due to the stage of the genome assembly, sequence Ar120226.01 was thought to be part of a larger PKS sequence, maybe part of truncated proteins such as ArPKS01 or ArPKS04. Thus, we identified 13 putative PKS proteins encoded in 12 scaffolds of the draft version of the *A. rabiei* genome. Also, two PKSs were found to be side by side in the same scaffold. These were ArPKS05 which was determined to be a reducing PKS, and ArPKS06 which was found to be a non reducing PKS. A BLASTP search showed that all 13 putative PKSs had similarity with previously described fungal PKSs with an E-value of 0.0. Their total score varied from 800 to 5200 and their query coverage ranged from 78 to 100% (Table 3.2). The domain architecture analysis along with the phylogenetic analysis determined 10 of the putative PKSs were of reducing nature and three were of non-reducing (Table 3.2 and Figure 3.1). The domain architecture analysis also showed that ArPKS12 was a PKS/NRPS hybrid protein. Proteins ArPKS01, ArPKS03, ArPKS04, and ArPKS07 lacked the ACP domain and could be nonfunctional.

The identification of putative methyltransferases was conducted by creating a database of methyltransferases with emphasis in LaeA-like methyltransferases. This database was used as a query to search for scaffolds bearing similarity to methyltransferases in the genome of *A. rabiei* using TBLASTN algorithm. This search yielded six scaffolds that were analyzed using fgenesh as described before. This analysis yielded a total of 28 proteins that were further analyzed using a pHMM built with the SAM binding site derived from the methyltransferases used for the initial screening. The pHMM showed four proteins with bit score ≤ 0.0. The domain architecture analysis

Table 3.2. Identification, homology and protein domain architecture of putative PKS proteins from the predicted proteome of *A. rabiei*

Protein[1,2]	Scaffold and protein ID	pHMM analysis		Gene, transcript, and protein size	Number of Exons	BLASTP analysis				Domain architecture[3]
		Fungal KS domain (bit score)	Fungal AT domain (bit score)			Description, Accession number and organism	E-value	Total Score	Query coverage	
ArPKS01^{Re}	Ar110090.01	666.8	-161.7	2079 bp 1875 bp 625 aa	3	Polyketide synthase 2 ABB76806 *Cochliobolus heterostrophus*	0.0	1008	100%	KS-[AT]^{Tr}
ArPKS02^{Re}	Ar147596.06	863.4	515.4	7224 bp 6825 bp 2274 aa	8	Putative polyketide synthase CBI53318 *Sordaria macrospora*	0.0	2251	95%	KS-AT-DH-ER-KR-ACP
ArPKS03^{Re}	Ar148596.03	727.8	511.9	8833 bp 8160 bp 2719 aa	3	Polyketide synthase AAR90270 *Cochliobolus heterostrophus*	0.0	3883	78%	KS-AT-DH-MeT-PyrR-KR-KR^{NF}
ArPKS04^{Re}	Ar149017.01	890.4	-204.4	2295 bp 1905 bp 635 aa	2	DmbS (PKS/NRPS hybrid) ADN43685 *Beauveria bassiana*	0.0	800	98%	KS-[AT]^{Tr}
ArPKS05^{Re}	Ar149451.01	867.8	489.2	7671 bp 7455 bp 2484 aa	5	Polyketide synthase AAR90258 *Cochliobolus heterostrophus*	0.0	3884	100%	KS-AT-DH-MeT-ER-KR-ACP
ArPKS06^{NR}	Ar149451.02	641.2	225.9	8009 bp 7668 bp 2555 aa	4	Polyketide synthase AAR90275 *Cochliobolus heterostrophus*	0.0	3139	83%	KS-AT-ACP-MeT-KE/TE
ArPKS07^{Re}	Ar149630.01	878.6	477.5	5755 bp 5571 bp 1857 aa	4	Polyketide synthase AAR85531 *Peyronellaea zeae-maydis*	0.0	2233	99%	KS-AT-DH^{NF}
ArPKS08^{NR}	Ar149802.05	777.4	329.9	7269 bp 6777 bp 2258 aa	6	Polyketide synthase AAR92210 *Gibberella moniliformis*	0.0	2755	97%	KS-AT-ACP-ACP-KR/TE
ArPKS09^{Re}	Ar150329.02	786.3	472.4	8054 bp 7863 bp 2620 aa	4	Solanapyrone polyketide synthase BAJ09789 *Alternaria solani*	0.0	5200	100%	KS-AT-DH-MeT-ER-KR-ACP
ArPKS10^{Re}	Ar151747.03	765.1	460.9	8363 bp 8004 bp 2667 aa	5	Solanapyrone polyketide synthase BAJ09789 *Alternaria solani*	0.0	2440	99%	KS-AT-DH-MeT-ER-KR-ACP
ArPKS11^{NR}	Ar155444.04	816.4	400.5	6520 bp 6448 bp 2155 aa	2	Melanin polyketide synthase BAD22832 *Bipolaris oryzae*	0.0	3643	100%	KS-AT-ACP-ACP-TE
ArPKS12$^{Re, Hy}$	Ar155635.06	881.4	479.9	12533 bp 12162 bp 4053 aa	7	Putative PKS/NRSP hybrid CAG28798 *Magnaporthe grisea*	0.0	2408	99%	KS-AT-DH-MeT-KR-ACP-C-A-ACP-KR
ArPKS13^{Re}	Ar155751.01	686.0	248.5	7906 bp 6462 bp 2153 aa	8	Polyketide synthase AAR90279 *Cochliobolus heterostrophus*	0.0	1838	81%	KS-AT-DH-KR-ACP

[1]Reducing nature of the PKS protein. Re indicates reducing PKS. NR indicates non-reducing PKS.

[2]Hy indicates PKS/NRPS hybrid

[3]Protein domain abbreviations: KS=beta-ketoacyl synthase, AT= acyltransferase, DH= dehydratase, MeT= C-methyltransferase, ER= enoylreductase, KR= ketoreductase, ACP= acyl carrier protein, TE= thioesterase, C= condensation, A= adenylation, Tr= truncated gene, NF= non-functional protein.

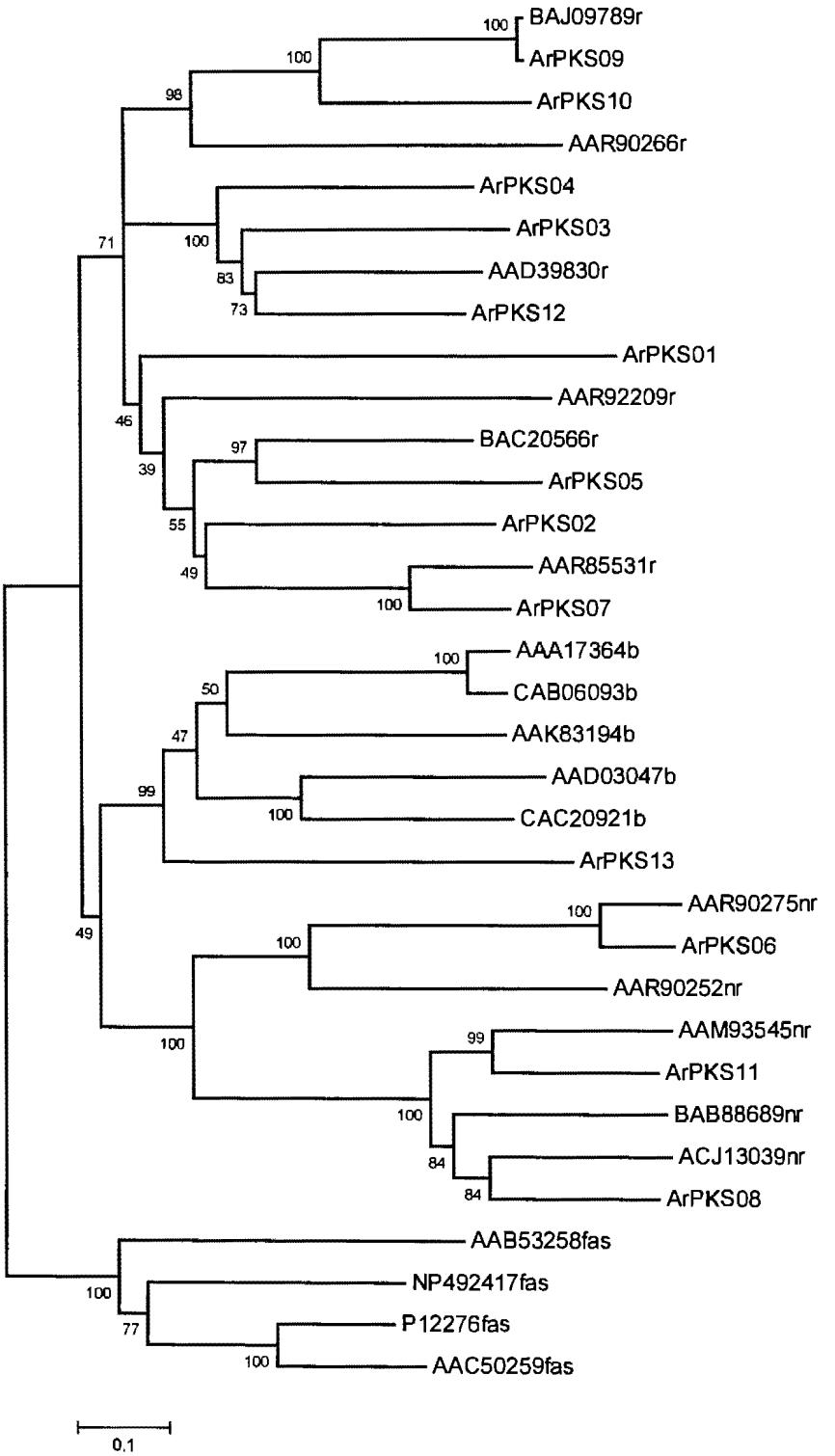

Figure 3.1. Phylogenetic analysis of the KS domain of the putative *Ascochyta rabiei* PKS proteins. Abbreaviations: r indicates reducing PKS, nr indicates non-reducing PKS, b indicates bacterial PKS, and fas indicates animal fatty acid synthase.

of these four proteins determined that all four proteins only contained the SAM binding site. The protein length of these putative methyltransferases ranged from 320 to 404 amino acid residues. The BLASTP analysis showed that these putative methyltransferases are in fact similar to methyltransferases. But no clear similarity to LaeA-like methyltransferases was observed (Table 3.3).

