

AN ABC TRANSPORTER GENE FROM *FUSARIUM VERTICILLIOIDES*, *FvABC1*, MAY
CONFER TOLERANCE TO CORN ANTIMICROBIAL COMPOUNDS

by

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(Under the Direction of Anthony Glenn)

ABSTRACT

An ABC transporter gene, *FvABC1*, was cloned and sequenced from the corn pathogen *Fusarium verticillioides* in order to study non-degradative tolerance to corn antimicrobial compounds. The predicted protein encoded by *FvABC1* showed the typical topology of ABC transporters from the pleiotropic drug resistance (PDR) family: two nucleotide-binding domains (NBDs) and two transmembrane domains (TMDs) in the [NBD-TMD]₂ orientation. *FvABC1p* showed the same topology as the ABC transporter from *Magnaporthe grisea* (*ABC1*) and *Gibberella pulicaris* (*Gpabc1*). Both *ABC1* and *Gpabc1* transporters were classified as pathogenicity factors and were needed for colonization of host tissues. A southern blot analysis showed that several *Fusarium* species from the *Gibberella fujikuroi* species complex possessed an *FvABC1* homolog. Other species outside the complex but associated with corn also possessed the homolog. An analysis of *F. verticillioides* genomic data identified thirteen additional ABC transporters and all of them belonged to the PDR family.

INDEX WORDS: *Fusarium verticillioides*, corn, maize, hydroxamic acids, ABC transporters, non-degradative tolerance, DIMBOA, DIBOA, MBOA, BOA

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DEDICATION

To Eduarda del Rosario and Jose Victor for their love.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

The non-obligate ascomycete *Fusarium verticillioides* (synonym = *F. moniliforme*; teleomorph = *Gibberella moniliformis*, mating population A) is commonly associated with maize (*Zea mays*) and maize-based products around the world (Leslie et al., 1990). This pathogen is of particular interest in the Southeastern United States because of its economical damage to corn production. Although it is consistently associated with maize, *F. verticillioides* has been isolated from other cereal crops, including barley, wheat, rice and sorghum (Bacon and Hinton, 1996). This ubiquitous cosmopolitan plant pathogen can live as an asymptomatic endophyte but has been associated either with asymptomatic infections or with maize diseases including seedling blight, seed rot, root rot stalk rot and ear rot (Kaldau and Yates, 2000; Kommendahl and Windels, 1981; White, 1999).

The main concern with *F. verticillioides* infection is the production of toxic compounds known as mycotoxins, including , fusarin C, moniliformin, fusaric acid, and fumonisins (Bacon et al., 2004; Cerdá-Olmedo et al., 1994; Marasas et al., 1986; Reynoso et al., 2004). Mycotoxins are secondary metabolites produced by fungi and decrease the quality of corn kernels because of their association with animal diseases. While all these toxins have been studied and investigated widely, within the last decade the study of fumonisins, especially fumonisin B₁, has caught the attention of many investigators (Cabrera et al., 2004; Castells et al., 2005; Cleveland et al., 2003; Missmer et al., 2006; Segvic and Pepeljnjak, 2001). Fumonisins are a family of secondary

metabolites produced by *F. verticillioides* and other related species. They are produced under certain environmental conditions such as drought and late season rain. Fumonisin as a group comprise several families (A,B,C,D) but fumonisin B1 (FB₁) is the most significant since it is most prevalent in contaminated maize and is considered the most toxic (Kouadio et al., 2005; Marasas, 2001; Mckean et al., 2006). The International Agency for Research on Cancer (IARC, <http://www.iarc.fr/>), has classified FB₁ as a potential carcinogenic substance to humans. The high incidence of FB₁ in corn and corn-based products has been associated with animal diseases including esophageal cancer (Jaskiewicz et al., 1987) and neural tube defects in humans (Missmer et al., 2006), porcine pulmonary edema (Osweiler et al., 1992), cancer in laboratory rats (Norred et al., 1996) and equine leukoencephalomalacia (Marasas et al., 1988; Wilkins et al., 1994).

Cycle of infection

At any stage of maize plant development, *F. verticillioides* can be pathogenic. The first study on *F. verticillioides* systemic infection (Foley, 1962) stated that such infection resulted from symptomless infection of seed, while stalk infection was a consequence of leaf sheath infections. Since then, many researchers have demonstrated potential systemic movement of *F. verticillioides* using various methods (Bush et al., 2004; Cotten and Munkvold, 1998; Glenn et al., 2001; Munkvold et al., 1997; Oren et al., 2003). The infection cycle of *F. verticillioides* is very complex and has been controversial in the past, with a number of questions that remain unanswered. The symptoms of infection can be variable ranging from lacking of any visible damage to complete rotting and wilting (Oren et al., 2003). During the asymptomatic phase, microscopic studies of *F. verticillioides* reveal that intercellular growth of hyphae are restricted to limited areas. For years, the controversy of *F. verticillioides* symptomless infection and any

potential advantages and disadvantages of this relationship has attracted many researchers to perform several studies. The advantages for maize plants to have an endophytic relationship is one of the questions that been remained unanswered. Yates et al (1997) performed an experiment in order to understand the possible benefits of this plant-pathogen association. In general, they found that *F. verticillioides* endophytic strains may benefit early seedlings growth. Seedlings infected with *F. verticillioides* showed an accelerated growth to exceed, in some cases, non-infected seedlings. Studies have elucidated the infection routes and their role in pathogenic interactions, including the transmission of *F. verticillioides* from corn seeds to kernels (Bush et al., 2004; Kederea et al., 1992). Systemic infection contributes to kernel infection in about 10-30% of the total number of kernels infected and it seems that the movement from roots to crown is the limiting factor in the transmission of the fungus (Lawrence et al., 1981; Munkvold et al., 1997; Nelson, 1992; Oren et al., 2003). Munkvold et al., (1997) showed that while infecting corn seeds with *F. verticillioides* resulted in systemic infection, infection via silks resulted in more infected kernels. In terms of systemic infection, *F. verticillioides* is able to move to above ground parts in the beginning of the interaction but fungal biomass remains low (Oren et al., 2003). These results showed that initial asymptomatic *F. verticillioides* root infections are characterized by intercellular growth, with hyphae infecting few cells, but as the plant ages necrosis of root tissue begins with unorganized fungal and extensive mycelia production (Oren et al., 2003).

After harvest *F. verticillioides* can survive for a long time in plant debris in cultivated fields and is a source of inoculum for the next growing season (Cotten and Munkvold, 1998). Oren et al. (2003) compared infected soil and infected inoculated seeds and found that infected soil resulted in a greater number of diseased plants than seed inoculation, suggesting that this source of inoculum may play an important role in seedling development. *F. verticillioides* in soil

penetrates the plant through lateral roots and the mesocotyl (Murillo et al., 1999; Oren et al., 2003). The constant production of airborne conidia from plant residues reduces the significance of long-term survival in *F. verticillioides* because during the growing season the fungus grows rapidly, infecting and spreading all over the corn fields (Cotten and Munkvold, 1998; Ooka and Kommedahl, 1977).

F. verticillioides can produce airborne microconidia and macroconidia (Nelson, 1992). The sources of these two types of conidia can be either crop residues or sporulation of the fungus on the aerial parts of the plant (Logrieco and Bottalico, 1987). These airborne conidia can be either water splashed or wind-blown and reach maize silks which can result in kernel infections. Another pathway for *F. verticillioides* infection is wounds created by insects on any of the above-ground parts of the maize plant (Sobek, 1996).

Mycotoxin production

Fumonisin, the secondary metabolites produced by *F. verticillioides*, were discovered in the late 1980's (Marasas, 2001). However the toxic effects of corn contaminated with this fungus have been recorded for almost a hundred years (Munkvold and Desjardins, 1997). In 1970, a moldy corn survey showed that *F. verticillioides* was the predominant fungus from samples taken from a field outbreak of leukoencephalomalacia in horses (Kellerman et al., 1972). A group of researchers in South Africa associated the incidence of esophageal cancer in the Southern Transkei region with high consumption of corn which is a staple in the diet in this geographical area. The most prevalent fungus in the corn samples from Transkei was *F. verticillioides* (Marasas et al., 1981). However, the elucidation of the chemical structure and isolation of fumonisin was not revealed until 1988. Since that date many studies have shown the high toxicity of fumonisin in horses (Marasas et al., 1988), and in swine (Harrison et al., 1990).

In 1990, an outbreak of leukoencephalomalacia in horses and pulmonary edema in swine triggered the interest of the United States researchers in fumonisins (Ross et al., 1990).

Plant chemical defenses

In general, plants are exposed to attacks by many pathogens. In response, plants have developed chemical defense mechanisms which are generally comprised of low molecular weight compounds not involved in the primary metabolism of the plants. The classification of these small compounds depends upon when they are produced (van Etten et al., 1994). When the compounds are produced *de novo* in response to pathogenic attack, they are classified as phytoalexins. In contrast, phytoanticipins are low molecular weight compounds produced prior to pathogen attack. Phytoalexins are structurally diverse and accumulate to high levels in wounded plant tissues (Ebel, 1986). In the Gramineae, the major cereal crops corn (*Zea mays*), rye (*Secale cereale*) and wheat (*Triticum aestivum*.) produce phytoanticipins called cyclic hydroxamic acids (Barnes and Putnam, 1987; Rice et al., 2005; Wahlroos and Virtanen, 1959). The most abundant hydroxamic acids in cereals crops are 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), produced by maize and wheat, and 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), produced by rye (Frey et al., 1997). These compounds were first reported on rye and associated with resistance to fungal infections in corn and wheat (Barnes and Putnam, 1987). Their main biological activities are to protect cereal crop grasses from many pests such as insects and plant pathogens (Niemeyer, 1988).

DIMBOA and DIBOA are naturally present in intact plants as glucosides, inactive compounds which have an attached glucose molecule. Glucosides are produced in the cytosol of cells (Frey et al., 1997; Massardo et al., 1994; Virtanen and Hietala, 1960). When plant tissue is damaged by any means (i.e. mechanical damage, pathogenic attack or insect attack) β -

glucosidases are released from the vacuole resulting in cleavage of the glucosides and liberation of the biologically active compounds (Tang et al., 1975). Interestingly, these hydroxamic acids have been associated with detoxification of herbicides and iron acquisition by roots (Hamilton, 1964; Tipton and Buell, 1970). DIMBOA and DIBOA are highly reactive and very unstable. Due to their high reactivity, DIMBOA and DIBOA are spontaneously converted into more stable benzoxazolinones: 6-methoxy-2-benzoxazolinone (MBOA) and 2-benzoxazolinone (BOA), respectively (Argandoña and Corcuera, 1985; Hashimoto and Shudo, 1996; Niemeyer, 1988; Richardson and Bacon, 1993). In corn plants, the amount of DIMBOA produced is higher than DIBOA. DIMBOA concentrations are greatest during seedling development with concentration decreasing as the plant matures (Cambier et al., 2000). The peak production of hydroxamic acids in corn occurs during the first week after germination and is affected by different environmental factors, such as long photoperiod, high temperatures and elevated light intensity (Epstein et al., 1986; Thompson et al., 1970). Richardson and Bacon et. al, (1993) concluded that hydroxamic acids produced by corn are stress metabolites due to the high levels of these compounds produced in corn seedlings under water stress conditions.

Chemical detoxification in phytopathogenic fungi

Although plants in general may produce compounds to protect themselves from pathogen or herbivore attack, some plant pathogens have developed specific mechanisms to overcome these chemical defenses. *F. verticillioides* is able to tolerate MBOA and BOA by transforming these corn antimicrobials into the non toxic compounds *N*-(2-hydroxy-4-methoxyphenyl) malonic acid (HMPMA) and *N*-(2-hydroxyphenyl) malonamic acid (HPMA), respectively (Yue et al., 1998). Genetic analysis of *F. verticillioides* showed evidence of two loci involved in this chemical detoxification (Glenn et al., 2002). Data suggested the formation of an intermediate, 2-

aminophenol (Glenn et al., 2003). Comparison between detoxifying and non-detoxifying strains of *F. verticillioides* showed that detoxification of MBOA and BOA is not a major virulence factor (Glenn et al., 2002) but may enhance ecological fitness of this plant pathogen. Detoxification was not needed for endophytic colonization of corn seedlings or development of seedling blight (Glenn et al., 2002).

The chemical degradation of host plant antibiotics by phytopathogenic organisms has been broadly studied (Friebe et al., 1998; Sandrock et al., 1995; Turner, 1961). An antibiotic compound synthesized by *Lycopersicon* and some *Solanum* species, the saponin α -tomatine, can inhibit growth of plant pathogens (Eltayeb and Roddick, 1984). However, the tomato pathogens *Phytophthora infestans* and *Pythium aphanidermatum* are capable of degrading α -tomatine to a non toxic compound known as tomatidine (Sandrock and van Etten, 1998). The oat pathogen, *Gaeumannomyces graminis* var. *avenae* tolerates avenacin saponin, another type of phytoanticipin, by production of a detoxifying enzyme (Crombie et al., 1986). Another example is the phytoalexin resveratrol, which is produced in grapevines and has been associated with antifungal activity against the phytopathogenic organisms *Plasmopara viticola*, *Rhizopus stolonifer*, and *Botrytis cinerea* (Adrian et al., 1997; Sarig et al., 1996). The isoflavonoid phytoalexins maackiain and medicarpin are produced by pea (*Pisum sativum*) and chickpea (*Cicer arietinum*), however *Nectria haematococca* mating-population VI (MPVI) is able to detoxify these phytoalexins (Lucy et al., 1988; Sarig et al., 1996).

F. verticillioides-corn interactions are of particular interest in the Southeastern United States because the environmental conditions in this part of the country are favorable for the fungus to cause deleterious effects in corn plants (Munkvold, 2003). A better understanding of plant pathogen-interactions, i.e. population dynamics, physiological activity, and gene

expression, will help to find strategies to manage the impact of *F. verticillioides* on corn (Munkvold, 2003). Genetic analyses led to the identification of two loci involved in the metabolic detoxification of the corn antimicrobials BOA (Glenn et al., 2002). However the previous analyses did not assess the possibility of other mechanisms of tolerance in this fungus, including non-degradative tolerance.

