Cloning, \textit{in silico} structural characterization and expression analysis of \textit{MfAtr4}, an ABC transporter from the banana pathogen \textit{Mycosphaerella fijiensis}

Y. Couoh-Uicab\textsuperscript{1}, I. Islas-Flores\textsuperscript{1*}, N. Kantún-Moreno\textsuperscript{2}, L.-H. Zwiers\textsuperscript{3}, M. Tzec-Simá\textsuperscript{2}, S. Peraza-Echeverría\textsuperscript{2}, L. Brito-Argáez\textsuperscript{1}, L. Peraza-Echeverría\textsuperscript{2}, R. Grijalva-Arango\textsuperscript{2}, A. James\textsuperscript{2}, C. Rodríguez-García\textsuperscript{2} and B. Canto-Canché\textsuperscript{2}

\textsuperscript{1}Unidad de Bioquímica y Biología Molecular de Plantas, Centro de Investigación Científica de Yucatán A.C., Calle 43 No. 130, Colonia Chuburná de Hidalgo, C.P. 97200, Mérida, Yucatán, México.
\textsuperscript{2}Unidad de Biotecnología, Centro de Investigación Científica de Yucatán A.C., Calle 43 No. 130, Colonia Chuburná de Hidalgo, C.P. 97200, Mérida, Yucatán, México.
\textsuperscript{3}CBS-KNAW, Fungal Biodiversity Centre, Utrecht, The Netherlands.

Accepted 2 December, 2011

ABC transporters are membrane proteins that use the energy released from the hydrolysis of ATP to drive the transport of compounds across biological membranes. In some plants, pathogenic fungi ABC transporters play a role as virulence factors by mediating the export of plant defense compounds or fungal virulence factors. \textit{Mycosphaerella fijiensis}, the causal agent of black Sigatoka disease in banana, is the main constraint for the banana industry worldwide. So far, little is known about molecular mechanism that it uses to infect the host. In this study, degenerated primers designed from fungal ABC transporters known to be involved in virulence were used to isolate homologs from \textit{M. fijiensis}. Here, we reported the full cloning of \textit{MfAtr4} a putative ortholog of \textit{MgAtr4}, an ABC transporter of the related \textit{Mycosphaerella graminicola} with a function in virulence. Similarities and differences with its presumed ortholog \textit{MgAtr4} are described, and the putative function of \textit{MfAtr4} are discussed. Analysis of \textit{MfAtr4} gene expression in field banana samples exhibiting visible symptoms of black Sigatoka disease indicated a higher expression of \textit{MfAtr4} during the first symptomatic stages in comparison to the late necrotrophic phases, suggesting a role for \textit{MfAtr4} in the early stages of pathogenic development of \textit{M. fijiensis}.

Key words: ABC transporters, virulence factors, \textit{MgAtr4} ortholog, \textit{Mycosphaerella fijiensis}, black Sigatoka, \textit{Musa} sp.

INTRODUCTION

The ATP-binding cassette (ABC) protein family constitutes one of the largest and ancient protein families. Currently, more than 10,000 members are known and it is expected that this number increases as new genome sequences become available (Kovalchuk and Driessen, 2010). ABC proteins are present in all organisms, from archaea to higher eukaryotes (Davidson and Maloney, 2007). Most of the ABC proteins characterized are classified as transmembrane proteins involved in the active transport of a broad range of substrates across biological membranes (Higgins, 1992; Laleh et al., 2008). However, to a lesser extent, some ABC proteins act as ion channels or receptors or are involved in ribosome biogenesis (Kovalchuk and Driessen, 2010). Based on the topology and ordering of specific domains normally present within ABC transporters, they can be divided into several subfamilies (ABC-A to ABC-H; Jie et al., 2010).
The structure of typical ABC transporters consists of four core domains, two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs). The domains TMD-NBD may be expressed as TMD-NBD in separate polypeptide chains or alternatively, as TMD-NBD in multidomain proteins. Based on the number of TMD-NBD domains inside polypeptides, two arrangements are common for eukaryotic ABC transporters. The functional unit is either composed of two “half transporters”, each containing its own TMD and NBD, or consists of one large polypeptide chain (“full transporters”) that includes all four domains (Del Sorbo et al., 2000; Kovalchuc and Driessen, 2010). ABC proteins containing the NBD but lacking TMDs are generally not involved in membrane transport (Kovalchuc and Driessen, 2010).