Table 3.3. Identification and homology of putative methyltransferase proteins from the predicted proteome of *Ascochyta rabiei*

Protein	Scaffold and protein ID	SAM pHMM analysis (bit score)	Gene, transcript, and protein size	Number of Exons	BLASTP analysis	
					Description, Accession number and organism	E-value
ArMet01	Ar147778.01	193.0	1180 bp 1056 bp 351 aa	2	Methyltransferase XP_001930674 *Pyrenophora tritici-repentis* Pt-1C-BFP	0.0
ArMet02	Ar152156.01	178.7	963 bp 963 bp 320 aa	1	Methyltransferase family protein XP_001391224 *Aspergillus nidulans* FGSC A4	4.0×10^{-130}
ArMet03	Ar153620.10	189.6	1359 bp 1215 bp 404 aa	3	Methyltransferase family protein XP_00139016 *Aspergillus niger* CBS 513.88	2.0×10^{-126}
ArMet04	Ar155827.01	75.4	1404 bp 999 bp 332 aa	5	Putative methyltransferase XP_001264635 *Neosartorya fischeri* NRRL 181	1.0×10^{-147}

RT-PCR characterization of putative PKS and methyltransferase genes. PCR analysis of the putative PKS and methyltransferase genes identified *in silico* in the genome of the *A. rabiei* isolate ME14 was conducted to detect the presence of these putative genes in the genome of the *A. rabiei* isolate Ar666, which is the isolate used throughout this study. PCR analysis was performed using gene-specific primers. This analysis showed that this particular isolate of *A. rabiei* contained all putative PKS and methyltransferase genes previously identified in *A. rabiei* isolate ME14.

Gene expression of the putative PKS and methyltransferase genes (Figure 3.2) was studied using mycelia collected from *A. rabiei* cultures grown in the presence of four divalent cations and

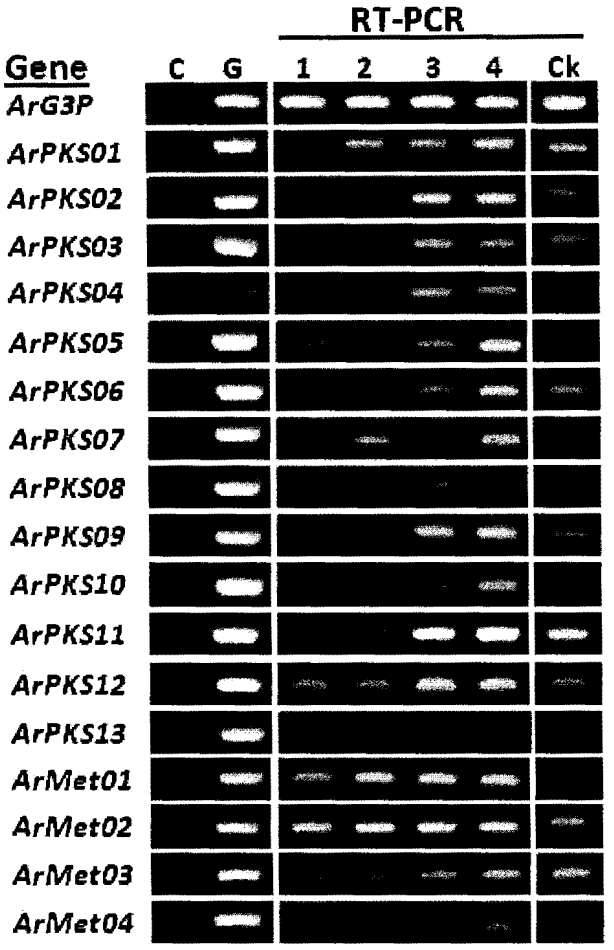

Figure 3.2. RT-PCR analysis of PKS and methyltransferase genes from *Ascochyta rabiei* grown in axenic cultures and inoculated chickpea plants using the *Ascochyta rabiei* isolate Ar666. Abbreviations, C indicates water control, G indicates genomic DNA, 1 is cDNA from culture grown in CDM kept in the dark, 2 is cDNA from CDM maintained at 12-hour light regimen, 3 is cDNA from CDCLM kept in the dark, 4 is cDNA from CDCLM maintained at 12-hour light regimen, and Ck indicates cDNA from inoculated chickpea seedlings. *G3P* is the glyceraldehydes-3-phosphate gene used as assay control.

light conditions described earlier. The RT-PCR profiles of the putative PKS and methyltransferase genes showed that the expression of some genes was influenced by presence of specific divalent cations or specific light conditions. The expression of *ArPKS06*, *ArPKS10*, *ArPKS11*, and *ArPKS12* seemed to be constitutive; however, the expression of *ArPKS10* and *ArPKS11* appeared to be enhanced by the divalent cations stimulus. The expression of *ArPKS01* and *ArPKS07* was stimulated

either by divalent cations or light stimulus, while the expression of *ArPKS05* was stimulated by either divalent cations stimulus or the absence of light. The expression of *ArPKS02*, *ArPKS03*, *ArPKS04*, *ArPKS08*, and *ArPKS09* seemed to be triggered by divalent cation stimuli. On the other hand, the gene expression of *ArMet01*, *ArMet02* and *ArMet03* seemed to be constitutive, while the gene expression of *ArMet04* was elicited by either the light or the divalent cation stimulus. The expression of the solanapyrone synthase gene (*ArPKS09*) appeared to be dependent on divalent cations, while the melanin synthase gene (*ArPKS11*) was found to be constitutively expressed in these axenic cultures. The analysis of expression of PKS and methyltransferase genes of *A. rabiei* when they are actively growing in chickpea plants showed that all PKS and methyltransferases are transcribed *in planta* with the exception of *ArPKS04*.

A second RT-PCR experiment was conducted to determine the influence of calcium, cobalt, copper, manganese, and zinc on the expression of selected PKS genes when these divalent cations are individually added to the CDM. For this experiment, *ArPKS02*, *ArPKS03*, *ArPKS04*, *ArPKS08*, and *ArPKS09* genes were selected because their expression was found to be dependent on the addition of all five divalent cations to the CDM. This experiment showed that *ArPKS02*, *ArPKS03*, and *ArPKS08* genes were only expressed in CDCLM, i.e. their expression was dependent on the exposure to the mixture of all five cations. However, *ArPKS04* and *ArPKS09* genes were found to be expressed on CDCLM and on CDM amended with zinc (Figure 3.3).

Phytotoxic potential, fungal biomass, and conidial production of *A. rabiei*. Isolate Ar666 of *A. rabiei* was grown under four different conditions to study the phytotoxic potential of the corresponding crude extract when exposed to divalent cations and/or light stimuli. The four conditions used to grow *A. rabiei* Ar666 in this study were 1) CDM liquid media kept in the dark, 2)

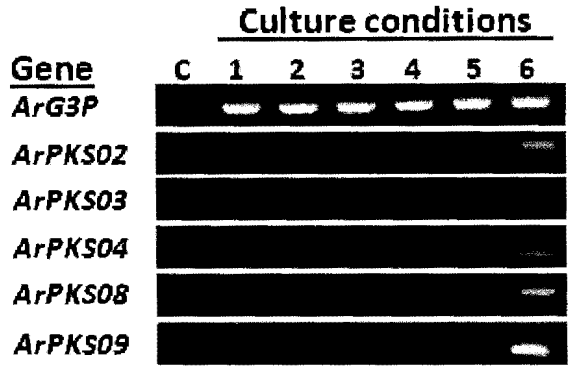

Figure 3.3. RT-PCR analysis of selected PKS genes from *Ascochyta rabiei* grown in axenic cultures in the presence of single divalent cations. Abbreviations, C indicates water control, 1 indicates cDNA from culture exposed to Ca^{++}, 2 is cDNA from culture exposed to Co^{++}, 3 is cDNA from culture exposed to Cu^{++}, 4 is cDNA from culture exposed to Mn^{++}, 5 is cDNA from culture exposed to Zn^{++}, and 6 is cDNA from culture grown in CDCLM. All cultures were maintained at 12-hour light regimen.

CDM liquid media maintained in a 12-hour light regimen, 3) CDCLM liquid media kept in the dark, and 4) CDCLM liquid media maintained in a 12-hour light regimen. The CDCLM liquid media contained the divalent cations that have previously been shown to be important for the production of the polyketide phytotoxin solanapyrone. The vegetative growth of *A. rabiei* in these four media was evaluated in two independent trials. These trials proved to be statistically similar and were combined for further analysis. The phytotoxicity assays in *C. reinhardtii* were also conducted in two independent trials. The trials performed using culture filtrates as well as the trials conducted using organic extracts were also found to be statistically similar and were combined for further analysis. Similarly, the two independent trials for the assessment of conidial production of *A. rabiei* were also combined for ANOVA analysis after Levene's test of variance homogeneity showed that they were not significantly different.

Fungal biomass was shown to be significantly different in cultures exposed to divalent cations stimuli; and it was not affected by light stimulus. Vegetative growth was 1.9x higher in CDCLM cultures as compared to CDM cultures (Figure 3.4A). The effect of the divalent cations

and/or light stimuli in conidial production was also evaluated. Conidial production was found to be significantly higher in solid media not exposed to light stimulus. The conidial production was observed to be 37.3 to 109.5x higher in cultures grown in the dark as compared to the cultures exposed to light stimulus. However, it was only 2.9x higher in cultures grown in the dark on media amended with divalent cations than in cultures grown in the dark on non-amended media (Figure 3.5).