Non-metabolic tolerance of antimicrobials

In the last 10 years, the study of ATP-binding cassettes (ABC) transporters in plant pathogens has increased. ABC transporters are transmembrane proteins involved in non-degradative tolerance to fungicides and antimicrobials. There is strong evidence suggesting that ABC transporters are involved in multidrug resistance to a broad number of commercial fungicides in plant pathogens (Schoonbeek et al., 2001; Stergiopoulos et al., 2003; Vermeulen et al., 2001; Zwiers et al., 2003; Zwiers and De Waard, 2000). The resistance to fungicides is an agricultural problem that has been escalating and it is imperative to develop new classes of fungicides and approaches to manage plant diseases (Gardner et al., 1999). The mechanism of resistance is a main concern due to the implications in crop production. Better understanding of this resistance mechanism could result in the development of efflux pump inhibitors. ABC transporters in the ubiquitous ascomycete *F. verticillioides* have not been fully evaluated. This project is concerned with of an ABC transporter gene of this fungus that might be involved in host-pathogen interactions.

ATP Binding Cassette Transporters

The ABC transporters comprise the largest known protein superfamily in prokaryotes and eukaryotes. ABC transporter proteins are widely distributed from bacteria to man (Holland and Blight, 1999) and are located in the plasma membrane of cells and in different cell compartment

membranes such as vacuoles, mitochondria, endoplasmic reticulum, and peroxisomes (Stergiopoulos et al., 2002). The ABC proteins require energy to translocate specific substrates across membranes. The main source of energy is ATP hydrolysis. This superfamily is divided into 3 categories: uptake permeases comprised of 22 different families; prokaryotic efflux, with 21 families; and eukaryotic efflux, with 11 families. The members of the latter family are classified, based on their functionality, into two types of pumps: pleiotropic-glycoprotein (multidrug resistance P-gp) and multidrug resistance protein (MDR) (Borges-Walmsley et al., 2003; Borges-Walmsley and Walmsley, 2001).

The first report of an ABC transporter sequence was published in the early 1980's (Higgins et al., 1992). Since then, many studies on these plasma membrane proteins have associated them with a broad range of physiological functions. A main function of ABC transporters in plant pathogens is to confer resistance to a wide range of chemical control compounds (Del Sorbo et al., 2000). For example, plant pathogen transporters may have roles for fungal cell protection by efflux of exogenous defense compounds produced by plants (i.e. hydroxamic acids in corn) and may enhance virulence by secretion of endogenous pathogenic factors such as toxins (De Waard, 1997).

In the last decade, it has been reported the association between ABC transporter proteins with resistance to therapeutic drugs, especially cancer treatments. In humans, mutations in these ABC proteins are associated with many genetic diseases such as cystic fibrosis, Tangier disease, Stargardt disease, Wegener's granulomatosis and Dubin-Johnson syndrome (Higgins and Linton, 2004). The ABC transporters not only function as efflux pumps but also play an important role in some other specific functions. In the yeast fungi *S. cerevisiae* and *S. pombe*, ABC proteins are mating factor transporters (Christensen et al., 1997; McGrath and Varshavsky, 1989). ABC

transporters can play an important role in the maintenance of the membrane integrity by extruding hydrophobic compounds capable of disturbing the membrane integrity (Mahe et al., 1996).

The uptake ABC proteins pumps are confined to prokaryotes, while the efflux pumps are found in both prokaryotes and eukaryotes (Higgins and Linton, 2004). There is some evidence suggesting the existence of bidirectional transport in prokaryotes but this is more an exception rather than the rule (Higgins and Linton, 2004). Organisms having ABC transporters functioning as importers need an additional extra-cellular protein called periplasmic binding protein (Tomii and Kanehisa, 1998). Most of these ABC transporters are involved in primary membrane translocation but they can work as channel regulators, receptors, proteases and sensors (Bauer et al., 1999). The main feature of ABC transporters is the broad and unrelated range of substrates that can be transported by them against a concentration gradient, from small molecules such as ions, vitamins, mycotoxins, and heavy metals to more complex molecules such as carbohydrates, steroids, phospholipids, glucocorticoids, antibiotics, peptides and whole proteins (Theodoulou, 2000).

Classification of ABC transporters

Transporter proteins are classified using a five-criteria classification system which is analogous to the classification system for enzymes (Transporter classification database <http://www.tcdb.org/tcdb/>). Every transporter classification number has five components as follows: 3.A.1.205.1. The first number corresponds to the transporter class which is divided into six categories and numbered as follows: (1) channels and pores, (2) electrochemical potential-driven transporters, (3) primary active transporters, e.g. ABC transporters, (4) group

translocators, (5) transport electron carriers, (8) accessory factors involved in transport, and (9) incompletely characterized transporters.

The second classification component is a letter and corresponds to the subclass. ABC transporters are subclass A because they can hydrolyze the diphosphate bond of any nucleoside triphosphate, including ATP. For a primary active transporter, this refers to the energy source. The third component is represented by a number corresponding to the superfamily. The ABC transporters are in the ATP-binding cassette superfamily (3.A.1). The family is represented by a number, such as 3.A.1.205, which is the pleiotropic drug resistance (PDR) family. The last component of the classification number corresponds to the substrate or range of substrates. For example, 3.A.1.205.1 refers to ABC transporters such as *AtrB* from *Aspergillus nidulans*, which confers resistance to a range of fungicides (Andrade et al., 2000).

Structure of ABC transporters

In order to understand how these efflux pumps actually transport toxic compounds out of the cell, it is imperative to perform studies to elucidate the structural conformation of these proteins. Most of what is known about ABC protein structure has come from crystallography studies showing that their molecular architecture is normally composed of four core domains which together comprise the minimal functional unit for ABC proteins (Higgins and Linton, 2004). This unit contains two transmembrane domains (TMD) with multiple predicted transmembrane segments (TMS) in each TMD, and two nucleotide binding domains (NBD) (Higgins, 1992). These four domains can be encoded either separately as individual polypeptides or as multidomain proteins (Higgins et al., 1986). Transporters in the pleiotropic drug resistance family are multidomain proteins typically with an structure [NBD-TMD]₂ (Taglicht and Michaelis, 1998).

The amino acid sequences of nucleotide-binding domains in all ABC transporters are highly conserved and comprised of three different motifs: the Walker A and Walker B motifs which are found in all nucleotide binding proteins, and the ABC signature motif which is found only in ABC transporters (Higgins and Linton, 2001). The NBDs are located in the cell membranes facing the cytoplasmic space and it is unlikely they are exposed extracellularly (Blott et al., 1999). Some researchers have proposed that NBDs may not contribute to transport directly, but are most likely involved in conformational changes in the transmembrane segments during the hydrolysis of ATP (Borges-Walmsley et al., 2003).

The transmembrane domains typically contain six transmembrane segments but a variation of this formula can be found in some organisms (Chang and Roth, 2001; Locher et al., 2002). Some scientists believe that this variation in number can be due to the role of the segments in transport. The transmembrane segments in ABC transporters are more closely grouped than those in other ATP-dependent transporters which are more spaced (Higgins and Linton, 2004). The predicted number of membrane-spanning segments may not be crucial *per se* in the transportation of compounds but may play a role in auxiliary functions such as insertion or regulation (Higgins and Linton, 2001; Higgins et al., 1982).

Mechanism of transport

Although the studies of ABC transporters in different organisms have increased in the last decade, there are some questions that remain unanswered about the mechanism involved in the transport of different compounds by ABC proteins. Mechanisms of transport are similar in both prokaryotes and eukaryotes. Some recent evidence suggests that ATP binding provides the power for transport rather than ATP hydrolysis (Higgins and Linton, 2004).

One of the models, called ATP-switch model, involves a cyclic-bidirectional communication between the NBDs and TMDs (Higgins and Linton, 2004). The conformational changes initiated by the presence of the hydrophobic drug compound in the cell membrane, generates the formation of a closed dimer between the two NBDs upon the binding two ATP molecules. One of the questions is whether both nucleotide-binding domains are functionally equivalent. The line of evidence suggests that the ATP binding step is the one that provides energy to actively transport compounds through the membranes (Mitchell, 1957). Biochemical evidence suggests that both NBDs can hydrolyze ATP, but a cooperative interaction between the two halves of the molecule is needed for transport (Loo and Clarke, 1994). The dissociation of the dimer is facilitated by ATP hydrolysis and eventually will release ADP and organic phosphate (Borges-Walmsley et al., 2003).

ABC transporters in plant pathogenic fungi

ABC transporters play an important role in pathogenicity in plant pathogenic fungi since they comprise a protection system for pathogens (Del Sorbo et al., 2000). In *Botrytis cinerea* (teleomorph *Botryotinia fuckleliana*), the gray mold pathogen, ABC transporters have been classified and functionally characterized (Schoonbeek et al., 2001; Schoonbeek et al., 2003; Schoonbeek et al., 2001; Vermeulen et al., 2001). A PDR-type ABC transporter, *BcatrB*, was capable of transporting phytoalexins. Gene replacement mutants showed increased sensitivity to the grapevine phytoalexin resveratrol and reduced virulence in plant assays (Schoonbeek et al., 2001). This evidence suggests that *BcatrB* might be a pathogenicity factor in *B. cinerea*.

Dry rot disease in potatoes is caused by the necrotrophic fungus *Gibberella pulicaris*. Recently, an ABC transporter from *G. pulicaris*, *Gpabc1*, was characterized (Fleissner et al., 2002). *Gpabc1*, a member of the pleiotropic drug resistance family, is rapidly induced by the

potato phytoalexins rishitin and lubimin. In pathogenicity tests, *Gpabc1* mutants were not able to colonize potato tissues and were sensitive to both potato phytoalexins (Fleissner et al., 2002). *Gpabc1* is classified as pathogenicity factor due to its essential role in *G. pulicaris* pathogenicity.

An ABC transporter from the rice blast disease fungus, *Magnaporthe grisea* (*ABC1*) has been characterized and classified as pathogenicity factor. *ABC1* mutants did not show increased sensitivity to the rice phytoalexin sakuranetin, but they were not capable of forming appresoria and infecting the plant (Urban et al., 1999). Although it is clear the important role of *ABC1* in pathogenicity, further analysis need to be performed in order to establish how this ABC transporter protein is involved in *M. grisea* pathogenicity.

In this work, I report the characterization of an ABC transporter from *F. verticillioides*, *FvABC1*, which might play a role in fungal virulence.

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CHAPTER 2

FvABC1, AN ABC TRANSPORTER FROM *FUSARIUM VERTICILLIOIDES*

INTRODUCTION

Fusarium verticillioides (synonym, *Fusarium moniliforme*; teleomorph, *Gibberella moniliformis*) is a pathogenic ascomycete with a broad host range and commonly associated with corn around the world (Leslie, 1996). *F. verticillioides* can produce asymptomatic infections but it has also been reported to cause severe rotting in different plant tissues, including in seedling stages (Kommendahl and Windels, 1981; Oren et al., 2003). This species is the main cause of *Fusarium* ear rot, a disease characterized by a white-pink mycelium causing kernel rot (Miller, 1994), which is favored by warm and dry environmental conditions with a temperature optima about 30°C (Munkvold, 2003; Reid et al., 1999). Although *Fusarium* ear rot can be a problem for the corn industry, because it can reduce kernel quality, it normally does not drastically affect corn yield. The main concern with *F. verticillioides* infection is the production of mycotoxins, especially fumonisins (Gelderblom et al., 1991). The combination of high levels of fumonisins and symptomless infections in corn plants is a serious threat to animal and human health (Munkvold and Desjardins, 1997; Nelson et al., 1993).

Fumonisin are a group of closely related secondary metabolites produced by *F. verticillioides* and related species, which have been detected in corn and corn-based products (Shepard et al., 1996). Fumonisin B1 is the most significant and abundant of this group, with fumonisin B2 and B3 also occurring but less abundantly (Thiel et al., 1992). Fumonisin B1 has been associated with many animal diseases. In horses fumonisins cause a fatal disease called

leukoencephalomalacia, which is characterized by liquefactive necrosis in the brain cortex (Ross et al., 1991). In swine, fumonisin B1 causes pulmonary edema, an abnormal presence of fluids in the lungs (Marasas, 2001). Fumonisin B1 has been associated with esophageal cancer in humans (Sydenham et al., 1991), and with high prevalence of neural tube defects among the Mexican-American population in the USA and other human populations in Guatemala, South Africa and China (Marasas et al., 2004; Missmer et al., 2006).

As with many fungi, *F. verticillioides* is likely to be challenged by a variety of fungistatic and fungitoxic compounds. Plant species of the Gramineae are known to produce low molecular weight compounds that have antibiotic activity against insects and microbial pathogens (Niemeyer, 1988; Rhoades, 1979). These low molecular weight compounds can be classified as either phytoalexins, which are produced *de novo* in response to a pathogen attack, or phytoanticipins, which are produced constitutively as part of normal plant development (VanEtten et al., 1994). Corn plants produce the phytoanticipins DIMBOA (2,4 dihydroxy-7-methoxy-1,4-benzoxazin-3-one) and DIBOA (2,4-dihydroxy-1,4-benzoxazin-3-one) that may play an important role in plant protection (Argandoña and Corcuera, 1985; Richardson and Bacon, 1993). These hydroxamic acids are produced as inactive glucosides, but in response to either pathogen attack or cell damage, glucosidases are released from vacuoles and cleave DIMBOA-Glc and DIBOA-Glc to produce the bioactive aglycone compounds (Hashimoto and Shudo, 1996; Massardo et al., 1994). During early stages of corn plant development the concentration of cyclic hydroxamic acids is high, suggesting that these compounds play an active role in seedling protection. DIMBOA and DIBOA are highly reactive and unstable compounds which spontaneously degrade to MBOA (6-methoxy-2-benzoxazolinone) and BOA (2-benzoxazolinone) (Hashimoto and Shudo, 1996). Both MBOA and BOA have been reported to

retain antimicrobial activity (Glenn et al., 2001; Richardson and Bacon, 1995). *F. verticillioides* tolerates MBOA and BOA by metabolizing these compounds into non-toxic compounds. In *F. verticillioides* and some other related species, MBOA is degraded into HMPMA (*N*-(2-hydroxy-4-methoxyphenyl) malonamic acid) and HPMA (*N*-2(2-hydroxyphenyl) malonamic acid) (Glenn et al., 2001; Yue et al., 1998). This detoxification pathway involves two different loci and the formation of an intermediate called 2-aminophenol (Glenn et al., 2003). Although this degradation may play a role in the ecological fitness of this fungal species, it is not a major virulence factor in *F. verticillioides* (Glenn et al., 2002).