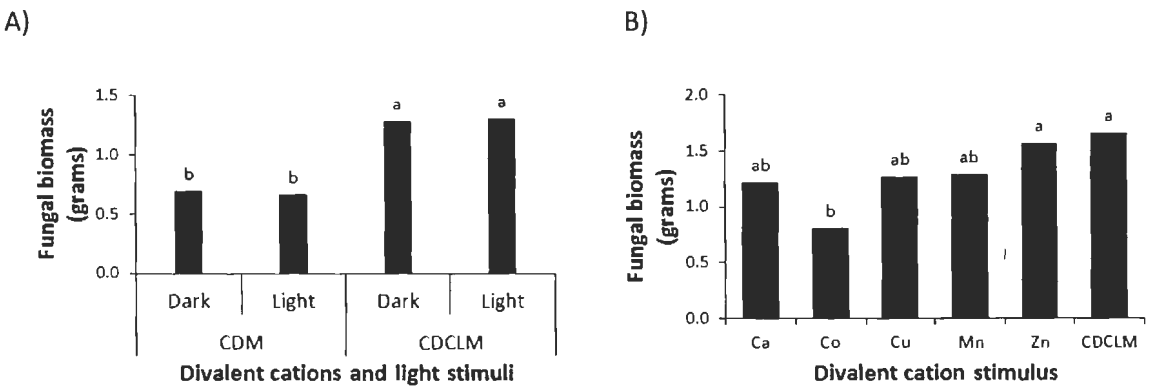

Figure 3.4. Vegetative growth of *Ascochyta rabiei* cultures grown under different divalent cation and light stimuli. The graph on the left (A), fungal biomass of cultures grown on the following conditions: CDM liquid media kept in the dark (CDM Dark), CDM liquid media maintained in a 12-hour light regimen (CDM Light), CDCLM liquid media kept in the dark (CDCLM Dark), and CDCLM liquid media maintained in a 12-hour light regimen (CDCLM Light). The graph on the right (B), fungal biomass of cultures grown on CDM liquid media maintained in a 12-hour light regimen individually amended with calcium, cobalt, copper, manganese, and zinc.

A second experiment using isolate Ar666 of *A. rabiei* was conducted to determine which divalent cations had the greatest effect on the production of phytotoxic compounds by cultures of *A. rabiei*. This fungus was grown separately in CDM liquid media amended with each individual divalent cation (calcium, cobalt, copper, manganese, and zinc). The vegetative growth of *A. rabiei* in these media was assessed in two independent trials. These trials proved to be statistically similar and they were combined for further analysis. Likewise, the phytotoxicity assays in *C. reinhardtii* were conducted in three independent trials. They were also shown to be statistically similar and

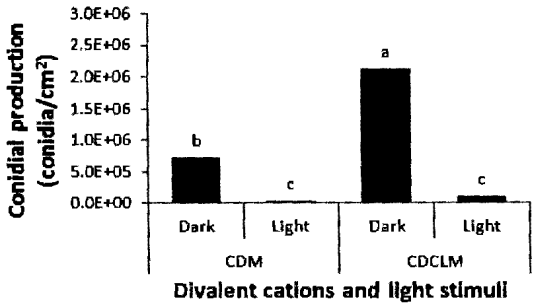

Figure 3.5. Conidial production by *Ascochyta rabiei* cultures grown under different divalent cation and light stimuli. The graph shows conidial concentration per area of cultures grown on solid media and incubated as follows: CDM liquid media kept in the dark (CDM Dark), CDM liquid media maintained in a 12-hour light regimen (CDM Light), CDCLM liquid media kept in the dark (CDCLM Dark), and CDCLM liquid media maintained in a 12-hour light regimen (CDCLM Light).

were combined for further analysis. The fungal biomass assessment showed that zinc is an important divalent cation for the vegetative growth of *A. rabiei*. The vegetative growth of *A. rabiei* in CDM amended with zinc was statistically the most similar to the vegetative growth of the culture cobalt was the poorest and statistically different from *A. rabiei* grown on CDCLM (Figure 3.4B). The phytotoxicity of organic extracts of *A. rabiei* cultures grown in the presence of individual divalent cation (Figure 3.6C) showed that addition of calcium, cobalt, copper, manganese, and zinc individually to CDM liquid media did not stimulate the synthesis of phytotoxic compounds as compared to the organic extract from *A. rabiei* cultures grown in the presence of all five divalent cations (CDCLM). In fact, the organic extracts derived from *A. rabiei* cultures grown in the presence of individual divalent cation were not found to differ significantly from the control. The pH of CDM amended with single divalent cations and CDCLM varied between 7.30 to 7.50; however the pH of the inoculated CDCLM crude extract (after 14 days of incubation) was approximately 8.5 while the pH of the inoculated CDM with single divalent cations did not surpass 7.8 (data not shown).

A third experiment was conducted to study the effect of the pH on the vegetative growth and phytotoxic potential of *A. rabiei* cultures. The initial pH of the media was 7.5 for CDM, 7.2 for

CDCLM, and 8.5 for CDM with pH adjusted to 8.5 (CDMpH). The vegetative growth and phytotoxic potential of the *A. rabiei* grown on CDMpH were significantly different from the vegetative growth and phytotoxic potential of *A. rabiei* grown on CDCLM and no different from CDM cultures (Figure 3.7). Therefore, the pH does not have any effect in the accumulation of phytotoxic compounds or in the vegetative growth of *A. rabiei*.

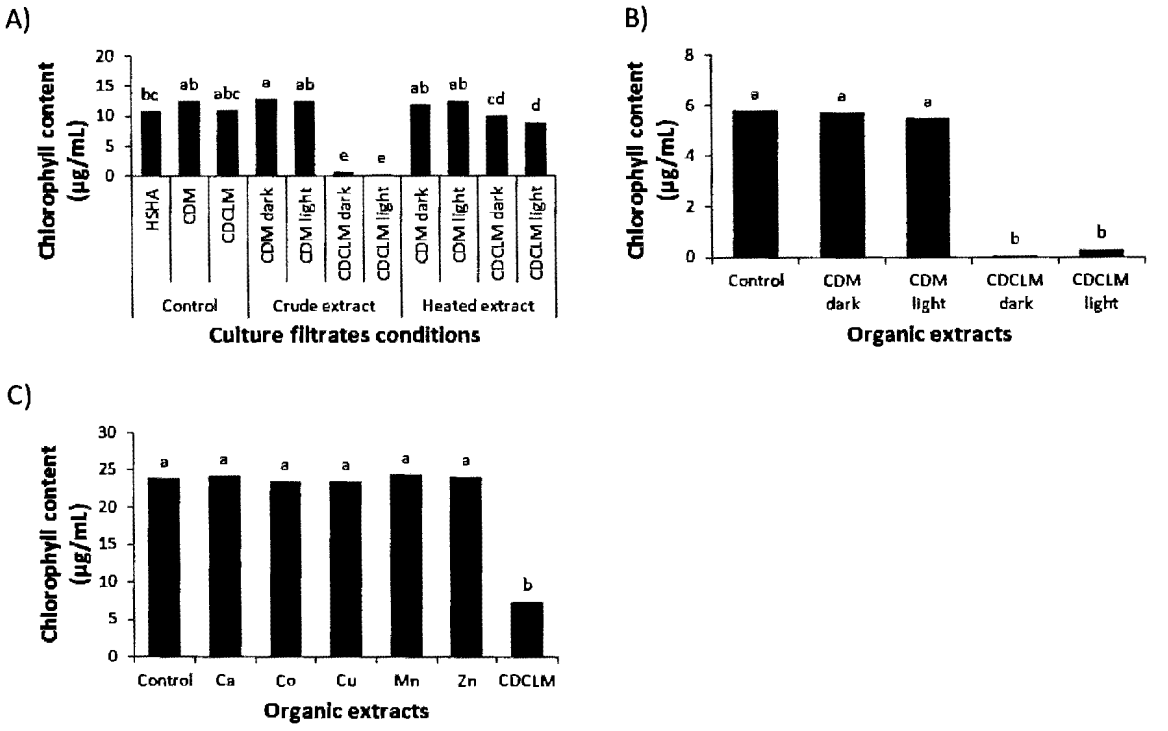

Figure 3.6. Phytotoxicity assay of *Ascochyta rabiei* culture filtrates on *Chlamydomonas reinhardtii*. The top left graph (A) shows the phytotoxicity of culture filtrates of *A. rabiei* grown under divalent cations and light stimuli. The top right graph (B) shows the phytotoxic of the organic extracts of *A. rabiei* grown under divalent cations and light stimuli. The bottom graph (C) shows the phytotoxicity of the organic extracts of *A. rabiei* cultures grown on CDM liquid media amended with individual divalent cations.

In conclusion, phytotoxicity of the culture filtrates and normalized organic extracts of *A. rabiei* isolate Ar666 on chickpea plants and in liquid cultures of *C. reinhardtii* was determined to be dependent on the combined effect of calcium, cobalt, copper, manganese, and zinc. Likewise, the

gene expression of *ArPKS02*, *ArPKS03*, *ArPKS04*, *ArPKS08*, and *ArPKS09* (solanapyrone synthase) was also dependent on the combined effect of these divalent cations as described earlier. Therefore, we believe that these PKSs may be involved in the biosynthesis of phytotoxic polyketide compounds such solanapyrone.

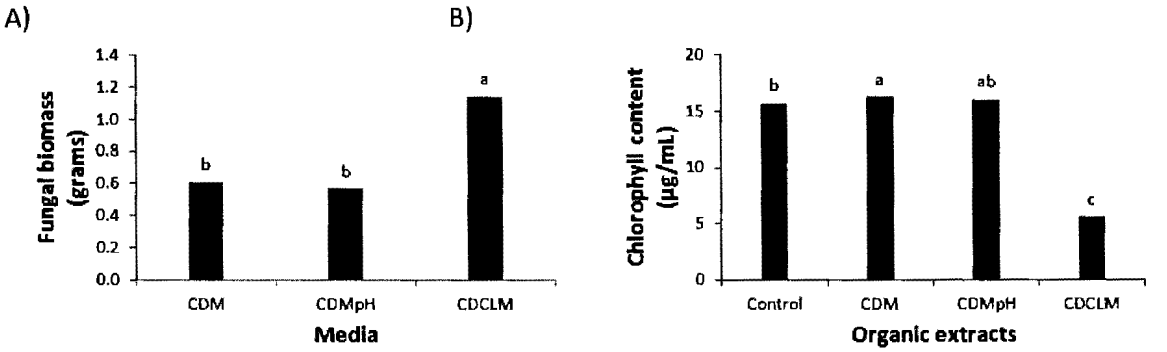

Figure 3.7. Effect of pH in the vegetative growth and phytotoxic potential of *Ascochyta rabiei*. The left graph (A) shows the vegetative growth, and the right graph (B) shows the phytotoxic potential.