In contrast to degradative tolerance of phytoalexins and phytoanticipins, another mechanism for tolerance involves the use of ATP to remove the toxic compounds from the cytosol and plasma membranes. The ATP-binding cassette (ABC) transporters may be candidates for non-metabolic tolerance of cyclic hydroxamic acids. In general, ABC transporters are one of the largest protein families which have been found from bacteria to man (Dassa, 2003). Certainly, within the last decade ABC transporters have attracted the attention of many researchers from medical and agricultural areas because of their role in conferring chemical resistance. A better understanding of how these proteins work could lead to the design of inhibitors of these efflux pumps (Borges-Walmsley et al., 2003). ABC proteins hydrolyze ATP, the source of energy, to transport toxic compounds out of the cells (Borges-Walmsley and Walsmley, 2001; Dassa, 2003). The structure of ABC transporters consists of two similar halves, each half consisting of a nucleotide domain (NBD) and transmembrane domain (TMD) with typically six transmembrane segments (Holland and Blight, 1999). Full length transporters are comprised of two NBD and two TMD. They are classified into two families: pleiotropic drug resistance (PDR) family with a structure [NBD-TMD]₂ and the multidrug resistance protein

(MRP) with a structure of [TMD-NBD]₂. ABC transporters have a broad range of substrates, yet these substrates are generally highly hydrophobic and toxic. The biological functions of ABC transporters range from general efflux pumps to pathogenicity factors (Stergiopoulos et al., 2002).

In fungi, so far, four characterized ABC transporters have a demonstrated role in pathogenicity and/or fitness (Fleissner et al., 2002; Schoonbeck et al., 2002; Stergiopoulos et al., 2003; Urban et al., 1999). The first ABC transporter in pathogenic fungi shown to be related to pathogenicity was *ABC1*, an efflux pump in the rice blast fungus *Magnaporthe grisea*. Disruption of the *ABC1* promoter by insertional mutagenesis led to inhibited growth and eventually death of mutants (Urban et al., 1999). Transcriptional studies in *M. grisea* showed that *ABC1* was expressed in response to xenobiotic compounds and sakuranetin phytoalexins produced by rice (Urban et al., 1999). *ABC1* null were able to penetrate de plant cells but die shortly in the initial infection. The *MgAtr4* gene, an ABC transporter from the causal agent of septoria blotch on wheat, *Mycosphaerella graminicola*, has been characterized and disruption of *MgAtr4* resulted in decreased efficiency in colonization (Stergiopoulos et al., 2003). *MgAtr4* transcription was enhanced in the presence of a wheat phytoalexin, resorcinol, suggesting a role in protection of *M. graminicola in planta* (Zwiers et al., 2003a). The *BcatrB* gene, encoding an ABC transporter in *Botrytis cinerea*, had enhanced transcription in the presence of *Pseudomonas* antibiotics, suggesting an important role in the ecological fitness of *B. cinerea* (Schoonbeck et al., 2003). An ABC transporter from *Gibberella pulicaris*, *Gpabc1*, was characterized and related to pathogenicity in potato tubers (Fleissner et al., 2002). Gene deletion mutants showed increased sensitivity to the phytoalexin rishitin compared to the wild type. *Gpabc1* mutant strains were not able to colonize potato tubers. *PMR5*, an ABC transporter from *Penicillium*

digitatum has been reported to play a role in fungal protection, since null mutants showed decreased, tolerance to natural and chemical compounds (Nakaune et al., 2002).

I report the cloning and analysis of *FvABC1*, an ABC transporter in the endophytic fungus *F. verticillioides* that may have a role in efflux of benzoxazolinones, specifically, BOA. The putative protein encoded by *FvABC1* showed the [NBD-TMD]₂ topology typical of PDR type ABC transporters. Members of the PDR family such as *ABC1*, *Gpabc1*, *MgAtr4*, and *BcatrB* showed significant identity with *FvABC1* (38%, 37%, 37%, and 69% respectively); BLAST analysis of the *F. verticillioides* genome identified 14 additional putative ABC transporters 13 of which appear to be in the PDR family. Phylogenetic assessments are presented. Collectively, these data will facilitate future studies on the role of ABC transporters in ecological and host-pathogen interactions.

MATERIALS AND METHODS

cDNA subtractive library

In order to identify a *F. verticillioides* genes which are up-regulated in the presence of BOA, a suppression subtractive hybridization (SSH) technique was previously performed using a BD PCR-Select™ cDNA subtraction kit (Clontech, Palo Alto, CA). This technique compared two mRNA populations, one derived from *F. verticillioides* cultures exposed to BOA (Sigma-Aldrich, St Louis, MO; 1mg/ml final concentration) and the other from cultures exposed to 1% ethanol only (the stock solution of BOA added to the cultures was dissolved in ethanol). *F. verticillioides* cultures were harvested after 2, 4, and 6 hours incubation and the mRNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). The mRNA samples were pooled for each condition. The resulting SSH cDNA library was cloned into the pCR® II-TOPO® cloning

vector (Invitrogen) for sequencing. Putative protein coding sequences were identified by BLASTX searches based on the resulting expressed sequence tag (ESTs).

Cosmid macroarray hybridization

An aliquot of a *F. verticillioides* genomic cosmid library was obtained from the Fungal Genetics Stock Center (<http://www.fgsc.net/>). This library was made by using *F. verticillioides* strain M3126 and the pCosP1-9 vector. The vector contained ampicillin and hygromycin resistance gene cassettes. The library was plated onto 12-250 ml LB agar plates amended with ampicillin (50 µg/ml final concentration), and the resulting colonies were picked using robotics and arrayed into 50 96 well microtiter plates containing 300 µl of LB plus ampicillin (50 µg/ml) and 10% glycerol per well. Clones were then stamped on a nitrocellulose membrane to create a macroarray used for hybridization to identify cosmids containing *FvABC1*.

ESTs having homology to fungal ABC transporters were identified in the subtracted cDNA library. Probes were designed from ESTs 1G7 and 1G8. Primers for 1G7 (1G7for, 5'-CTATGCTACCGTCCGTGAG-3' and 1G7rev, 5'-CTTACCACCCTTAGCGAG-3') and 1G8 (1G8for, 5'-CAGCACGGTACTGGCTACTTT-3' and 1G8 rev, 5'-AGCGACAAAATGAA GTTGAAGAC-3') were used in combination with a DIG PCR labeling kit (Roche, Indianapolis, IN) to generate two probes of 397 bp and 433 bp, respectively to screen the *F. verticillioides* genomic library for cosmid clones containing a full length ABC transporter gene. PCR amplification for both 1G7 and 1G8 was performed as follows: initial denaturation at 95°C for 2 min, then 30 cycles of 95°C for 30 s, 57°C for 30 s, 72°C for 40 s and finally 72°C for 7 min. Total volume of 5µl of each PCR products was separated in a 1.2% agarose gel and visualized using ethidium bromide solution (0.5 µg/ml) to verify probe length. Probes (120 µl total volume) were individually diluted with 15 ml of DIG easy hyb solution (Roche) and incubated at 95°C

for 5 minutes. Macroarray membranes were placed into incubation tubes and incubated with diluted probes overnight at 37°C in a rotary incubator. The macroarray hybridization detection was performed using a chemiluminescence detection kit (Roche). The membrane was washed twice with 2X SSC buffer (Amresco, Solon, OH) for 15 minutes at 37°C followed by two washes of 0.5X SSC buffer for 15 minutes at 65°C. Membranes were then washed for 10 minutes using 1X washing buffer (Roche) and incubated at room temperature with blocking solution (Roche) for 30 minutes on a rotary shaker followed by an antibody solution (Roche) for 30 minutes. The membrane was washed twice in washing buffer for 10 minutes followed by a 1X detection solution for 15 minutes and finally a CDP star solution (Roche) was added to the membrane. An Alpha Innotech FC8000 (San Leandro, CA), was used to detect the membrane hybridization (50 minute exposure).

DNA sequencing and genome analysis

DNA manipulations and other basic molecular biology techniques were performed according to standard procedures (Sambrook et al., 1989). Primer walking and EZ::TN <Kan-2> Insertion Kit (Epicentre, Madison, WI) were used to sequence the 12A2 cosmid clone, which was identified from the macroarray hybridization. Two DNA sequencing facilities were used. First was the Nucleic Acid Facility, Integrated Biomolecular Resources, USDA, ERRC, Wyndmoor, PA. This sequencing facility used an Applied Biosystems 3730 Genomic Analyzer (Applied Biosystems, Foster city, CA). The second facility was Southeast Poultry Research Laboratory Sequencing Unit, USDA, Athens, GA which used an ABI 3700 DNA analyzer (Biorad, Hercules, CA) for sequencing. DNA sequences were analyzed using Sequencher software version 4.2 (Genes Codes Corporation, Ann Arbor, MI). Exon and intron prediction was performed using FGENESH with *Fusarium graminearum* as the comparative organism

(<http://sun1.softberry.com>). Genomic sequence was also compared to ESTs from the subtracted cDNA library and from the *F. verticillioides* gene index EST sequencing project (<http://www.tigr.org/tdb/tgi/cw/cwgi2/>). A TBLASTn search was conducted to obtain genomic sequences from the *F. verticillioides* database at the Broad Institute website (<http://www.broad.mit.edu/>) using a cutoff E-value 1×10^{-3} cutoff. Only loci with complete amino acid sequence were used for further analysis (see table 2.1 for more details). An ABC transporter from *F. verticillioides* (FVABC GenBank accession number CAA60778) has been already annotated (Lopez_Errasquin, 2005) and was designated *FvABC7* in this analysis. A BLASTp search (<http://www.ncbi.nlm.nih.gov/BLAST/>) was performed to identify ABC transporters from other species having significant similarity to *FvABC1*. In addition to this data, a BLASTp search was performed using *Fusarium graminearum* and *Magnaporthe grisea* databases (Broad Institute) in order to obtain protein sequences with high similarity to FvABC1 protein. Protein sequences with an E-value equal to 0.0 were selected for further comparative analyses. ClustalW version 1.8 was used to create a multiple protein sequence alignment (<http://www.ebi.ac.uk/clustalw>). The neighbor-joining phylogenetic analysis was conducted using MEGA version 3.1 (Kumar et al., 2004) and PAUP version 3.1.1 (Swofford, 1993). The Transporter Classification Database (TCDB, <http://www.tcdb.org/>) was used to classify the *FvABC1* gene and to predict the transmembrane segments using the Hidden Markov Model for Topology Prediction (HMMTOP; (Tusnady and Simon, 1994). Hydropathy plots were created using TopPred (Claros and von Heijne, 1994; von Heijne, 1992) and the TCDB analysis tools.

***FvABC1* Southern blot of various *Fusarium* species**

The following *Fusarium* species were used in the *FvABC1* genomic Southern analysis: *Fusarium verticillioides* MRC826, Medical Research Council, Tygerberg, South Africa; *F.*

verticillioides, AEG3-1-9 lab strain from genetic crosses; *F. verticillioides* NRRL 25059, Honduras, banana; *F. beomiforme* NRRL 13606, Australia, soil; *F. brevicatenulatum* NRRL 25446, Madagascar, *Striga asiatica*; *F. cerealis* (= *Fusarium cookwellense*) RRC 449, Canada, maize; *F. circinatum*, NRRL 25333, South Africa, *Pinus patula*; *F. circinatum* NRRL 26431, Japan, *Pinus sp*; *F. graminearum*, NRRL 5885, Ohio, maize; *F. graminearum*, NRRL 26916, South Africa, maize; *F. oxysporum* f. sp. *lycopersici*, NRRL 22544, unknown, tomato; *F. oxysporum* f. sp. *chrysanthemic* NRRL 22539, Florida, *chrysanthemum sp*; *F. oxysporum* NRRL 13307, Florida, tomato; *F. proliferatum*, JFL D02877, Missouri, sorghum; *F. pseudoanthophilum*, NRRL 25206, Zimbabwe, maize; *F. pseudoanthophilum* NRRL 25209 Zimbabwe, maize; *F. thapsinum* JFL F00921, Kansas, sorghum; *F. subglutinans* JFL E01583, China, maize; *F. dlamirii* NRRL 13164, South Africa, maize field soil, *Fusarium sp.*, NRRL 25221, Zimbabwe, maize.

All the strains were grown individually in 50 ml potato dextrose broth and incubated at room temperature for 4 days using a rotary shaker at 200 rpm. The cultures were collected and ground into fine powder in liquid nitrogen and stored at -80° C until ready for DNA extraction, which was performed by using a DNeasy Plant Mini kit (QIAGEN Sciences, Maryland USA). The amount of DNA was measured using a spectrophotometer and a total of 1 µg of genomic DNA from each sample was digested overnight using EcoRV (New England Biolab, Beverly, MA) at 37°C. The enzyme was inactivated at 65°C for 15 minutes and each sample was loaded onto a 1.0% agarose gel and ran for 3 hours at 85 volts. The gel was placed in a 0.25 N HCl solution for 10 minutes and then washed with denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 10 minutes. The gel was then transferred to a Hybond-N+ nylon membrane (Amersham Biosciences, England) overnight. The nylon membrane was washed in a 2X SSC solution for 5

minutes then was baked at 98°F in a conventional oven. Hybridization and detection were performed as described earlier for the macroarrays. A DIG labeled probe was created using primers ABC-P4 (5'-CAACAACAAGTTCGCTCTCC) and ABC-P8 (5'-CGGGTAGAATCACTTGGGAA-3') that amplified a 330 bp amplicon in the third exon of the *FvABC1* gene in a TMD region that is not highly conserved. PCR conditions for this reaction were as follows: 95°C for 2 min, then 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 40 s and finally 72°C for 7 min.