Identification of the solanapyrone biosynthesis cluster. The identification of scaffolds Ar150329 and Ar153167 from the genome of *A. rabiei* isolate ME14 corresponding to the solanapyrone cluster was conducted using TBLASTN with the predicted proteins of the solanapyrone cluster from *Alternaria solani* (Genbank accession numbers BAJ09784 to BAJ09789). Scaffold Ar150329 was 12,496 bp in length and it aligned towards the 5'-end of the solanapyrone cluster. Likewise, scaffold Ar153167 was 10,270 bp in length and it aligned towards the 3'-end of the cluster. These two scaffolds were then aligned to the nucleotide sequence of the solanapyrone cluster from *A. solani* (20 kb in length) using TBLASTX at the NCBI website (Figure 3.8A and 3.8B). Scaffold Ar150329 was determined to match the solanapyrone cluster with an E-value of 0.0, a total score of 4.72×10^4, and query coverage of 58%. Scaffold Ar153167 was determined to align to the solanapyrone cluster with an E-value of 0.0, a total score of 3.75×10^4, and query coverage of 50%.

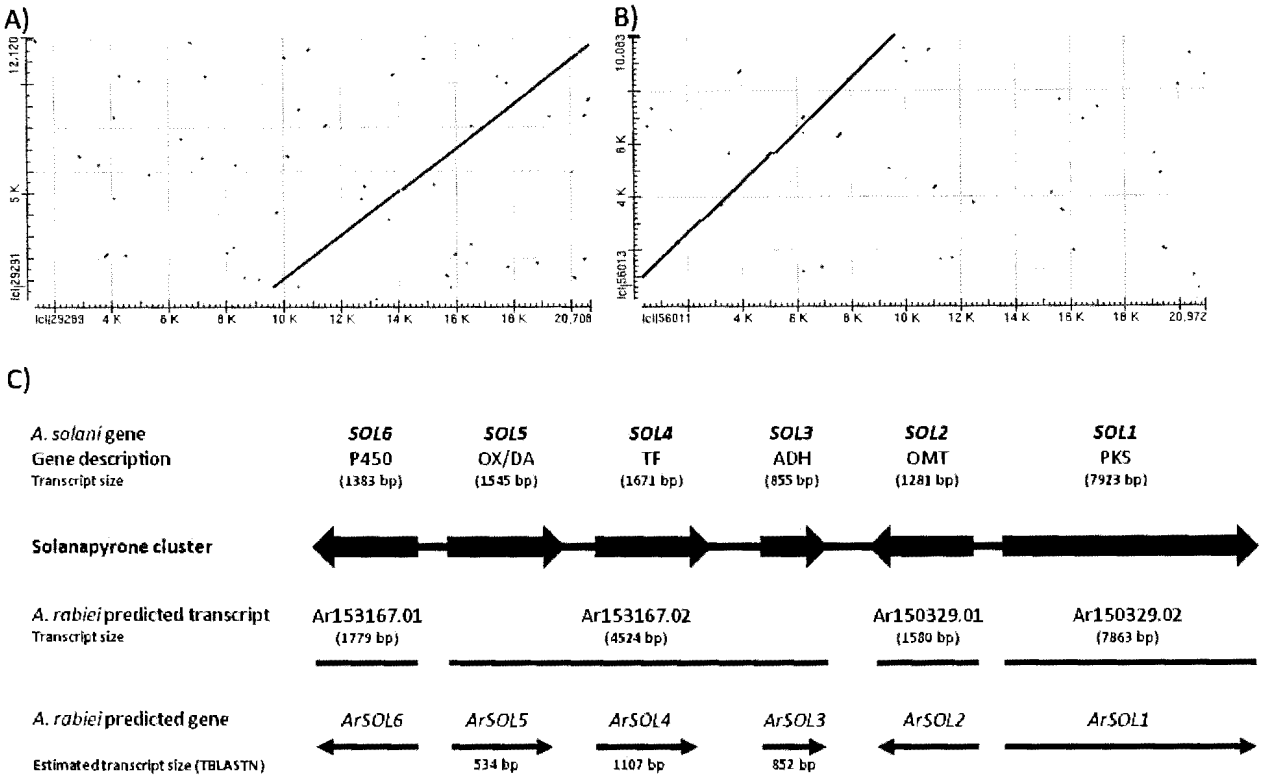

Figure 3.8. Identification of the solanapyrone gene cluster in the genome of *A. rabiei*. (A) Dot matrix for Ar150329 scaffold against the solanapyrone cluster from *A. solani*. (B) Dot matrix for Ar153167 scaffold against the solanapyrone cluster of *A. solani*. (C) Homology of the putative solanapyrone gene cluster from *A. rabiei* against the solanapyrone gene cluster from *A. solani*.

Four predicted proteins were identified from these two scaffolds and were analyzed using BLASTP with the non-redundant protein database at NCBI (Table 3.4). Protein Ar150329p01 was identified as an *O*-methyltransferase (Sol2). Protein Ar150329p02 was previously determined to be a polyketide synthase and homolog to protein Sol1 from *A. solani*. Protein Ar153157p01 was predicted to be a cytochrome P450 and homolog to protein Sol6 from *A. solani*. Predicted mRNA Ar153167p02 was found to harbor homologs to three proteins belonging to the solanapyrone cluster from *A. solani*. These homologous proteins were Sol3, Sol4 and Sol5. The open reading frames of these six solanapyrone biosynthesis genes in the genome of *A. rabiei* were determined to be oriented in manner similar to their homologs in the genome of *A. solani* (Figure 3.8C).

Table 3.4. Identification and homology of genes and proteins from the predicted solanapyrone gene cluster of *Ascochyta rabiei*

Protein	Scaffold and protein ID	Gene, transcript, and protein size (Fgenesh)	Number of Exons	Location of the gene in the scaffold (bp)	Orientation of the gene in the cluster	BLASTP analysis			
						Description, Accession number and organism	E-value	Total Score	Query coverage
ArSol1	Ar150329.02	8054 bp 7863 bp 2620 aa	4	4266 to 11642	5' to 3'	Solanapyrone synthase (2641 aa) BAJ09789 *Alternaria solani*	0.0	5200	100%
ArSol2	Ar150329.01	1580 bp 1296 bp 431 aa	5	800 to 1705	3' to 5'	*O*-methyltransferase (427 aa) BAJ09788 *Alternaria solani*	0.0	803	95%
ArSol3[1]	Ar153167.02	6150 bp 4524 bp 1507 aa	12	8745 to 9645	5' to 3'	Dehydrogenase (285 aa) BAJ09787 *Alternaria solani*	0.0	548	18%
ArSol4[1]	Ar153167.02	Same as previous	Same as previous	7152 to 8258	5' to 3'	Transcription factor (557 aa) BAJ09786 *Alternaria solani*	0.0	1110	36%
ArSol5[1]	Ar153167.02	Same as previous	Same as previous	4566 to 5099	5' to 3'	Oxidase/Diels-Alderase (515 aa) BAJ09785 *Alternaria solani*	0.0	969	33%
ArSol6	Ar153167.01	2523 bp 1779 bp 592 aa	6	1658 to 2809	3' to 5'	Cytochrome P450 (461 aa) BAJ09789 *Alternaria solani*	0.0	880	83%

[1]ArSol3, ArSol4 and ArSol5 were predicted as one protein by fgenesh in the genome of *A. rabiei*

Discussion

In this study, we used a genome-mining approach to identify 13 putative PKSs and four methyltransferase genes in the genome of the *Ascochyta rabiei* isolate ME14 from Australia. The PKS genes were identified with the previously validated fungal KS and AT pHMMs (Delgado *et al.*, 2011). This method helped to distinguish true PKS proteins from eukaryotic fatty acid synthases which is often difficult as these two protein families are structurally related (Keller *et al.*, 2005). Methyltransferase genes were also identified using another pHMM built using the SAM binding site domain from methyltransferases that have similarity to LaeA proteins. We targeted the identification of PKSs in this fungal plant pathogen because of the strong evidence that the polyketide phytotoxin solanapyrone A is a major virulence factor in chickpea plants affected by Ascochyta blight (Hamid and Strange, 2000; Kaur, 1995; Latif and Strange, 1993; Zerroug *et al.*,2007). However, it has also been suggested that other factors could be involved in the phytotoxic potential of *A. rabiei* (Zerroug *et al.*, 2007). Fungal methyltransferase LaeA homologs were targeted because LaeA has been described as a global regulator of secondary metabolism (Bok and Keller, 2004; Bok *et al.*, 2005). The expression of these PKS and methyltransferase genes was studied in the presence of divalent cations such as calcium, cobalt, copper, manganese, and zinc as these have been reported to be required for the production of solanapyrone A in cultures of *A. rabiei* (Chen and Strange, 1991). The effect of light stimulus was studied because LaeA is a member of the velvet complex, which is known to respond to light stimulus in *A. nidulans* (Bayram *et al.*, 2008).

Analysis of the predicted proteome of *A. rabiei* genome showed that there were 13 predicted PKS proteins according to the fungal KS pHMM, and 12 putative PKS proteins according to the fungal AT pHMM. The high number of PKS genes within the genome of this fungal pathogen

agrees with the high number of PKSs found in the genomes of the members of subphylum Pezizomycotina, especially from members of the class Dothiodeomycetes (Kroken *et al.*, 2003). Of the 13 predicted PKSs in *A. rabiei*, only 11 predicted proteins had both the KS and AT domains according to the fungal KS and AT pHMMs. However, analysis of the protein domain architecture showed that ArPKS01 and ArPKS04, identified by the fungal KS domain but not by the fungal AT domain, had a truncated AT domain. These protein sequences abruptly ended when the corresponding scaffolds ended. These scaffolds only contained one predicted protein. The truncated AT domain of these PKSs were 85 and 48 amino acid in length, respectively. The fungal AT pHMM predicted one putative protein that was not predicted by the fungal KS pHMM (Ar120226.01). This protein sequence was determined to be related to PKS protein and to contain the AT and DH domain, but this protein sequence was believed to be part of an already identified putative PKS by the fungal KS pHMM. Therefore, it was not characterized as the putative proteins predicted by the fungal KS pHMM. These differences in the predictions between the pHMMs could be attributed to the early stage of the genome assembly used for this study.

The degree of reduction of the β-carbon in polyketide compounds varies according to the reducing nature of the corresponding PKS (Keller *et al.*, 2005). Phylogenetic analysis of the KS domain of these putative PKSs showed that ten were reducing PKSs and three were non-reducing PKSs. Reducing PKSs synthesize polyketides with linear carbon chains such as alternapyrone and T-toxin, while non-reducing PKS produce polyketides with aromatic carbon chains such as melanin and cercosporin (Choquer *et al.*, 2005; Fujii *et al.*, 2005; Gaffoor *et al.*, 2005; Loppnau *et al.*, 2004; Yang *et al.*, 1996). However, the final product of non-reducing PKSs may also be reduced by enzymes other than PKSs (Kroken *et al.*, 2003). Reducing domain identified in the putative PKSs of *A. rabiei* include KR, DH, and ER (Fujii *et al.*, 2004; Gaffoor and Trail, 2006; Kroken *et al.*, 2003)

These reducing domains were only seen in ArPKS02, ArPKS03, ArPKS05, ArPKS07, ArPKS09, ArPKS10, ArPKS12, and ArPKS13; which were also identified as reducing PKSs by phylogenetic analysis. ArPKS13 protein was determined to be closer to bacterial PKSs than fungal PKSs according to the phylogenetic analysis. Two PKSs from *Cochliobolus heterostrophus* were also found to be closer to bacterial PKSs. These *C. heterostrophus* PKSs were speculated to have been acquired through horizontal gene transfer events (Kroken *et al.*, 2003). ArPKS13 was not expressed in any of the axenic conditions but it was found to be expressed *in planta*. This is similar to the PKS named PLSP1 in *Fusarium graminearum* that was found to be expressed only *in planta* (Gaffoor *et al.*, 2005).

Gene prediction analysis showed that ArPKS05 and ArPKS06 are located side by side in scaffold Ar149451. Phylogenetic domain architecture analysis showed that ArPKS05 and ArPKS06 were reducing and nonreducing PKSs, respectively. These proteins also differed in their gene expression profile but they both were expressed under the stimulus of divalent cations. The synthesis of fungal polyketides such as zearalenone, T-toxin, and botcinic acid requires two PKSs (Baker *et al.*, 2006; Dalmais *et al.*, 2011; Gaffoor and Trail, 2006; Kim *et al.*, 2005). These two PKSs are in opposite orientation in the scaffold. ArPKS05 is in a negative chain orientation, while ArPKS06 is in a positive chain orientation. In *F. graminearum*, the two PKSs required for the biosynthesis of zearalenone are also side by side and in opposite orientation in the genome of this wheat pathogen. The disruption of any of these two PKS genes in *F. graminearum* completely abolishes the production of zearalenone (Gaffoor and Trail, 2006; Kim *et al.*, 2005). The scaffold also contains genes that presented similarity with zinc-binding oxireductase, trichothecene 3-*O*-acetyltransferase, and fungal Zn_2Cys_6 binuclear DNA-binding transcription factor.

The protein domain architecture of the ArPKS09 and ArPKS10 proteins was found to be the same as the PKS involved in the synthesis of solanapyrone (Sol1) from *Alternaria solani* (Kasahara *et al.* 2010). *A. rabiei* and *A. solani* are known producers of solanapyrones A, B and C, which are non-host specific toxins believed to act primarily by inhibiting active plant defenses such as detoxification and/or stimulation of membrane-localized H^+-ATPase activity (Chen *et al.*, 1991; Ichihara *et al.*, 1983; Knogge, 1996; Švábová and Lebeda, 2005). Both, ArPKS09 and ArPKS10 also had high similarity to Sol1 from *A. solani* according to the BLASTP analysis with an E-value of 0.0. However, ArPKS09 was found to have the highest total score and query coverage. Also, the phylogenetic analysis of the KS domain of these 13 predicted PKSs in the genome of *A. rabiei* showed that the KS domain of ArPKS09 is the most similar to the KS domain of Sol1 from *A. solani*. TBLASTN analysis using the protein sequences corresponding to the solanapyrone cluster proteins against the scaffolds of *A. rabiei* showed the highest similarity with two scaffolds (Ar150329 and Ar153167). Scaffold 150329 was shown to bear protein homologs to solanapyrone synthase (Sol1) and to *O*-methyltransferase (Sol2). Scaffold Ar153167 was shown to bear homologs to the rest of the proteins of the solanapyrone cluster such as dehydrogenase, transcription factor, oxidase/diels-alderase, and cytochrome P450. These two scaffolds seemed to contain the entire solanapyrone cluster. Moreover, the orientation of the solanapyrone genes in the *A. rabiei* scaffolds were the same as in the solanapyrone cluster of *A. solani* (Kasahara *et al.*, 2010), suggesting the conservation of the cluster between these two Pleosporales species. To our knowledge, this is the first description of the solanapyrone cluster in the genome of *A. rabiei*. The expression of this solanapyrone synthase (*ArPKS09*) gene in the *A. rabiei* isolate Ar666 was also shown to be dependent on divalent cations, which supports previous reports that the accumulation of

solanapyrones in *A. rabiei* cultures is decreased in the absence of calcium, cobalt, copper, manganese, and zinc (Chen and Strange, 1991).

Culture filtrates and culture organic extracts of *A. rabiei* were demonstrated to be phytotoxic on chickpea shoots and in liquid cultures of *C. reinhardtii* only when grown in still liquid CDM amended with calcium, cobalt, copper, manganese, and zinc divalent cations. The phytotoxicity assay on chickpea shoots, as described by Hamid and Strange (2000), was found to be difficult for an unbiased rating of the symptoms. Therefore, we decided to use the single-celled alga as a model system for phytotoxicity as proposed by Alexander *et al.*, (1999). Earlier studies have shown that the divalent cations calcium, cobalt, copper, manganese, and zinc simulate the effect of aqueous extract of chickpea seeds for the synthesis of solanapyrones (Chen and Strange, 1991). The elimination of each of the above mentioned divalent cations from the CDCLM was previously reported to reduce the production of solanapyrone A, and the elimination of zinc from the media completely abolished the production of solanapyrones (Chen and Strange, 1991). The absence of manganese was also shown to significantly reduce the levels of this toxin (Chen and Strange, 1991). However, in our study where we added each cation independently instead of removing them, we found that the addition of each individual divalent cation to the CDM did not increase the phytotoxicity of the organic extracts (Figure 3.6C). Organic extracts from *A. rabiei* grown in CDM amended solely with either zinc or manganese were not phytotoxic in *C. reinhardtii* cultures. RT-PCR analysis using cDNA from *A. rabiei* cultures grown in the presence of single divalent cations showed that *ArPKS04* and *ArPKS09* were also expressed in cultures grown in the presence of zinc. However, the expression signal of *ArPKS09* was weaker than in CDCLM. The organic extract of cultures grown in the presence of zinc was not phytotoxic in *C. reinhardtii*. At the moment, we know that *ArPKS09* is expressed in zinc but we do not have information of the expression the other

five genes in the cluster of solanapyrone in response to zinc. The solanapyrone synthase (ArPKS09) synthesizes desmethylprosolanapyrone and the synthesis of solanapyrone A requires the intervention of *O*-methyltransferase (Sol2), cytochrome P450 monooxygenase (Sol6), and flavin-dependent oxidase/Diels alderase (Sol3). A dehydrogenase (Sol4) is required for the conversion of solanapyrone A into solanapyrone B (Kasahara *et al.*, 2010). It seemed that all five divalent cations were necessary for the synthesis of phytotoxic compounds by *A. rabiei* isolate Ar666. The possibility exists that a combination of two or more divalent cations could be required for the synthesis of phytotoxic compounds by *A. rabiei*. Divalent cations have been reported to be required for total and messenger RNA levels, fungal biomass, aflatoxin biosynthesis, among other changes axenic cultures of *Aspergillus* species (Cuero *et al.*, 2003; Yasmin *et al.*, 2009). During different total RNA isolations, we noticed that the total RNA concentration was always lower in cultures grown in CDM in the dark, followed by CDM grown under light regimen, then by CDCLM in the dark, and the highest yield of total RNA was obtained from cultures grown in CDCLM under light regimen (data not shown). These reduced levels of total RNA in the *A. rabiei* cultures grown in the absence of divalent cations could be responsible for the lower vegetative growth compared to cultures grown the presence of divalent cations.