***FvABC1* gene deletion constructs**

A traditional approach developed by Butchko et al.(2003) and the split marker technique (Catlett et al., 2003) were attempted. The strategy by Butchko et al (2003) consisted on amplifying two regions of the *FvABC1* coding sequence. The 3' region flanking *FvABC1* was amplified with primers P-3 (5'-TGCTCTAGATTCCACGAGCTACTCCAGAA-3)' and P-4 (5'-GACTCTAGATTCCACGAGCTACTCCAGAA-3') which had EcoRI and XbaI sites (indicated as bold nucleotides) added to the 5' end of each primer, respectively. The 3' flank was cloned into pBluescript II KS+ (Stratagene, LaJolla, CA), to create pBs-3' vector. The 5' region flanking *FvABC1* was amplified with P1 (5'-CGGGGTACCCTCAAGCGGTCTTTTCATCA-3') and P2 (5'-GACGGTACCCTCAAGCGGTCTTTTCATCA-3'), which had KpnI and EcoRI sites added to their 5' ends, respectively. The PCR product of the 5' flank and pBs-3' were both double-digested with KpnI and EcoRI and then fused to form pBs-5'3'. Finally, the hygromycin-resistance cassette from pCB1003 was amplified with M13For (5'-TGTA AACGACGGCCAGT-3') and M13Rev (5'-AGCGGATAACAATTTACACAGGA-3') and then digested with EcoRI. The cassette was then ligated into EcoRI digested pBs-5'3'. PCR conditions for 5' and 3' fragments and for hygromycin cassette were as follows: 95°C for 5

min, 35 cycles of 95°C for 15s, 56°C for 15 s, 72°C for 1 min, and a final step of 72°C for 5 min. This final knock-out construct (pBs-5'Hyg-3') was sequenced to verify the position of the hygromycin cassette.

Split marker approach, which consists of amplifying two regions flanking the *FvABCI* gene. These flanks are each fused via PCR to overlapping segments of the hygromycin resistance cassette. The flanking regions were upstream and downstream of the start and stop codons of the *FvABCI* sequence, respectively. The upstream flanking region (5' fragment) was amplified using primers F1 and F2, and for the downstream region (3' fragment) primers F3 and F4 were used. See table 2.1 for the list of primers and their sequences. Similar conditions for 5' and 3' fragment amplifications were used: 95°C for 5 min, then 35 cycles 95°C for 15s, 55°C for 15 s, 72°C for 1 min, and a final step 72°C for 10 min. The first fragment from the hygromycin cassette (HY fragment) and the second fragment (YG fragment) were amplified using the primer sets M13Rv2/HygRev and M13Fv2/HygFor, respectively using the hygromycin cassette from pCB1003 (Carroll et al., 1994) as template. PCR conditions for HY and YG fragments were: 95°C for 5 min, then 35 cycles 95°C for 15 s, 58°C 15 s, 72°C for 2 min, and a final step 72°C for 10 min. In individual reaction, 5' and HY fragments, and 3' and YG were fused. Primers F1 and HygRev were used for 5'-HY fusion, and primers HygFor and F4 were used for HY-3' fusion. Both 5'-HY and HY-3' fusions were loaded into a 1% agarose gel and visualized by ethidium bromide solution (0.5 µg/ul) and purified using QIAquick gel extraction kit (QIAGEN, Sciences; Maryland, USA). Fragments 5'-HY and HY-3' were used for *F. verticillioides* transformation.

Protoplast formation and transformation of *F. verticillioides*

Wild-type *F. verticillioides* MRC826 was inoculated in two 125 ml baffled flasks, each containing 50 ml potato dextrose broth (Becton, Dickinson and Company, Sparks, MD). The flasks were incubated at room temperature on an orbital shaker at 200 rpm for 3 days in the dark. Spores were harvested and filtered using sterile cheesecloth and collected in a 50 ml centrifuge tube (Becton, Dickinson and Company). Conidia were centrifuged at 3000x g and washed in water. A total amount of 1×10^9 spores were inoculated in 50 ml potato dextrose broth and incubated at room temperature on a rotary shaker for 12 hours at 120 rpm. The germinating spores were transferred to a 50 ml conical tube and centrifuged at 1500x g for 15 min. The conidia were suspended in a lytic mixture which consisted of SCS solution (1.0 M Sorbitol, 50mM sodium citrate), 40 mg β -glucuronidase (Sigma-Aldrich, St Louis, MO) and 260mg glucanex (Sigma-Aldrich). After 6 hours of incubation at 30° C and 120 rpm in an incubator shaker, 20 ml chilled ST buffer (0.6 M Sorbitol, 100mM Tris-HCl pH 7.4) was carefully added to the tube so that a clear interface was maintained between solutions. After centrifugation at 1950x g for 20 minutes at room temperature, protoplasts were carefully recovered from the interface and transferred to a 50 ml centrifuge tube. The protoplasts were suspended carefully in 40ml of pre-chilled STC buffer (1.2 M sorbitol, 50 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 10 mM Tris-HCl pH 8.0) and collected by centrifugation at 1500 x g for 15 minutes. Protoplasts were frozen at -80°C in 100 μl aliquots after adding 500 μl of freezing solution (8:2:0.1 ratio of STC solution, SPTC [40% PEG 8000, 0.8M sorbitol, 50 mM Tris-HCl pH 8.0, 50 mM CaCl_2], and DMSO, respectively). For transformations 100 μl of frozen protoplasts were thawed on ice, then 10 μl were added to the protoplasts, along with 100 μl of STC buffer and 50 μl of 30% PEG solution (30% PEG 8000, 0.8 M sorbitol, 50 mM Tris-HCl pH 8.0, 50 mM CaCl_2) and incubated at room

temperature for 20 minutes, followed by addition of 2 ml of 30% PEG solution and continued incubation at room temperature for 5 minutes. Finally, 4 ml of STC solution were added and gently mixed followed by combining 600 μ l of the protoplast solution with 4.5 ml molten overlay medium. The overlay medium consisted of 2 g yeast extract, 2 g casein enzymatic hydrolysate, 20 g agarose per liter, mixed an equal volume of 1.6 M sucrose. The protoplast mixture was poured over 20 ml of regeneration medium. The regeneration medium consisted of the same components of the overlay medium but instead of using 20 g/L of agarose, 32 g/L agar was used. Plates were incubated at 27°C overnight and overlaid with 10 ml of 1% amended with hygromycin (525 μ g/ml). The final concentration of hygromycin in each 100 mm petri palates was 150 μ g /ml. After 4 days, transformants were transferred to potato dextrose agar amended with hygromycin (150 μ g/ml), and incubated at 27°C for 6 days. Transformation using constructs from the split marker technique was performed as described above but instead of using pBs-5'Hyg3' vector, 5 μ l of both 5'-HY and YG-3' were mixed with 100 μ l of protoplasts. In both transformation protocols, individual transformants were single spore isolated on PDA plates. A small sample of each transformants was diluted in 1ml of autoclaved water, and from this suspension sample 50 μ l was distributed on a PDA plate using a glass spreader. Inoculated PDA plates were incubated at 27°C for 3 days and transferred into PDA plates amended with BOA (0.5 mg/ml) and grown for 5 days. The final concentration of BOA was used in order to make the BOA medium less stringent.

Transformants from the pBs-5'Hyg-3' transformation were used to verify homologous integration. Primers WFor (5'-TCTCTGGACATCTCTCGCAA-3') and P-1 (5'-AGGAATTAGCCCGAGACTTG-3') were used, with WFor annealing to a region within the *FvABC1* coding and P-1 annealing to the region downstream of the 3' flank. The set of primers P-2 (5'-

AACTCAACTGGACCAACAAACGGC-5') and WTRev (5'-ACGACGTCAATCATGTGCTC-3') were used, with WTRev annealing to a region within the *FvABC1* coding sequence and P-2 annealing to the region upstream of the 5' flank, then only the native allele should amplify.

To confirm homologous integration in the split marker technique, three set of primers were used. Primers M13Fv2 and M13Rv2 were used to confirm the hygromycin cassette insertion (see table 2.1). To confirm homologous integration, two sets of primers were used. Primer 5'-outer (5'-AACTCAACTGGACCAACAAACGGC-3'), and WTRev (see sequence above) were used. The primer set WTRFor (see sequence above) and 3'outer (5'-ATTCAGGGTATGCCAATGGAGGGT-3') were used to verify homologous integration.

RESULTS

Characterization of *FvABC1*

In order to identify clones with an ABC transporter from a genomic cosmid library, two DIG labeled probes, 1G7 (397 bp) and 1G8 (433 bp), were designed based on ESTs from the previously constructed subtractive cDNA library. The screening process of the *F. verticillioides* genomic cosmid library macroarray followed by subsequent PCR verification using the same primers for probes 1G7 and 1G8 resulted in identification of cosmid 12A2. The two probes hybridized two different regions in the ABC transporter sequence (Fig. 2.1 A). The sequence of a 12473-bp contig from 12A2 cosmid revealed an ORF for a full-length ABC transporter of 4323 bp with three predicted exons (781, 891, and 2535 bp respectively), and interrupted by two introns of 49 and 53 bp respectively. This ORF, designated *FvABC1*, encodes a putative protein of 1406 amino acids, with a calculated a weight of 156.144 kDa and isoelectric point of 6.14 as predicted by CLC protein workbench software v 2.5.1. The predicted structure of FvABC1p is typical of ABC transporter proteins in the pleiotropic drug resistance family, with two

homologous halves in a [NBD-TMD]₂ orientation with transmembrane segments predicted by HMMTop software (Tusnady and Simon, 2001). The hydropathy analysis revealed that each half of the predicted protein consists of a N-terminal hydrophilic domain followed by a hydrophobic domain (see Fig 2.1 B for indicated domains). Each hydrophobic domain was predicted to have five (residues 468 to 488, 512 to 537, 551 to 576, 584 to 602, and 690 to 709) and six (residues 1115 to 1133, 1141 to 1159, 1183 to 1208, 1222 to 1241, 1253 to 1273, and 1377 to 1396) transmembrane segments, respectively. An additional transmembrane segment in the N-terminal hydrophobic domain was expected to maintain the protein symmetry and is indicated in Figs 2.1 A and 2.1 B.

Southern blot analysis

A southern blot analysis was performed in order to find *FvABC1* homologs across *Fusarium* species. A DIG PCR labeled probe (designated SB probe in Fig 2.1 A) was created in order to identify *FvABC1* homologs. The SB probe (330 bp) was created to hybridize a less conserved region in the *FvABC1* sequence at the end of the exon 3 to reduce non-specific hybridization. In a study conducted by Glenn et al. (2001) a tolerance survey was assessed using 29 *Fusarium* species. A subset of these taxa was selected for their relationship to *F. verticillioides* within the *Gibberella fujikuroi* species complex or their association with corn, wheat or rye. The objective was to assess if any relationship existed between the presence of a *FvABC1* homolog and the capacity to tolerate BOA. The amount of DNA of each *Fusarium* species was spectrophotometrically measured and 1µg of each sample was digested with EcoRV. The total volume was loaded into a 1.2% agarose gel (see Fig. 2.2 A) and was ran for 3 hours. As shown in Fig. 2.2 B, *FvABC1* seems to be single copy gene; however a faint band (around 2.0 Kb) on each strain was seen. All the analyzed samples from the *G. fujikuroi* species complex

were predicted to have an *FvABC1* homolog. The same pattern was shown for species that are associated with cyclic hydroxamic acids producing hosts (corn, wheat or rye). In each case, a single band was seen for any of these species. *Fusarium* species showing a degree of tolerance to PDA amended with BOA (1mg/ml). Glenn et al. (2001) showed the presence of an *FvABC1* homolog except for *F. oxysporum* strain NRRL 13307. Two *F. oxysporum* strains (NRRL 22544 and NRRL 22539) did not show tolerance to BOA, but possessed the *FvABC1* homolog. Interestingly, *F. oxysporum* NRRL 13307 showed a slight tolerance to BOA however did not possess the *FvABC1* homolog.

Characterization of other putative *F. verticillioides* ABC transporters

A TBLASTn analysis of the *F. verticillioides* genome at the Broad Institute Database (http://www.broad.mit.edu/cgi-bin/annotation/fungi/fusarium_verticillioides) resulted in the identification of nineteen putative ABC transporters including *FvABC1*; however five of these genomic sequences were incomplete and were not included in our analysis. A total of fourteen putative ABC transporters were analyzed (Table 2.2) and thirteen showed the typical structure of the PDR family with [NBD-TMD]₂ orientation (data not shown). A selection of the hypothetical genes is presented in Figures 2.3 to 2.5. *FvABC14* was a member of The Eye Pigment Precursor Transporter (EPP) Family (ABCG) and was not characterized further. *FvABC1*, *FvABC2*, and *FvABC3* were found in the same contig 2.2, *FvABC9* and *FvABC10* in the supercontig 2.23 and finally *FvABC11* and *FvABC12* were found in supercontig 2.28 A ClustalW alignment of the entire amino acid sequences of *FvABC1* through *FvABC13* were compared to characterized ABC transporters from filamentous fungi. An alignment of the motifs is shown in Fig. 2.3. *FvABC1* showed identity with ABC transporters from *A. nidulans* (*atrB*, 69%), *B. fuckeliana* (*BcatrB*, 69%), *G. pulicaris* (*Gpabc1*, 37%) and *M. grisea* (*ABC1*, 40%). *FvABC1* through *FvABC13*

possessed, at the nucleotide binding domain, the Walker A (GsSGxGKT), Walker B (XXXXD, with X representing a hydrophobic residue), and ABC signature (SGGERKRVxI) motifs (Fig. 2.3). The ABC signature was highly conserved at the N-terminal portion of the amino acid sequences; however the ABC signature at the C-terminal portion was degenerate.

A diverse subset of ABC transporters (*FvABC3*, *FvABC4*, *FvABC8*, and *FvABC9*) was analyzed to predict the exon-intron structure (Fig 2.4). Phylogenetic analysis indicated these genes were widely distributed (see below). As seen in Fig 2.4, genes showed the typical structure of the PDR family with a [NBD-TMD]₂ structure. The predicted ORF for *FvABC3* consisted of two exons (854 and 2810 bp, respectively), with *FvABC4* having with four exons (3784, 358, 23, and 104 bp, respectively, *FvABC8* having four exons (1044, 1527, 1233, and 651 bp, respectively), and *FvABC9* with two exons (2878 and 1894 bp respectively). Predicted transmembrane segments are indicated (Fig 2.4 and Fig. 2.5).

Phylogenetic analysis

An analysis was conducted to infer the phylogenetic relationships among PDR-type ATP transporters from a range of fungi. The alignment of the entire protein sequences was generated using ClustalW. Neighbor-joining analysis of the alignment (Fig 2.6) showed that the 13 putative ABC transporter proteins from *F. verticillioides* were distributed in different clades. *FvABC1*, *FvABC3*, *FvABC6*, and *FvABC11* showed interesting phylogenetic relationships with other already characterized ABC transporters in phytopathogenic fungi. *FvABC1* and *FvABC11* were grouped in the same clade. *FvABC1* showed close relation to a hypothetical ABC transporter from the corn pathogen, *G. zae* (EAA717570). *FvABC1* and *FvABC11* were grouped with *PMR5*, *atrB*, *MgATr5* and *BcatrB*, which are characterized ABC transporter genes that play an important role in fungal protection against chemical compounds (Del Sorbo et al., 1997;

Nakaune et al., 2002; Vermeulen et al., 2001; Zwiers et al., 2003b). The high bootstrap values support the consistency of this data. The protein encoded by *FvABC3* was clustered with a hypothetical ABC transporter protein from *G. zeae* (FG8312) and with *ABC1* from *M. grisea*, which has been classified as pathogenicity factor (Urban et al., 1999). *FvABC6* was grouped in the same clade as a hypothetical protein from *G. zeae* (FG04580) and *Gpabc1* from *G. pulicaris*. Again, this is a case of a phylogenetic relationship between an ABC transporter protein from *F. verticillioides*, and an ABC transporter classified as pathogenicity factor, *Gpabc1* (Fleissner et al., 2002). The other *F. verticillioides* ABC transporters were distributed across the phylogenetic tree. *FvABC9* formed a clade with two hypothetical ABC transporters from *G. zeae* and *M. grisea*. The bootstrap value for the *FvABC9* clade supports its consistency. *FvABC8* was grouped with hypothetical ABC transporter from *G. zeae* and *M. grisea*. *FvABC4* was consistently placed in a clade with a hypothetical ABC transporter in *G. zeae*. (FG07383). Three of the genes in the same clade as *FvABC4* (*atrF*, *BcatrA* and *MgAtr1*) have been already characterized and showed to play a role in fungal protection (Slaven et al., 2002; Zwiers and De Waard, 2000). An ABC transporter in *F. verticillioides* (CAI60778) has been annotated in GenBank but was not named and has not been published, so it was herein designated *FvABC7*. *FvABC7* was shown to be closely related to *FvABC3* and *FvABC6* (Fig. 2.6). Overall tree topology was similar between the neighbor-joining and most parsimonious tree. The clades containing *FvABC1*, *FvABC3*, *FvABC4*, *FvABC8*, and *FvABC9* were consistent in both analyses.

***F. verticillioides* transformation**

In order to functionally characterize *FvABC1*, attempts were made to generate gene replacement mutants. *F. verticillioides* was transformed by using plasmid pBs-5'Hyg-3'. Ten independent transformations yielded 486 hygromycin-resistant colonies, and all of them were

able to grow on PDA with hygromycin. All the colonies were single-spore isolated and screened on PDA amended with BOA (0.5 mg/ml). Hygromycin-resistant colonies with decreased growth on BOA were assessed by PCR to differentiate integration types. The PCR analysis (data not shown) showed that 120 of them had ectopic integration. Primers WTFor/P1 and P2/WTRev indicated of intact wild type allele (1921 and 3281 bp, respectively).

The split marker technique was employed to reduce the number of transformants that must be screened. Ten hygromycin-resistant strains were recovered from single transformation. Two of these strains, designated T1 and T2, produced visible mycelia after a day of inoculation in PDA slants amended with hygromycin (150 µg/ml). All the hygromycin resistant colonies were used in a phenotype assay using PDA amended with BOA (0.5 mg/ml) and PDA with ethanol (1% final concentration). Strains T1 and T2 showed the same phenotype as the wild type MRC826 in both PDA+BOA and PDA+ethanol plates. The other eight hygromycin-resistant strains showed restricted growth on both medium. In order to identify homologous integrants, the DNA was extracted and used as template for PCR confirmation. The first approach was to use primers M13Fv2/M13Rv2 (see table 2.1) to identify colonies with the entire hygromycin cassette. Only T1 and T2 strains possessed the entire hygromycin gene (1585 bp). To confirm ectopic/homologous integration, two set of primers were used (see Materials and Methods section). The first was set 5'-outer/WTRev and all of the hygromycin-resistant strains showed a band at 2.235 Kb. The next PCR confirmation was performed using primers WTFor and 3'-outer, and all the strains showed a band at 2.240 Kb. None of the hygromycin-resistant colonies was a true knock out.

DISCUSSION

The main objective of this work was to identify and characterize an ABC transporter from *F. verticillioides* which we designated *FvABC1* that may be involved in tolerance to phytochemicals. To our knowledge, *FvABC1* is the first reported PDR-type ABC transporter from *F. verticillioides*. The gene herein designated *FvABC7* has been annotated in GenBank (CAI60778) but was not given a name and has not been published. The ABC transporters mediate multidrug resistance by reducing the concentration of toxic drugs in the cytoplasm (Balzi et al., 1994; Higgins, 1992). Tolerance to chemical and natural compounds mediated by ABC transporters in phytopathogenic fungi may be indispensable in natural environments. In a previous study, *FvABC1* was highly represented in a cDNA subtractive library constructed under BOA conditions (Glenn, 2004). For this reason, it is possible to hypothesize that *FvABC1* might, at least, mediate tolerance to the corn antimicrobial BOA. Interestingly, some ABC transporter genes have been characterized in other phytopathogenic fungi as pathogenicity factors due to inhibited growth of gene deletion mutants *in planta* (Fleissner et al., 2002; Tremblay et al., 2003; Urban et al., 1999; Vermeulen et al., 2001). The ABC transporters *ABC1* from *M. grisea* and *Gpabc1* from *G. pulicaris* are needed for fungal colonization. *FvABC1* showed amino acid sequence similarity to both *Gpabc1* and *ABC1* but was more closely related to *atrB* and *BcatrB* which were shown to confer tolerance to fungicides and some natural toxic compounds. The predicted genes *FvABC3*, *FvABC6*, and *FvABC7* were more closely related to *ABC1* and *Gpabc1*.

FvABC1 homologs were found in all the species from the *Gibberella fujikuroi* species complex analyzed in this study. Two *F. verticillioides* strains, AEG3-1-6 and NRRL25059 were BOA sensitive since they do not possess the functional loci necessary for detoxification of cyclic

hydroxamic acids (Glenn et al., 2002), however pathogenicity assays showed that both AEG3-1-6 and NRRL25059 were still virulent even though they were not able to metabolize BOA. *FvABC1* was present in both *F. verticillioides* strains so based on this data it is hypothesized that *FvABC1* might confer a non-degradative tolerance mechanism against the maize phytochemicals. In the southern blot study, *FvABC1* homologs were found in *Fusarium* species associated with corn, wheat or rye. Here again, there is an association with *FvABC1* and cyclic hydroxamic acids. Additionally, *Fusarium* species showing a degree of tolerance to BOA (1 mg/ml) possessed this homolog. However the most interesting results came from the *F. oxysporum* strains. Two *F. oxysporum* strains (NRRL22544 and NRRL22539) possessed the *FvABC1* homolog yet were not tolerant to BOA (Glenn et al., 2001). In contrast strain NRRL13307 possessed some degree of tolerance to BOA but did not possess the *FvABC1* homolog. Further analysis should address this conflicting result to more thoroughly evaluate the role of *FvABC1* to BOA tolerance. Although the three *F. oxysporum* strains were not associated with either cyclic hydroxamic acid producing hosts or the *G. fujikuroi* species complex, the fact that they possess this *FvABC1* homolog might confer these strains an ecological advantage in some environments. The distribution of an *FvABC1* homolog in the *G. fujikuroi* species complex suggests a common ancestral origin for this gene. Future studies could include functional analysis of *FvABC1* homologs in multiple species.

Hydropathy analysis for a select group of the predicted *F. verticillioides* ABC transporters showed two hydrophobic domains. In *FvABC1*, *FvABC3*, and *FvABC8* the N-terminal TMD was predicted to have five transmembrane segments by HMMTop. Six transmembrane segments is standard for most of the ABC transporters characterized, but a TMD with five transmembrane segments is reported in multidrug resistance protein (MRP) which has

an extra TMD (Fernandez et al., 2002). So far, no PDR-type ABC transporter has been reported to have anything other than six transmembrane segments per TMD. For this reason the sixth TMS was estimated for *FvABC1*, *FvABC3*, and *FvABC8*. This discrepancy in TMS prediction might be solved by using more than one prediction tool with different algorithms and assumptions used to make such predictions.

A TBLASTn analysis identified 13 new putative ABC transporters from *F. verticillioides* in addition to *FvABC1*. An amino acid sequence alignment demonstrated the presence of Walker A and B and the ABC signature motifs for *FvABC1* through *FvABC13*. The presence of specific amino acid sequence motifs defines the classification of ABC transporter from *S. cerevisiae* (Decottingnies and Coffeau, 1997). The presence of cysteine (C) in the Walker A of the N-terminal NBD, glutamic acid (E) and lysine (K) in the ABC signature of the N terminal NBD, and the motif valine, glutamic acid and glutamine (VEQ) in the ABC signature of the C-terminal NBD is typical of characterized genes in *S. cerevisiae* (*PDR5*, *SNQ*, and *PDR12*) and filamentous fungi (*atrB*, *BcatrB*, *Gpabc1*, and *ABC1*) which might be associated with functional homology.

In our *F. verticillioides* genomic analysis, *FvABC14* was identified but not appear to be a PDR-type ABC transporter. It is predicted to be a member of the Eye Pigment Precursor Transporter (EPP) family (ABCG) with homology to *BCRP* of *Homo sapiens* (AAC97367), a breast cancer resistance related protein as predicted by the transporter classification database. BCRP analyses suggest that this gene is likely to be a major cellular defense mechanism elicited in response to exposure to anticancer treatments (Ross et al., 1999). Therefore it is possible that *FvABC14* might be involved in chemical protection in *F. verticillioides*.

Some of the *F. verticillioides* predicted genes were found on the same supercontig. Gene duplication is a mechanism in evolution to create paralogous genes that may have different or overlapping functions. This might be the case for putative ABC transporters *FvABC1*, *FvABC2*, and *FvABC3*, which were found on the same supercontig, yet they were distributed over the phylogenetic tree. Likewise *FvABC9* and *FvABC10*, and finally *FvABC11* and *FvABC12* might have resulted from duplication in *F. verticillioides*.

The identification of different ABC transporters is interesting from both functional characterization and evolutionary points of view. A phylogenetic tree was created using the 13 putative ABC transporters from *F. verticillioides* and other PDR-type ABC transporter proteins from filamentous fungi. Phylogenetic analyses can be used to formulate a hypothesis about biological functions of proteins (Hall, 2001). The analyses in Fig 2.6 and Fig. 2.7 showed that the 13 *F. verticillioides* predicted proteins were distributed over the phylogenetic trees, suggesting different functions for different clades of ABC transporters from *F. verticillioides*. Interestingly, *FvABC1* belonged to the same clade as well characterized fungal ABC transporters. One of them, *BcatrB* from *B. cinerea*, is involved in protection against the phytoalexin resveratrol in grapevine (Schoonbeck et al., 2001). *FvABC3* showed high similarity with *ABC1* from *M. grisea*. Further analysis of *FvABC3* could be worthwhile to assess what role, if any, it may have on *F. verticillioides*-corn interactions. Similarly, *FvABC6* grouped with *Gpabc1*, another pathogenicity factor. Here again, this phylogenetic relationship allows us to hypothesize *FvABC6* might play a role in fungal pathogenesis. The importance to create *FvABC3* and *FvABC6* null mutants rely on the fact that the deletion of the corresponding genes could cause changes in such mutants that might have quantitative differences in *F. verticillioides* virulence and/or fitness.

The study of gene function at the molecular level is important since it allows deleting the function of a specific gene under controlled conditions. However some techniques have to be adapted for different organisms. The first knock-out approach resulted in only ectopic integration. High frequency of ectopic integration has been reported in *A. fumigatus* (Aufauvre-Brown et al., 1993) and is due to promiscuous integration events. Colonies from the transformation were transferred from hygromycin and then were screened on BOA. The reasoning behind this BOA screening, in principle, was to select only those colonies with restricted growth in the presence of BOA based on the assumption that a gene deletion mutant would have reduced growth. This assumption may have actually resulted in the failure to detect a knock-out mutant since all the hygromycin-resistant colonies that were screened were the result of ectopic integration.

The split marker technique has claimed to increase homologous integration in fungal organisms (Catlett et al., 2003; Fu et al., 2006; Skov et al., 2004). In theory, all the hygromycin resistant colonies obtained should be gene replacement mutants. For this reason the split-marker technique approach was utilized to attempt gene deletion of *FvABC1*. Results showed that 2 of 10 hygromycin-resistant colonies recovered from a single transformation did have recombination between the two hygromycin fragments but were shown to be the result of ectopic integration. The remaining 8 colonies had very restricted growth on hygromycin, and PCR indicated they did not contain the intact hygromycin cassette. They were likely not untransformed colonies that were not suppressed by the hygromycin selection.

The identification of ABC transporters in *F. verticillioides* is important for understanding the impact of non-degradative tolerance on host-pathogen interactions. Future analysis could involve the molecular manipulation of one or more of the *F. verticillioides* genes

identified have in to create null mutants. This would allow for direct comparisons of different genes relative to tolerance and virulence. The inability to create knock out mutants of *FvABC1* might suggest the gene is essential for *F. verticillioides*, however, so far, no PDR-type ABC transporters in fungi have been reported to be essential.