Gene expression studies showed that the *A. rabiei* PKSs responded to the divalent cations and light stimuli differently. Several PKSs were found to be expressed constitutively such as the melanin synthase *ArPKS11*, previously identified by Akamatsu *et al.*, (2010). However, five PKS genes were found to be expressed only in cultures containing divalent cations, including *ArPKS02*, *ArPKS03*, *ArPKS04*, *ArPKS08*, and *ArPKS09*. These were therefore thought to be associated with the phytotoxicity of *A. rabiei*. The ArPKS09 protein was identified as solanapyrone synthase as discussed above. Solanapyrone is probably the major phytotoxic compound present in the organic

extract because this phytotoxin has been correlated with aggressiveness of this pathogen in chickpea plants (Hamid and Strange, 2000; Kaur, 1995; Latif and Strange, 1993; Zerroug *et al.*, 2007). The gene expression studies did not find any exclusive association between the identified methyltransferases and divalent cations stimulus. The studied divalent cations appeared to increase the fungal biomass of *A. rabiei* by 1.9x. We speculate the some of the PKSs that are expressed under the stimulus of divalent cations may be involved in the synthesis of polyketide compounds that serve as small signaling molecules for developmental events such as vegetative growth (Kroken *et al*, 2003) as previously reported in *F. graminearum*, where the disruption of the PKS genes *GRS1* and *PKS2* resulted in the reduced mycelial growth. These *F. graminearum* PKSs were thought to be regulators of mycelia proliferation (Gaffoor *et al.*, 2005). Gene expression studies also found an association between the absence of light and the conidial production, which is similar to the study where the disruption of the PKS gene *FLUP* in *Aspergillus parasiticus* resulted in elimination of asexual sporulation, reduced aflatoxin accumulation, and inhibition of mycelial proliferation (Zhou *et al.*, 2000). The melanin synthase gene (*ArPKS11*) was found to be expressed constitutively in this study; however, conidiospores are not melanized (Akamatsu *et al.*, 2010). Melanin is accumulated mainly in the pycnidia protecting the conidiospores from the UV light (Akamatsu *et al.*, 2010). We found also that the conidial production was enhanced in the presence of all five divalent cations. Divalent cations may interact with *A. rabiei* in host recognition signaling, sensing the host for conidial dispersal. Conidiation and differentiation have also been linked to divalent cations, especially zinc, in fungi such *Aspergillus flavus* and *F. graminearum* (Cuero and Ouellet, 2005; Kroken *et al*, 2003).

In this study, we identified PKSs and methyltransferase genes and their expression in response to divalent cations, and to the presence and absence of light, which have been associated

with production of phytotoxic compounds and mycelial growth. These stimuli were shown to have an effect in the expression profile the genes evaluated, in mycelial growth of the pathogen, conidial production, and phytotoxic potential of culture filtrates of *A. rabiei*. Conidial production was higher in the absence of light, which was not surprising since the conidiospores of *A. rabiei* are not melanized; and therefore, sensitive to UV radiation. The stimulus of divalent cations had an effect in phytotoxicity, fungal biomass and in the expression of five PKS genes. We believe that the production of these polyketide metabolites maybe involved with these biological processes. The fact that these five PKSs are only expressed under the stimulus of divalent cations may be due to physiological costs of the synthesis of these polyketides and other metabolites (Challis and Hopwood, 2003; Gaffoor *et al.*, 2005) associated with the phytotoxicity and mycelial growth of *A. rabiei*. The divalent cations included in this study were derived from previous studies of the composition of aqueous extract of chickpea seeds for the synthesis of solanapyrone (Chen and Strange, 1991). We think that these divalent cations may act as small signaling molecules for the recognition of the host (chickpea) (Cuero and Ouellet, 2005); and therefore the production of these expensive metabolites is triggered in order to colonize the host. In this regard, solanapyrones have been associated with the pathogen's aggressiveness and effect in cell wall degradation producing breakage of the chickpea stems (Hamid and Strange, 2000). Our study suggests that five ArPKSs are dependent on divalent cations and, perhaps, are involved in plant recognition in the *A. rabiei*-chickpea pathosystem. However, disruption of these five PKSs of *A. rabiei* must be conducted in order to further characterize the function and effect of these polyketide compounds during plant penetration and colonization.

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SUMMARY

The production of chickpeas in the United States and Canada in 2009 represented 0.7% and 5.6% of the world's harvested area and yield, respectively. This crop is affected by over 50 pathogens but the most destructive disease for this crop is Ascochyta blight, which is caused by the necrotrophic fungus *Ascochyta rabiei* (teleomorph *Didymella rabiei*). Several Ascochyta blight epidemics have been reported in the United States and Canada since the introduction of this pathogen in the chickpea production areas in 1986 posing severe yield and economic losses. To date, there is no chickpea cultivar available in the market that has complete resistance to this disease. However, there are several cultivars with partial resistance to isolates of *A. rabiei* pathotype I and II in North America. The most commonly found pathotype in this geographic region is pathotype II, which is more aggressive than pathotype I. Partial resistance to Ascochyta blight is quickly broken down by *A. rabiei* due to its great genetic and pathogenic variability. Therefore, seed and foliar chemical treatments form an integral part of the disease management recommendation for this crop. In North Dakota, several applications of foliar fungicides are recommended during the growing season to avoid *A. rabiei* infections, which can occur at any stage of the plant growth. Quinone outside inhibitor (QoI) fungicides were the only fungicides with preventative and post-infection/curative properties before boscalid and prothioconazole were labeled for their use in chickpeas in North Dakota in 2003 and and 2007, respectively. Resistance to QoI fungicides found in *A. rabiei* isolates from Montana, Nebraska, North Dakota, and South Dakota in the United States and from Saskatchewan in Canada has threatened the use of these chemistries. The application of QoI fungicides in these regions may lead to severe economic losses due to the inability of these fungicides to control disease.

The infection process of *A. rabiei* on chickpea has been well described; however, the pathogen's molecular mechanism of infection and colonization has not been extensively characterized. To date, the only two virulence factors involved in disease development that have been studied are the polyketides melanin and solanapyrone. Melanin has been shown to be important in the biology and virulence of *A. rabiei* isolates although the appreassorium-like structure formed by *A. rabiei* is not melanized. Melanin accumulates in the walls of *A. rabiei* pycnidia protecting pycnidiospores from UV irradiation. Also, spontaneous albino mutants appear to be unable to produce pycnidiospores and have been proven to be non-pathogenic in susceptible varieties of chickpeas.

Solanapyrones A, B, and C are non-host specific phytotoxins produced by *A. rabiei*, and also by *Alternaria solani*. These phytotoxins have been suggested to be the most important virulence factor in Ascochyta blight of chickpeas as it seems to aid penetration and colonization by weaking the plant cell membrane. Solanapyrones have been found to cause growth inhibition, blight-like lesions, and stem breakage. The solanapyrone gene cluster has been cloned and characterized in the genome of *A. solani*, but is yet to be studied in the genome of *A. rabiei*.

Both solanapyrone and melanin are synthesized by polyketide synthase (PKS) pathways, which belong to the secondary metabolism of fungal species. PKS enzymes are the backbone enzymes of these pathways. PKSs are multidomain proteins that require a minimum domain architecture of three protein domains to be functional. These protein domains are the β-ketoacyl synthase (KS), acyltransferase (AT), and acyl carrier protein (ACP). The KS domain is the most conserved domain and has been used to clone PKSs using different molecular techniques and also to differentiate PKS proteins from related eukaryotic proteins such fatty acid synthases through phylogenetic analysis. Conversely, the ACP domain has been found to be the least conserved and

unsuitable to differentiate PKSs from other proteins. Recently, gene clusters of secondary metabolism have been observed to be modulated by a master regulator called LaeA methyltrasferase, which is characterized for having the S-adenosylmethionone (SAM) binding site domain. This SAM domain is thought to be involved in the modification of chromatin structure. LaeA methyltransferase was found to be light regulated in *Aspergillus* species and to play a key role in the accumulation of fungal natural products.

In this dissertation, we focused in the characterization of the cytochrome b gene fragment that harbors mutation related to the development of resistance to QoI fungicides as well as the identification and characterization of PKS and methyltransferase genes in *A. rabiei*. We feel that this information will eventually enable development of a better understading of the biology of this fungus and serve for a better design of Ascochyta blight management practices. Isolates of *A. rabiei* from North Dakota were previously reported to be 300 and 600-fold less sensitive than baseline isolates to the QoI fungicides, azoxystrobin and pyraclostrobin, respectively. In the first paper of this dissertation, we cloned and characterized the fragment of the cytochrome b gene from eight isolates of *A. rabiei* with different sensitivities to the QoI fungicides azoxystrobin and pyraclostobin. The cloned genomic DNA fragment was found to harbor a group I intron immediately after codon 131 that encoded for a GIY-YIC endonuclease. The cytochrome b gene is thought to be larger than 4.6 kb and its structure appeared to be similar to the gene structure of the cytochrome b gene of *Monilinia fructigena*. The cDNA fragments cloned from these eight isolates of *A. rabiei* were analyzed using multiple alignments which revealed a single nucleotide mutation at codon 143. This codon changed from GGT to GCT introducing an amino acid substitution from glycine to alanine (G143A). This mutation was confirmed by multiple alignments of the predicted protein sequences. This G143A mutation has been associated with resistance to QoI fungicides, conferring resistance

factor of over 100-fold. The cloned cDNA fragment was shown to be similar to the cytochrome b mRNA from *Alternaria solani*, while the predicted protein sequence seemed to be most similar to the cytochrome b protein from *Phaeosphaeria nodorum*. The single point mutation and the genomic DNA sequence was used to design simple PCR assay for diagnostic of QoI resistant isolates of *A. rabiei*. This diagnostic PCR was developed using an approach involving allele-specific PCRs known as mismatch amplification mutation assay (MAMA). This MAMA-PCR was conducted using two separate PCR assays with reverse primers specifically designed to detect the GGT- and GCT-alleles in *A. rabiei* isolates sensitive and resistant to QoI fungicides, respectively. This MAMA-PCR was validated using 56 *A. rabiei* isolates that were previously tested for QoI resistance using *in vitro* spore germination assay on fungicide amended solid media, and with isolates from other fungal species. A total of 70 isolates of *A. rabiei* were tested with this MAMA-PCR showing that only the alleles GGT and GCT were present in the studied individuals. This MAMA-PCR was found to be faster than the *in vitro* spore germination assay, providing a simple and accurate diagnostic tool for detection of QoI resistance in *A. rabiei* isolates.