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69. Zwiers, L. H., I. Stergiopoulos, M. M. C. Gielkens, S. D. Goodall, and M. A. De Waard. 2003b. ABC transporter of the wheat pathogen *Mycosphaerella graminicola* function as protectants against biotic and xenobiotic toxic compounds. *Molecular Genetics and Genomics* **269**:499-507.

Table 2.1. PRIMERS USED FOR GENE KNOCK OUT STRATEGY

Primer	First Round				Second Round		Sequence
	5' flank	3' flank	HY	YG	5'-HY	YG-3'	
M13Fv2				X			cgccagggtttcccagtcacgac
M13Rv2			X				agcggataacaatttcacacagga
HygFor				X		X	tcgtgcttcagcttcgatgtagg
HygRev			X		X		cattgtccgtcaggacattgttg
F1	X ^a				X	X	gacagctgcgtcttcgatt
F2 ^b	X						tctgtgtgaaattgttatccgcta agaggatagcctttaccgc
F3 ^b		X					gtcgtgactgggaaaacctggc ggggttacatggcaacctgat
F4		X					atatggacgctttgtgaggc

^a An “X” indicates that a primer is utilized in the indicated reaction.

^b For primer F2 and F3 highlighted regions are complementary to M13Fv2 and M13Rv2, respectively.

Table 2.2. ABC TRANSPORTERS FROM *FUSARIUM VERTICILLIOIDES*^a

Gene	Supercontig number	Location
<i>FvABC1</i>	2.2	323170 - 327494
<i>FvABC2</i>	2.2	2269000 - 2275100
<i>FvABC3</i>	2.2	2242000 - 2250900
<i>FvABC4</i>	2.5	374000 - 379500
<i>FvABC5</i>	2.6	730900 - 737000
<i>FvABC6</i>	2.9	363000 - 369500
<i>FvABC7</i>	2.11	1068000 - 1074000
<i>FvABC8</i>	2.15	7900 - 14000
<i>FvABC9</i>	2.23	407000 - 412100
<i>FvABC10</i>	2.23	247000 - 252700
<i>FvABC11</i>	2.28	4900 - 10500
<i>FvABC12</i>	2.28	67000 - 73420
<i>FvABC13</i>	2.34	120000 - 127000
<i>FvABC14</i>	2.31	469500 - 476000

^a Based on TBLASTn search of the *F. verticillioides* genome (Broad Institute) using *FvABC1* as query. Only loci with complete sequence data and having an E value < 1 x 10⁻⁰³ were included.

Fig. 2.1. FEATURES OF THE *FvABC1* GENE. **A.** The three predicted exons (781, 898, and 2535 bp, respectively) are indicated as white boxes which are interrupted by two introns (49 and 53 bp, respectively). The two NBDs are indicated as black boxes, and the 12 predicted transmembrane segments (TMS) are indicated by gray boxes. Probes 1G7 (397 bp) and 1G8 (433 bp) were used to screen *F. verticillioides* genomic cosmid library macroarrays. The EcoRV restriction sites and the probe (SB probe, 330 bp) used for the Southern blot analysis are indicated. Predicted TMSs, TopPred program (von Heijne, 1992), were marked with a gray box. While not predicted, one additional TMS in the TMD1 was expected in order to maintain symmetry and is indicated by red box in panel A. **B.** The hydropathy plot of the protein encoded by *FvABC1* with two NBDs (designated NBD1 and NBD2) and two TMDs (designated TMD1 and TMD2) are indicated. The gray and green lines in the hydropathy plot represent the upper and lower cutoffs to predict TMSs as designated by TopPred program. Predicted peaks for the TMSs were marked with a black dot, and the sixth TMS in TMD1 was marked with a red dot.

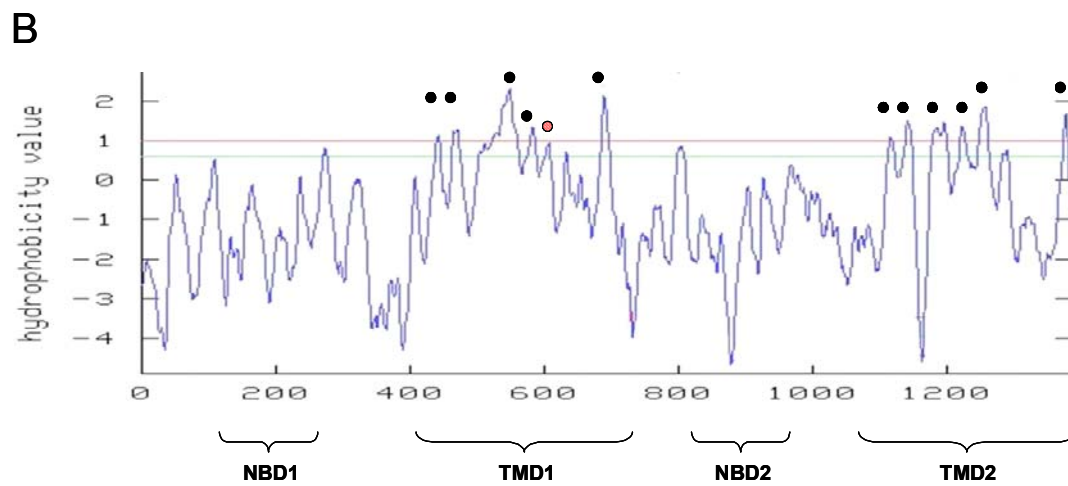
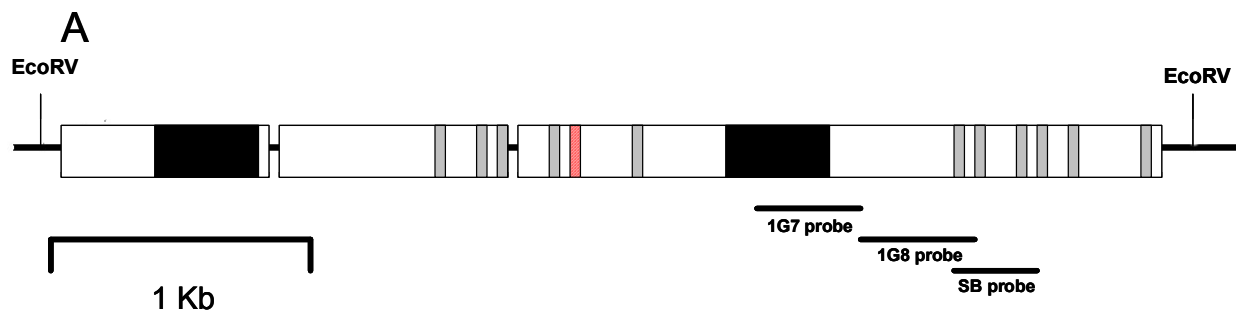


Fig. 2.2. SOUTHERN BLOT ANALYSIS FOR *FUSARIUM* SPECIES. **A.** Genomic DNA (1 µg) was digested with EcoRV and ran into 1.2% agarose gel, which was stained with ethidium bromide and irradiated with UV light. **B.** Southern blot analysis for *Fusarium* species from the *Gibberella fujikuroi* species complex (indicated by a black dot) and isolated from corn, wheat or rye (gray dot). Those species capable of growing on PDA amended with BOA (1mg/ml) (Glenn et al., 2001) were indicated with a dark gray dot. Each strain and the resulting blot was hybridized with SB probe (see Fig 2.1). DIG labeled Lambda Hind III marker is shown.

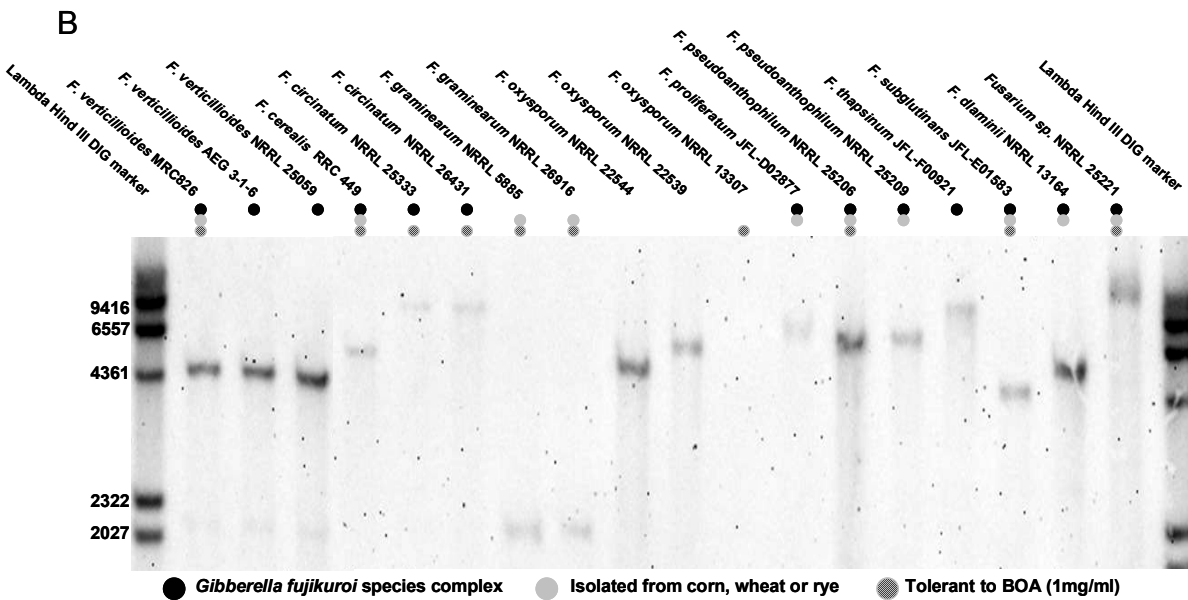
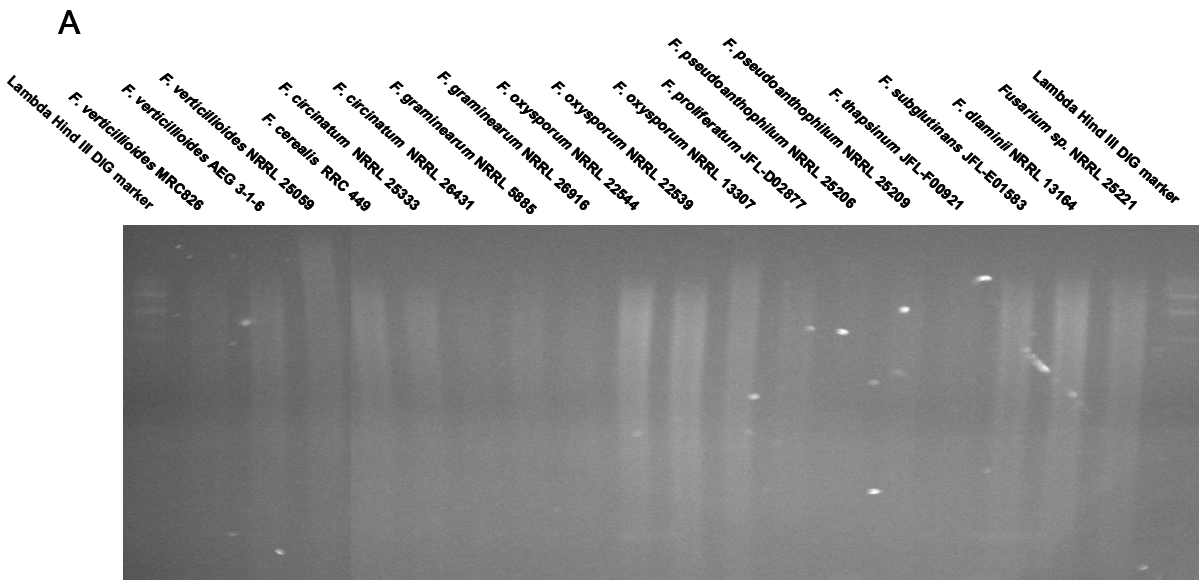


Fig. 2.3. MULTIPLE AMINO ACID SEQUENCE ALIGNMENT OF PDR-TYPE ABC TRANSPORTER PROTEINS FROM FILAMENTOUS FUNGI. The *FvABC1* through *FvABC13* amino acid sequences were aligned with the following ABC transporters (GenBank accession numbers are indicated): *Aspergillus nidulans*, *atrB* CAA93141; *Botryotinia fuckeliana*, *BcatrB* CAB52402; *Gibberella pulicaris*, *Gpabc1* CAC40023; *Magnaporthe grisea*, *ABC1* AAB86640. Bars above sequences indicate Walker A and B and the ABC signature motifs. Sequences were aligned using CLUSTALW (Thompson et al., 1994). Identical sequences within the motifs are indicated as shaded regions. *FvABC14* was not included since it is predicted to be member of the Eye Pigment Precursor Transporter (EPP) family (ABCG) and not a PDR-type transporter.