The goal of the second paper of this dissertation was to build a bioinformatic resource specifically designed for the identification type I iterative PKS proteins from the predicted proteomes of sequenced fungal species. This bioinformatic resource was developed using a statistical model known as profile Hidden Markov model (pHMM) with two of the three minimal protein domains necessary for PKS proteins to be functional. These pHMMs were constructed using the KS and AT domains retrieved exclusively from fungal PKS proteins deposited in GenBank. These pHMMs were referred to as fungi-based models. The performance of these newly built fungi-based pHMMs was compared to mixed-kingdoms models, which were constructed using type I PKS proteins from organisms belonging to different kingdoms. The pHMMs were first assessed on the

predicted proteome of *Fusarium graminearum* as the genome of this fungal pathogen has been well annotated and characterized. Both the fungi-based and mixed-kingdom models identified 15 PKSs that agreed with previous predictions and gene disruption studies. Subsequently, these pHMMs were used to identify a total of 54 type I iterative PKS proteins in the predicted proteomes of *Alternaria brassicicola*, *F. oxysporum* f. sp. *lycopersici*, *Verticillium albo-atrum* and *V. dahliae*. Both the fungi-based and mixed-kingdom models were found to identify the same number of fungal PKSs. However, these models were found to perform differently in terms of discrimination between true PKSs and non-PKSs. The fungi-based KS and AT pHMMs were shown to perform significantly better than the mixed-kingdom KS and AT pHMMs, respectively. We found that the fungi-based KS and AT pHMMs were able to identify true PKSs with a bit score greater than zero and non-PKS proteins with bit score lower than zero. In constrast, the mixed-kingdom models failed to achieve this clear bit score cut off that allowed discriminating true PKSs from non-PKSs. The fungi-based models were found to perform with significantly higher accuracy, sensitivity, specificity, and precision than the mixed-kingdom models. The predicted PKS proteins from the fungal pathogen *V. dahliae* were biologically confirmed to be PKSs by gene-specific PCR amplification and sequencing of the corresponding gene. Thus, we found that that our fungi-based PKS models were more suitable for the identification and annotation of fungal PKSs from predicted proteomes.

In the third paper of this dissertation, we identified 13 PKS genes and proteins from the first draft assembly of the *A. rabiei* genome. These PKS proteins were identified using the previously described fungi-based KS and AT pHMMs. Ten of these PKSs were found to be of reducing nature and three were non-reducing according to phylogenetic analysis and protein domains. The transcripts of all predicted PKSs were detected in either axenic cultures or infected chickpea plants or both. The expression of five PKS genes (*ArPKS02*, *ArPKS03*, *ArPKS04*, *ArPKS08*, and *ArPKS09*) was

found to be dependent on calcium, cobalt, copper, manganese, and zinc. Fungal vegetative growth and accumulation of phytotoxic compounds were also found to be dependent on these five divalent cations. However, the effect of single divalent cations and pH was not associated with phytotoxicity and fungal biomass of *A. rabiei*; except that the vegetative growth on media amended with these five divalent cations was not significantly different from the vegetative growth of the fungus grown on media amended with only zinc. The expression of the solanapyrone synthase gene was found to be dependent on divalent cations. The solanapyrone gene cluster was identified in the genome of *A. rabiei*. The cluster was found to be highly similar and in synteny to the solanapyrone gene cluster from *A. solani*, suggesting that horizontal gene transfer might have occurred between these pathogens. In the genome of *A. rabiei*, four methyltransferases were identified; however, none of these were found to be dependent on either divalent cations or light stimuli. Moreover, none of the identified methyltransferase was found to be highly similar to LaeA methyltransferase by BLASTP but they had the SAM domain. In this paper, we identified and characterized PKS and methyltransferase genes in response to divalent cations and light stimuli. We believe that the studied divalent cations modulate gene expression of not only PKSs but of other genes affecting the several biological processes such as accumulation of phytotoxic compounds, vegetative growth, host recongnition and colonization, amongst others.

APPENDIX A. GENBANK ACCESSION NUMBERS OF FUNGAL PKS PROTEINS

Table A.1. Accesion numbers of reducing polyketide synthases of fungal origin

Accession no.	Accession no.	Accession no.	Accession no.	Accession no.	Accession no.
AAB08104	AAR92208	BAE56814	CAK41642	CAP80780	EAL85129
AAB49684	AAR92209	BAE56830	CAK41692	CAP91516	EAL86424
AAC49814	AAR92212	BAE56924	CAK41928	CAP91938	EAL86540
AAD34559	AAR92213	BAE57016	CAK42316	CAP92707	EAL87227
AAD39830	AAR92214	BAE59642	CAK42483	CAP93050	EAL87813
AAD43562	AAR92216	BAE61265	CAK42679	CAP93159	EAL89230
AAK48943	AAR92217	BAE62480	CAK43489	CAP93818	EAL91103
AAO62426	AAR92219	BAE62485	CAK43811	CAP94063	EAL92117
AAR85531	AAR92220	BAE63250	CAK43813	CAP94993	EAQ70609
AAR90237	AAR92221	BAE63408	CAK44344	CAP95290	EAQ70662
AAR90238	AAR92222	BAE63932	CAK45416	CAP95296	EAQ83688
AAR90239	AAS46233	BAE64031	CAK46336	CAP95381	EAQ84243
AAR90240	AAS98200	BAE64579	CAK48258	CAP95404	EAQ86385
AAR90241	AAT28740	BAE65442	CAK49051	CAP95405	EAQ87449
AAR90242	AAT92023	BAE65683	CAK49105	CAP96141	EAQ87856
AAR90244	AAV66106	BAE66025	CAK49166	CAP96413	EAQ88528
AAR90246	AAX35547	BAE66270	CAK96260	CAP98105	EAQ90439
AAR90247	AAY32931	BAE66272	CAK96551	CAP99663	EAQ91166
AAR90252	AAY40862	BAE80697	CAK96944	EAA26702	EAQ91811
AAR90253	AAY41231	BAG82673	CAK97281	EAA28899	EAS33665
AAR90254	ABA02239	CAA39295	CAL69597	EAA28933	EAS34545
AAR90256	ABA02240	CAG28797	CAN87161	EAA34002	EAS34547
AAR90257	ABB76806	CAG28798	CAO91861	EAA36364	EAT77779
AAR90258	ABQ42548	CAG29113	CAP58786	EAA58453	EAT81575
AAR90259	ABS58604	CAH59193	CAP60208	EAA58609	EAT82888
AAR90260	ABS87601	CAH59194	CAP61891	EAA59820	EAT85332
AAR90261	ACB12550	CAK37791	CAP61938	EAA63131	EAT87259
AAR90262	ACD39758	CAK38394	CAP62086	EAA63354	EAT91803
AAR90263	ACD39767	CAK38698	CAP62330	EAA63960	EAU29410
AAR90264	ACD39774	CAK39062	CAP64486	EAA64124	EAU29417
AAR90265	ACM42406	CAK40124	CAP65099	EAA64652	EAU30237
AAR90266	ACM42412	CAK40131	CAP65540	EAA64864	EAU30794
AAR90267	BAA20102	CAK40147	CAP65896	EAA64867	EAU31345
AAR90268	BAC20564	CAK40660	CAP66437	EAA65602	EAU31921
AAR90269	BAC20566	CAK40714	CAP67467	EAA65604	EAU31923
AAR90270	BAD44749	CAK40750	CAP69528	EAA66622	EAU32451
AAR90271	BAD83684	CAK40752	CAP70096	EAA67005	EAU32663
AAR90275	BAE54587	CAK40845	CAP71783	EAA67034	EAU32666
AAR90277	BAE54637	CAK41106	CAP74149	EAL84933	EAU32819
AAR90279	BAE55815	CAK41112	CAP80186	EAL85113	EAU33224

Table A.1. (continued)

Accession no.	Accession no.	Accession no.	Accession no.	Accession no.	Accession no.
EAU33817	EAW19759	EDN31627	EEA18579	EED15402	EED48583
EAU35431	EAW19771	EDN32209	EEA18650	EED15547	EED49595
EAU36706	EAW19983	EDN93374	EEA18652	EED15709	EED49862
EAU36720	EAW20415	EDN93579	EEA19308	EED15802	EED49876
EAU38928	EAW21094	EDN95526	EEA20045	EED16635	EED51023
EAU38971	EAW23638	EDN96866	EEA20741	EED16637	EED52019
EAW07064	EAW23793	EDN98782	EEA22389	EED18001	EED52023
EAW07624	EDJ95592	EDN99357	EEA23570	EED18128	EED53171
EAW08895	EDJ95896	EDO04615	EEA23575	EED18841	EED57354
EAW08950	EDJ99000	EDP48973	EEA23602	EED19830	EEH02664
EAW09019	EDJ99064	EDP49155	EEA24143	EED21266	EEH04903
EAW09117	EDJ99646	EDP49172	EEA24841	EED21572	EEH10010
EAW10019	EDK02535	EDP52288	EEA25173	EED22580	EEH16752
EAW11760	EDK03178	EDP53389	EEA25568	EED22651	EEH19899
EAW12049	EDK04179	EDP53404	EEA25741	EED22678	EEH41414
EAW12170	EDK04299	EDP53518	EED11515	EED22683	EEH44268
EAW12336	EDK04353	EDP55391	EED11953	EED24614	EEH46751
EAW12416	EDK06282	EDU39548	EED12110	EED44780	EEH50245
EAW13520	EDN02239	EDU42159	EED12124	EED45116	EEP76517
EAW13531	EDN22443	EDU46521	EED12350	EED45460	EEP78969
EAW13625	EDN22562	EDU46982	EED13058	EED45665	EEP81156
EAW14856	EDN23222	EDU47082	EED13571	EED45667	EEP81634
EAW15062	EDN23517	EDU47225	EED13647	EED47354	P22367
EAW16387	EDN24214	EDU47346	EED14251	EED47649	Q0C8M3
EAW16886	EDN26866	EDU49219	EED14366	EED47890	Q9Y8A5
EAW17018	EDN29096	EDU49723	EED14393	EED48448	
EAW17052	EDN29884	EDU50339	EED14463	EED48486	

Table A.2. Accesion numbers of non-reducing polyketide synthases of fungal origin

Accession no.	Accession no.	Accession no.	Accession no.	Accession no.	Accession no.
1905375A	AAS48892	BAC45240	EAA31350	EAW20700	EEA25563
2123354A	AAS66004	BAD22832	EAA57749	EAW24682	EEA28350
AAA81586	AAS89999	BAE55957	EAA59563	EAW24697	EED11516
AAC23536	AAS90022	BAE58990	EAA61200	EAW25724	EED12747
AAC39471	AAS90047	BAE59509	EAA66023	EDK03173	EED15770
AAC41674	AAS90093	BAE61567	EAL84397	EDK03942	EED16360
AAC41675	AAS92537	BAE62229	EAL84875	EDN20835	EED17890
AAC49191	AAT69682	BAE65965	EAL89339	EDN26857	EED18910
AAD31436	AAZ95017	BAE71314	EAL94057	EDN28304	EED18976
AAD38786	ABD47522	CAA46695	EAQ86888	EDN97264	EED21054
AAM93545	ABG91136	CAA76740	EAQ92307	EDN98463	EED21099
AAN59953	ABU63483	CAB92399	EAS29823	EDP47078	EED45393
AAN74983	ACD39753	CAC88775	EAT76667	EDP47964	EED47975
AAN75188	ACD39762	CAC94008	EAT80393	EDP49937	EED53479
AAO60166	ACD39770	CAK38306	EAT83782	EDP55264	EED53907
AAR32704	ACH72912	CAK40778	EAT84550	EDU42706	EED57518
AAR90248	ACJ13034	CAK47960	EAU31624	EDU45231	Q03149
AAR90249	ACJ13035	CAL00851	EAU31762	EDU45846	Q12053
AAR90250	ACJ13039	CAM35471	EAU32763	EEA19397	Q12397
AAR90251	ACM42403	CAP61360	EAU37396	EEA22394	
AAR90272	BAA18956	CAP73289	EAU38791	EEA22952	
AAR90274	BAB88688	CAP73353	EAW13612	EEA23448	
AAR92210	BAB88689	CAP96142	EAW14609	EEA24206	
AAR92211	BAB88752	CAP96497	EAW19338	EEA25245	

APPENDIX B. BLASTP OF PREDICTED ITERATIVE PKS FROM FUNGAL PROTEOMES

Table B.1. Protein homologs of the putative PKS proteins from the predicted proteome of *Alternaria brassicicola*, *Fusarium graminearum*, *F. oxysporum* f. sp. *lycopersici*, *Verticillium albo-atrum*, and *V. dahliae*

Protein	Accession Number	Description	Organism	E-value
Ab001seq371	ACZ57548	Polyketide synthase	*Alternaria brassicicola*	0.0
Ab001seq648	BAD22832	Polyketide synthase	*Bipolaris oryzae*	0.0
Ab003seq685	AAR90270	Polyketide synthase	*Cochliobolus heterostrophus*	0.0
Ab003seq693	AAR90279	Polyketide synthase	*Cochliobolus heterostrophus*	3.4×10^{-87}
Ab004seq368	BAD83684	PKSN polyketide synthase for alternapyrone biosynthesis	*Alternaria solani*	0.0
Ab004seq469	XP_001937136	Phenolpthiocerol synthesis polyketide synthase ppsA	*Pyrenophora tritici-repentis*	0.0
Ab005seq505	AAR90263	Polyketide synthase	*Cochliobolus heterostrophus*	0.0
Ab008seq352	AAR90269	Polyketide synthase	*Cochliobolus heterostrophus*	0.0
FG01790	XP_003012265	Polyketide synthase	*Arthroderma benhamiae*	0.0
FG02395	ABB90282	Polyketide synthase	*Gibberella zeae*	0.0
FG03340	AAR92220	Polyketide synthase	*Gibberella moniliformis*	0.0
FG03964	XP_002486858	Polyketide synthase	*Talaromyces stipitatus*	0.0
FG04588	XP_001261880	Polyketide synthase	*Neosartorya fischeri*	0.0
FG05794	XP_002151741	Polyketide synthase	*Penicillium marneffei*	0.0
FG08795	AAR92214	Polyketide synthase	*Gibberella moniliformis*	0.0
FG10548	XP_002485500	Polyketide synthase	*Talaromyces stipitatus*	0.0
FG12040	AAU10633	Type I polyketide synthase	*Gibberella zeae*	0.0
FG12055	ABB90283	Polyketide synthase	*Gibberella zeae*	0.0
FG12100	ACS68554	Hybrid PKS/NRPS protein	*Metarhizium anisopliae*	0.0
FG12109	AAR90247	Polyketide synthase	*Botryotinia fuckeliana*	0.0
FG12121	XP_001270376	Polyketide synthase	*Aspergillus clavatus*	0.0
FG12125	AAR92210	Polyketide synthase	*Gibberella moniliformis*	0.0
FG12977	AAR92209	Polyketide synthase	*Gibberella moniliformis*	0.0
FOXT_02741	XP_001270321	Polyketide synthase	*Aspergillus clavatus*	0.0
FOXT_02884	ACZ57548	Polyketide synthase	*Alternaria brassicicola*	0.0
FOXT_03051	AAR92214	Polyketide synthase	*Gibberella moniliformis*	0.0
FOXT_03945	AAR92216	Polyketide synthase	*Gibberella moniliformis*	0.0
FOXT_04757	CAB92399	Polyketide synthase	*Gibberella fujikuroi*	0.0
FOXT_05816	AAR92210	Polyketide synthase	*Gibberella moniliformis*	0.0
FOXT_10805	XP_001936632	Mycocerosic acid synthase	*Pyrenophora tritici-repentis*	0.0

Table B.1. (continued)

Protein	Accession Number	Description	Organism	E-value
FOXT_11892	AAR92215	Polyketide synthase	*Gibberella miniliformis*	0.0
FOXT_11954	AAR92220	Polyketide synthase	*Gibberella moniliformis*	0.0
FOXT_11954	AAR92220	Polyketide synthase	*Gibberella moniliformis*	0.0
FOXT_14587	XP_002496604	Hybrid PKS/NRPS enzyme	*Talaromyces stipitatus*	0.0
FOXT_14850	AAR92219	Polyketide synthase	*Gibberella moniliformis*	0.0
FOXT_15248	AAR92213	Polyketide synthase	*Gibberella moniliformis*	0.0
FOXT_15296	AAR92208	Polyketide synthase	*Gibberella moniliformis*	0.0
FOXT_15886	AAR92219	Polyketide synthase	*Gibberella moniliformis*	0.0
VDAG_00190	XP_003008898	Conidial yellow pigment biosynthesis polyketide synthase	*Verticillium albo-atrum*	0.0
VDAG_01835	XP_003007526	Lovastatin nonaketide synthase	*Verticillium albo-atrum*	0.0
VDAG_01856	XP_003007505	Lovastatin nonaketide synthase	*Verticillium albo-atrum*	0.0
VDAG_03466	XP_003003869	Mycocerosic acid synthase	*Verticillium albo-atrum*	0.0
VDAG_04539	XP_003005392	Lovastatin nonaketide synthase	*Verticillium albo-atrum*	4.4×10^{-176}
VDAG_07270	XP_003000627	Lovastatin nonaketide synthase	*Verticillium albo-atrum*	0.0
VDAG_07928	XP_003009645	Lovastatin nonaketide synthase	*Verticillium albo-atrum*	0.0
VDAG_08448	CBI53318	Putative polyketide synthase	*Sordaria macrospora*	0.0
VDAG_09534	XP_003000081	Phenolpthiocerol synthesis polyketide synthase ppsA	*Verticillium albo-atrum*	0.0
VDBG_00162	CBI53318	Putative polyketide synthase	*Sordaria macrospora*	0.0
VDBG_00580	ACN32207	Polyketide synthase	*Glomerella graminicola*	0.0
VDBG_01329	XP_003069897	Putative polyketide synthase	*Coccidioides posadassi*	0.0
VDBG_01693	AAR92214	Polyketide synthase	*Gibberella moniliformis*	0.0
VDBG_01714	AAR92212	Polyketide synthase	*Gibberella moniloformis*	0.0
VDBG_04992	XP_002478535	Putative polyketide synthase	*Talaromyces stipitatus*	0.0
VDBG_04998	XP_002380243	Hybrid PKS/NRPS enzyme	*Aspergillus flavus*	0.0
VDBG_06312	XP_002151045	Putative polyketide synthase	*Penicillium marneffei*	0.0
VDBG_09122	ADF28668	Reducing type I polyketide synthase	*Peltigera membranacea*	0.0
VDBG_09801	XP_002377152	Putative polyketide synthase	*Aspergillus flavus*	0.0

APPENDIX C. HOW TO BUILD AND USE HMMER FOR PKS PROTEIN SEQUENCES SEARCH

Reprint from Delgado, J.A, Al-Azzam, O., Denton, A.M., Markell, S.G., and Goswami, R.S. 2011. A resource for *in silico* identification of fungal polyketide synthases from predicted fungal proteomes. Molecular Plant Pathology. Accepted for publication.

1. Construct a multiple alignment for each domain using clustalW software. This software can be found at (http://www.clustal.org/#Download). Save the alignment file with extension .aln for each domain.

2. Construct pHMM for each .aln file. The binary files for the software can be found in (http://hmmer.janelia.org/). Documentation of how to use the software is in (ftp://selab.janelia.org/pub/software/hmmer3/3.0/Userguide.pdf). To construct pHMM use the command: hmmbuild pHMM_File_Name.hmm Multiple_Alignment_File.aln

3. Calibrate the pHMM using the command: hmmcalibrate pHMM_File_Name.hmm

4. Search for the sequences that have the domain. Initially we have a database of sequences, and we want to know which sequences have the domain. To search for these sequences, we use the constructed pHMM. To search the databases use the command: hmmsearch pHmm_File_Name.hmm Database_Name.fasta. To save the output on an output file, add >Output_File_Name.out at the end of the command.

APPENDIX D. GENBANK ACCESSION NUMBERS OF FUNGAL METHYLTRANSFERASES

AAR01218
AAX68415
ABA87011
ACD50375
ADL63139
BAF74528
CBE54370
CBF88745
EAL84975
EAL87582
EAL90797
EAL92093
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