	<u>Walker A</u>	<u>ABC signature</u>	<u>Walker B</u>
FvABC1	GEMLLVVLGRPGSGCTILLNMI	VSGGERKRVS I IETLATQGSVFCWD	
FvABC2	GEMVLVLGKPGSGCTIFLKTI	VSGGERKRVS I AEAMICSGAVFSWD	
FvABC3	GEMLVVLGPPGAGCSTLLKTI	VSGGERKRVTI SE AALSGAPLQCWD	
FvABC4	GEMMLVVLGRPGAGCSTFLKAI	VSGGERKRVS I AETLASKSTVMAWD	
FvABC5	GEMLAVLGP PGSGCSTFLKTI	VSGGERKRVTI AE AALSY SPLQCWD	
FvABC6	GEMLVVLGPPGSGCSTFLKSL	VSGGERKRVTI AEATLSNAPFQCWD	
FvABC7_CAI60778	GEMLVVLGPPGSGCSTYLKTI	VSGGERKRVTI AEASLSGAPLQCWD	
FvABC8	GEMLCVLGPPGSGCSTLLRSI	ISGGERKRVTI AE AALGYSPLQCWD	
FvABC9	GELLLVLGRPGAGCSTFLKTF	VSGGERKRVS I AEAMITRASVQGW	
FvABC10	GELLLVLGPPGSGCSTLLKTI	VSGGERKRVS I AE AALTGAKLQCWD	
FvABC11	GEMLLVLGRPGSGCTILLSVL	VSGGERKRVS I VECLTTRASVFCWD	
FvABC12	GETLIV-----	VSGGERKRVS I AEMMLAQSPMAAWD	
FvABC13	GELLLVLGRPGSGVSTLLKTI	VSGGERKRVS I AEAVLNQSAIQCW	
atrB_CAA93141	GEMLLVLGRPGSGCTILLKML	VSGGERKRVS I IECLGTRASVFCWD	
BcatrB_CAB52402	GEMLLVLGRPGAGCTILLKML	VSGGERKRVS I IEMLASRG SVMCWD	
Gpabc1_CAC40023	GEMLVVLGPPGSGCSTFLKSI	VSGGERKRVTI AEATLSNAPFQCWD	
ABC1_AAB86640	GEMLVVLGPPGSGCSTFLKTI	vSGGERKRVTI AE AALSGAPLQCWD	
	<u>Walker A</u>	<u>ABC signature</u>	<u>Walker B</u>
FvABC1	GMLGAIMGSSGAGKTILLDVL	LSVEQRKRVTI GVELVSKP SILIFLD	
FvABC2	GELTAIMGASGAGKTILLDVL	ITVEQRKRVTI GVELAAKPEIMLFLD	
FvABC3	GTLTAIMGVS GAGKTILLDCL	LNVEQRKRLTI GVELAAKPPLLLFVD	
FvABC4	GVMVAIMGASGAGKTILLNIL	LGVEQRKRLTI GVELAAKP SLLIFLD	
FvABC5	GTLTAIMGASGAGKTILLDVL	LNVEQRKRLTI GVELAARPQLLIFLD	
FvABC6	GTLTAIMGVS GAGKTILLDCL	LNVEQRKRLTI GVELAAKPPLLLFVD	
FvABC7_CAI60778	GTMTAIMGVS GAGKTILLDCL	LNVEQRKRLTI GVELAAKPPVLLFVD	
FvABC8	GTLTAIMGVS GAGKTILLDVL	LNVEQRKRLTI AVELVARPQLLIFLD	
FvABC9	GKLTAIMGASGAGKTILLNGL	LNAEQRKRLTI GVELASKPELIMFLD	
FvABC10	GKSTAIMGVS GAGKTILLDVL	LNVEQRKRLTI GVELAARPQLLVFLD	
FvABC11	GMLGAIMGSSGAGKTILLDVL	LSIEQRKRLTI AVELVAKP SILIFLD	
FvABC12	GTLTAIMGVS GAGKTILLDVL	LNVEQRKLLTI GVELAAKPKLLIFLD	
FvABC13	GKLTAIMGATGAGKTILLDVL	LNVEQRKRLS I GVELVAKPEALIFLD	
atrB_CAA93141	GMLGAIMGSSGAGKTILLDVL	LSVEQRKRVTI GVELVSKP SILIFLD	
BcatrB_CAB52402	GMLGAIMGSSGAGKTILLDVL	LSVEQRKRLTI GVELVSKP SILIFLD	
Gpabc1_CAC40023	GTLTAIMGVS GAGKTILLDCL	LNVEQRKRLTI GVELAAKPPLLLFVD	
ABC1_AAB86640	GTLTAIMGVS GAGKTILLDCL	LNVEQRKRLTI GVELAAKPPLLLFVD	

Fig. 2.4. FEATURES OF A SUBSET OF ABC TRANSPORTERS FROM *F. VERTICILLIOIDES*. The ABC transporters sequences analyzed were *FvABC3*, *FvABC4*, *FvABC8*, and *FvABC9* were analyzed and are representative of the diversity of *F. verticillioides* genes identified (see Figs 2.6 and 2.7). Features are denoted as described in Fig. 2.1.

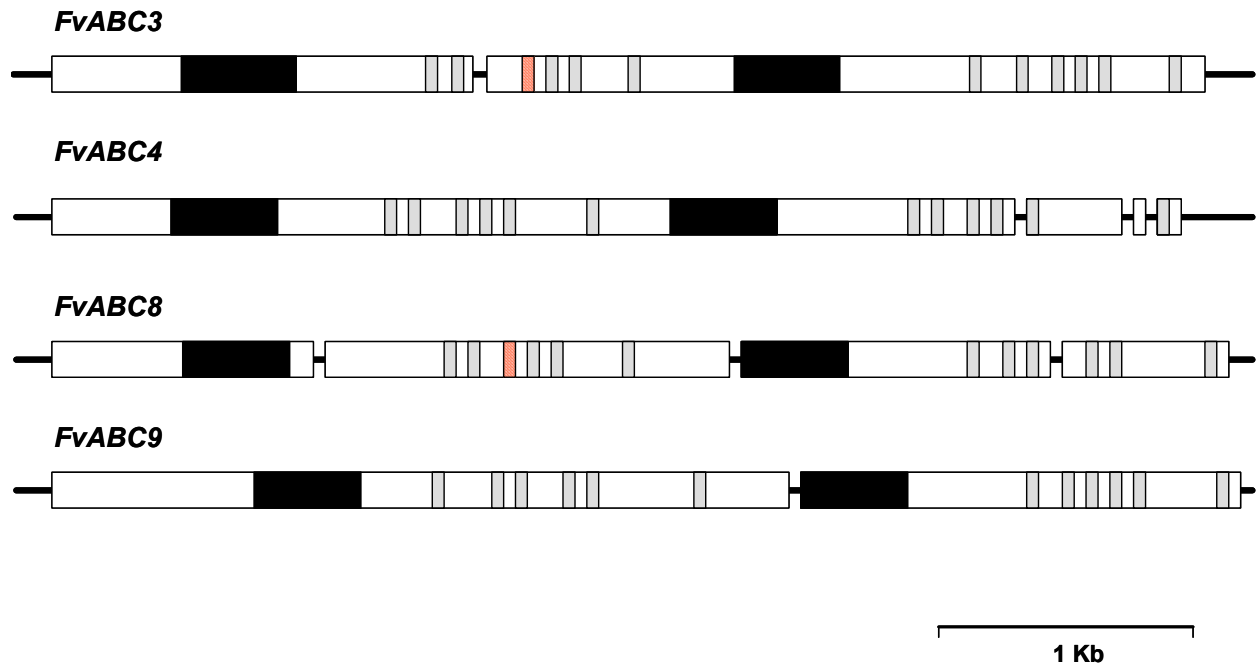


Fig. 2.5. HYDROPATHY PLOTS FOR A SUBSET OF *F. VERTICILLIOIDES* PROTEINS. Putative FvABC3p, FvABC4p, FvABC8p, and FvABC9p were analyzed using TopPred (Fleissner et al., 2002; von Heijne, 1992). Nucleotide binding domains, transmembrane segments and other notations are as described in Fig 2.1 B.

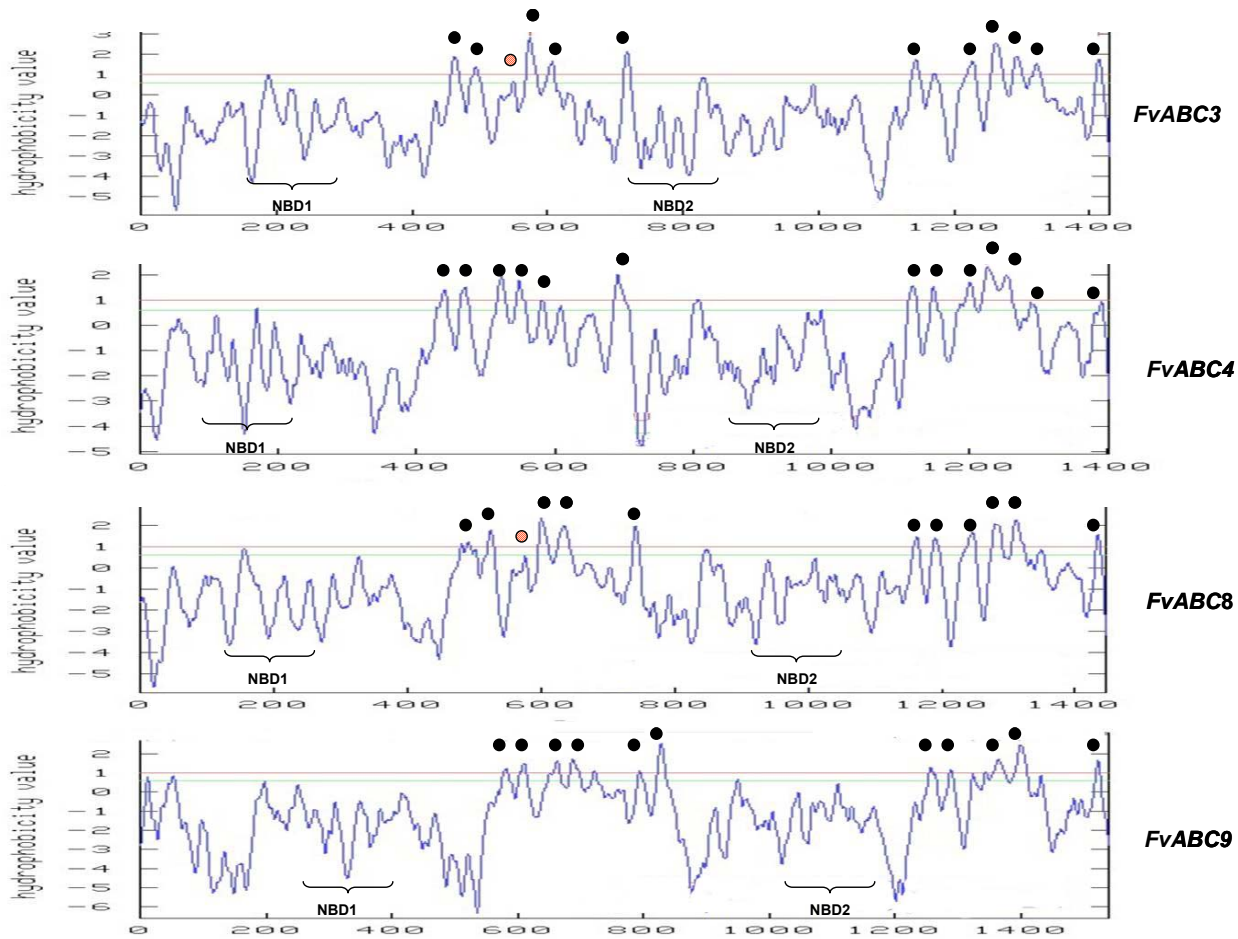


Fig. 2.6. NEIGHBOR-JOINING PHYLOGENETIC TREE. The analysis was based on entire protein sequence alignment. Bootstrap analysis performed with 1000 replications, pairwise deletion, and the Poisson correction model. The names and GenBank accession numbers of ABC transporters from other fungi includes: *Aspergillus fumigatus*, *abcA* CAD103327, *atrF* CAC36894; *Botryotinia fuckeliana*, *BcatrA* CAA93142, *BcatrB* CAB52402, *BcatrD* CAC41639; *Candida albicans*, *CDR1* CAA54692; *Emericella nidulans*, *atrB* CAA93141, *atrE* CAC42216, *atrF* CAC42217, *atrG* CAC42218; *Gibberella pulicaris*, *Gpabc1* CAC40023; *Gibberella zeae*, hypothetical protein CAA71570; *Magnaporthe grisea*, *ABC1* AAB86640; *Mycosphaerella graminicola*, *MgAtr1* CAB46279, *MgAtr2* CAB46280, *MgAtr3* AAK62341, *MgAtr4* AAK15314, *MgAtr5* AAK62340; *Neurospora crassa*, hypothetical protein, EAA29293; *Penicillium digitatum*, *PMR1* BAA31254, *PMR5* BAB59028; *Saccharomyces cerevisiae*, *PDR5* CAA99359; *Venturia inaequalis*, *ViABC1* AAK62810, *ViABC2* AAK62811. In addition *Magnaporthe grisea* and *Fusarium graminearum* (*Gibberella zeae*) hypothetical transporters were included from the Broad Institute genome sequencing projects. These accessions are indicated with “MG” and “FG” respectively. The 13 ABC transporters from *F. verticillioides* are indicated with a black dot. GenBank accession CAI60778 is herein designated as *FvABC7* since no previous gene name was assigned.

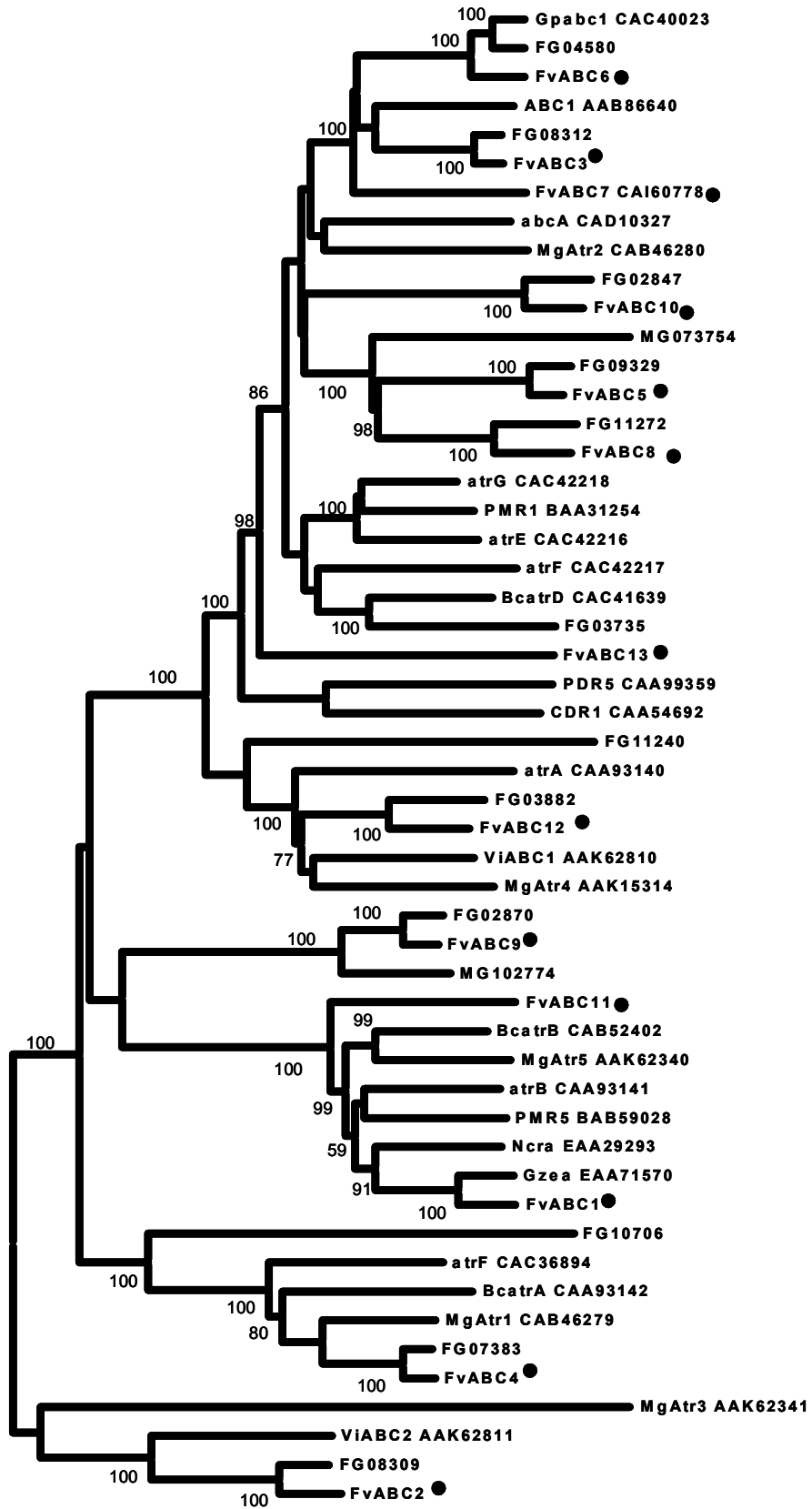
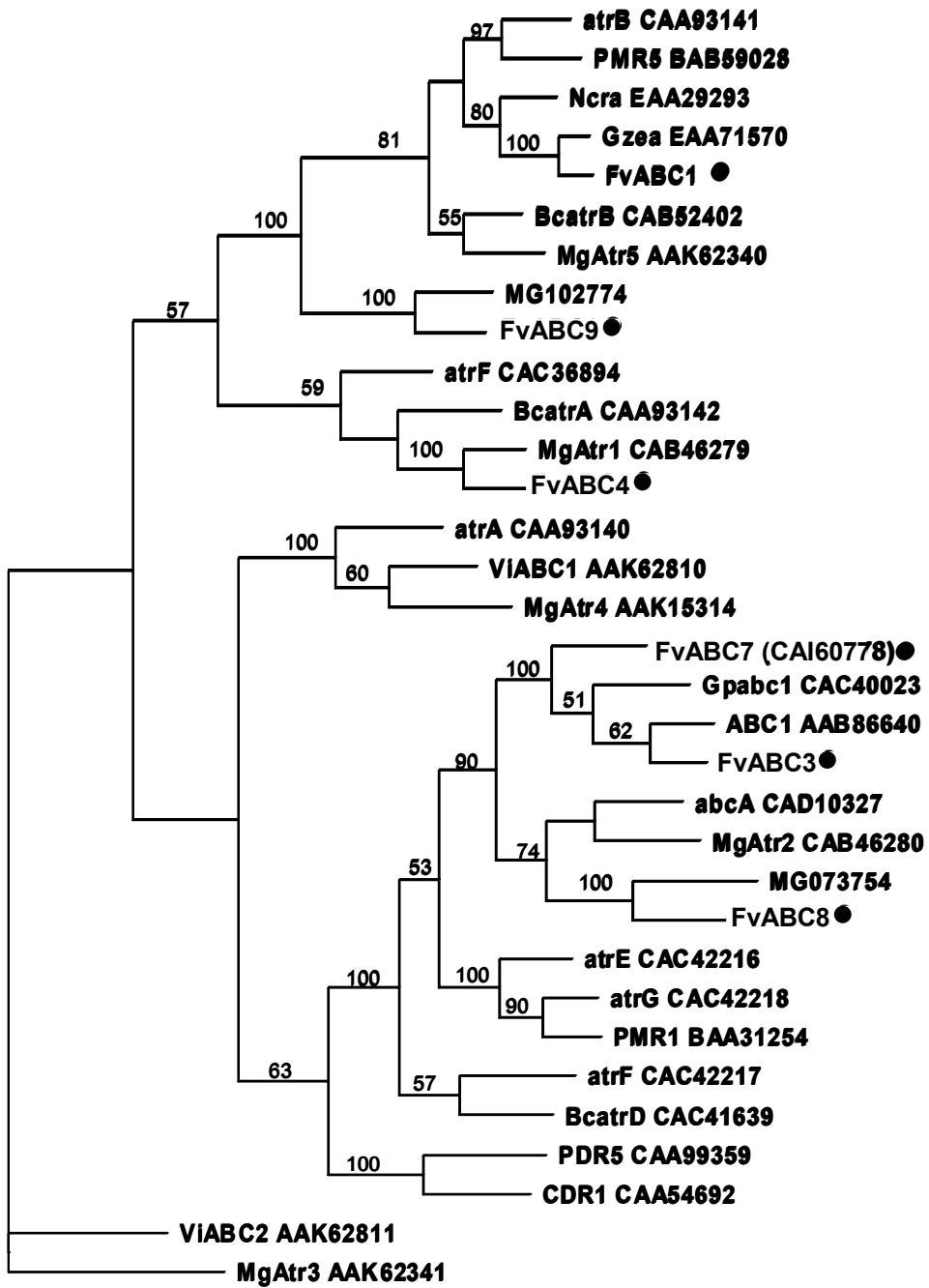


Fig. 2.7. PHYLOGENETIC TREE OF A SELECT SUBSET OF FUNGAL ABC TRANSPORTER OF PROTEINS. One of 4 most parsimonious trees generated by PAUP version 3.1.1. A Heuristic search was performed using random stepwise addition (10 reps). Bootstrap analysis was performed with 100 replications. The ABC transporters analyzed were: *Aspergillus fumigatus*, *abcA* CAD103327, *atrF* CAC36894; *Botryotinia fuckeliana*, *BcatrA* CAA93142, *BcatrB* CAB52402, *BcatrD* CAC41639; *Candida albicans*, *CDR1* CAA54692; *Emericella nidulans*, *atrB* CAA93141, *atrE* CAC42216, *atrF* CAC42217, *atrG* CAC42218; *Gibberella pulicaris*, *Gpabc1* CAC40023; *Gibberella zeae*, hypothetical protein CAA71570; *Magnaporthe grisea*, *ABC1* AAB86640; *Mycosphaerella graminicola*, *MgAtr1* CAB46279, *MgAtr2* CAB46280, *MgAtr3* AAK62341, *MgAtr4* AAK15314, *MgAtr5* AAK62340; *Neurospora crassa*, hypothetical protein EAA29293; *Penicillium digitatum*, *PMR1* BAA31254, *PMR5* BAB59028; *Saccharomyces cerevisiae*, *PDR5* CAA99359; *Venturia inaequalis*, *ViABC1* AAK62810, *ViABC2* AAK62811. *ViABC2* and *MgAtr3* were designated as outgroup sequences. *Fusarium verticillioides* transporter encoded by *FvABC1*, *FvABC3*, *FvABC4*, *FvABC8*, and *FvABC9* are indicated with a black dot, as is the GenBank accession CAI60778 which is herein designated *FvABC7*.

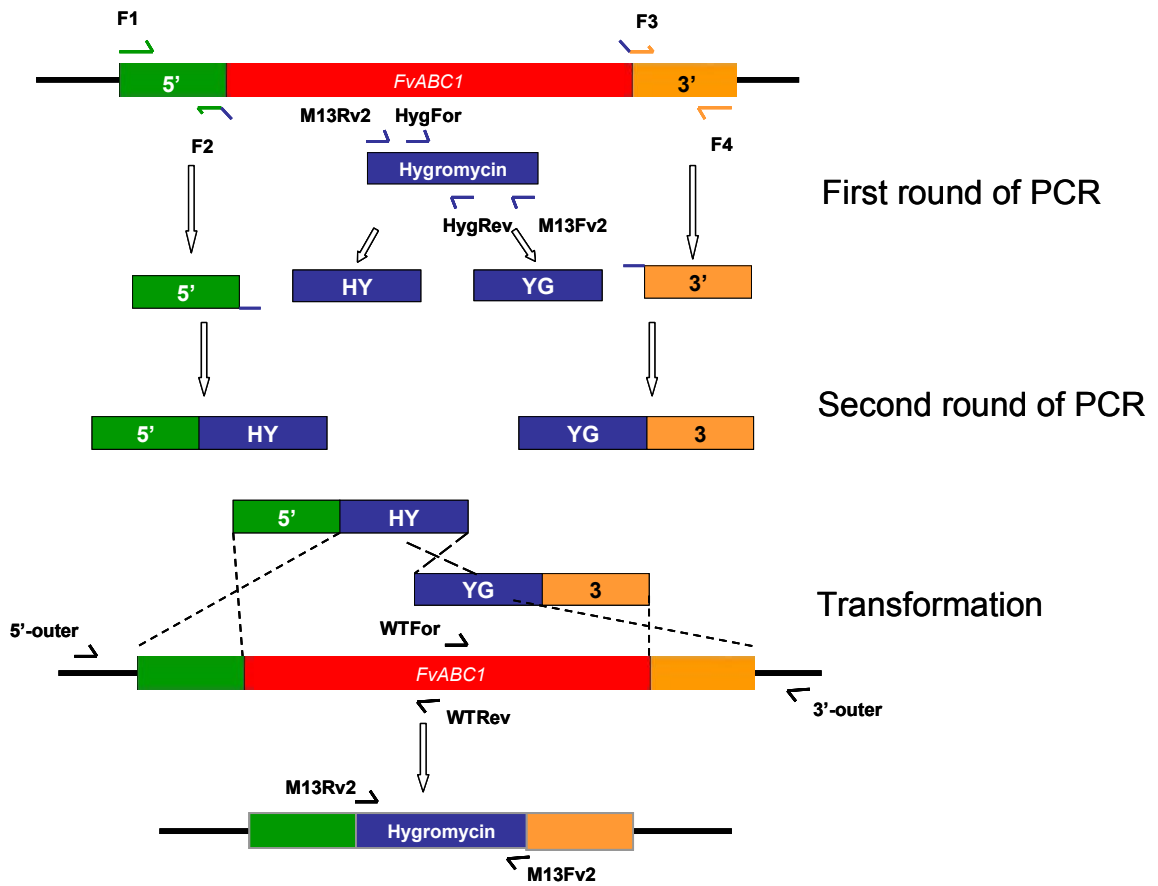


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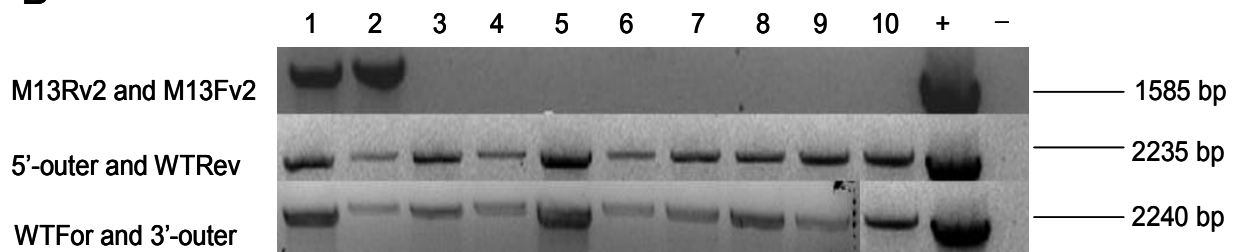
Fig. 2.8. SPLIT MARKER TECHNIQUE FOR GENERATING REPLACEMENT MUTANTS.

A. Primers F1/F2 and F3/F4 amplify flanking regions 5' and 3', respectively. The hygromycin cassette was divided into two fragments. Primers M13Rv2/HygRev were used to amplify the HY fragment and primers HygFor/M13Fv2 amplified the YG fragment. During the second round of PCR the 5' and HY fragments were fused using primers F1 and HygRev, while fragments 3' and YG were fused using primers HygFor and F4. Both 5'-HY and YG-3' amplicons were used in a transformation procedure for replacement of *FvABC1*. **B.** Ten hygromycin-resistant colonies were analyzed by PCR to confirm homologous integration. Primers M13Rv2 and M13Fv2 were used to identify homologous integrants. Primers 5'-outer and 3'-outer targeted upstream and downstream regions of the wild-type *FvABC1* locus. PCR using 5'-outer and WTRev amplified a 2235 bp amplicon, and WTFor and 3'-outer amplified a 2240 bp amplicon.

A



B



CHAPTER 3

CONCLUSIONS

The research conducted as part of this thesis involved the identification of an ABC transporter from *F. verticillioides*, designated *FvABC1*, as member of the pleiotropic drug resistance family. The structural analysis of *FvABC1* showed the typical topology of the PDR type of ABC transporters with [NBD-TMD]₂ orientation. The hydropathy plot and the conserved regions (Walker A, Walker B and ABC signature) confirmed that *FvABC1* gene is an active transporter of the pleiotropic drug resistance family. *FvABC1* is a single gene copy.

FvABC1 homologs were found in all the species from the *G. fujikuroi* species complex and species related with cyclic hydroxamic acid producing hosts, suggesting that *FvABC1* might be involved in non-metabolic tolerance of BOA. Phylogenetic analysis suggests that the 14 *F. verticillioides* ABC transporters identified herein may have different roles in plant pathogenesis. Specifically three ABC transporters, designated *FvABC1*, *FvABC3*, and *FvABC6*, and *FvABC7* showed high homology with ABC transporters from phytopathogenic fungi classified as pathogenicity factors. Further studies should address the functions of *FvABC1*, *FvABC3*, *FvABC6*, and *FvABC7*.