In fungi, the most common ABC transporters are the so-called full-size ABC transporters, in which all domains are contained in one polypeptide chain (Del Sorbo et al., 2000). The best characterized examples either belong to the ABC-A (multidrug resistance, MDR) or ABC-G (pleiotropic drug resistance, PDR) protein subfamilies. At the structural level, members of the ABC-A MDR subfamily exhibit the characteristic (TMD$_2$-NBD$_2$) topology, while members of the ABC-G PDR subfamily exhibit the reverse topology (NBD-TMD$_6$). The TMD impart ligand specificity and the NBDs are responsible for binding and hydrolysis of ATP needed to drive the transport of the substrate against a concentration gradient (Kenneth and Higgins, 2007). Fungal ABC transporters play key roles in many cell vital processes including toxin detoxification, secretion of mating peptides and the transport of a broad variety of substrates ranging from simple ions to complex polypeptides (Jones and George, 2004). These proteins can act as biological export machines (Stergiopoulos et al., 2002; De Waard et al., 2006) providing protection against endogenously produced toxic compounds, (example, secondary metabolites such as mycotoxins) and against exogenous toxic compounds from natural or man-made origin (example fungicides, antibiotics, and plant defense compounds), by preventing their cytoplasmic accumulation (De Waard et al., 2006; Coleman and Mylonakis, 2009).

ABC transporters can be involved in providing protection against fungicides. Characteristic for the involvement of ABC transporters in fungicide resistance is the development of multidrug resistance (MDR). MDR is the simultaneous development of resistance to structurally and functionally unrelated compounds. This phenomenon has originally been described in medicine where it is of great clinical significance. Since the early 1990’s, it was established that an active efflux-mechanism based on the ABC1 (Pgp1 glycoprotein) was preventing the adequate intracellular accumulation of anticancer drugs inside cancerous cells (Gottesman et al., 2002; Szakacs et al., 2006; Nikaido 2009; Kuo et al., 2010). Nowadays, MDR has also been widely described in filamentous fungi both of agricultural and medical relevance. AtrB of Aspergillus nidulans mediates resistance to camptothecin and resveratrol, natural toxic metabolites, but additionally AtrB confers resistance to all major classes of fungicides (Andrade et al., 2000). In Botrytis cinerea, a fungal pathogen with a broad host range, the ABC transporter BcatrB is upregulated by resveratrol, a grapevine phytoalexin, and also the fungicide fenpiclonil (Schoonbeek et al., 2001). ABC transporters from the wheat pathogen Mycosphaerella graminicola have substrates ranging from fungicides, plant secondary metabolites, bacterial antibiotics and fungal mycotoxins (Zwiers et al., 2003).

It has been found that in various fungal pathogens, ABC transporters can play a role in pathogenesis (Kretschmer et al., 2009). The first report was on Magnaporthe grisea in which the ABC1 gene, encoding an ABC transporter, was identified in a screening of pathogenicity mutants derived by insertional mutagenesis. Gene-replacement mutants of the ABC1 gene produced a mutant that was arrested in growth early in pathogenesis and unable to detoxify the rice-produced sakuranetin phytoalexin (Urban et al., 1999). Since this report, several papers have correlated the disruption or deletion of particular ABC transporters (especially belonging to the ABC-G subfamily) with a decrease in aggressiveness or loss of pathogenicity. Virulence-related ABC transporters have been described in Botrytis cinerea (Schoonbeek et al., 2001), the necrotrophic fungus Gibberella punicaris (Fleibner et al., 2002), the human pathogen Candida albicans (Theiss et al., 2002), the wheat pathogen M. graminicola (Stergiopoulos et al., 2003) and the causal agent of cereal blight and rot Fusarium culmorum (Skov et al., 2004). Recently, Gupta and Chattoo (2008) reported a second ABC transporter called ABC4, required for pathogenesis in M. grisea. Both virulence-associated ABC transporters, ABC1 and ABC4, are required during early steps in pathogenesis. Abc1 mutants formed appressoria that failed to elaborate extensive infection hyphae, while abc4 mutants were defective in appressoria formation. However, it cannot be ruled out that both transporters have a partial overlap in function. All these findings clearly show that fungal ABC transporters can be involved in pathogenesis and it is possible that multiple members of this large family could be involved in host-fungal interaction in the same species.

Mycosphaerella fijiensis, a hemibiotrophic pathogen, causes the disease known as black Sigatoka, the most important threat for the banana and plantain industry worldwide (Fahleson et al., 2009; Vásquez et al., 2009; Abiala et al., 2010). The fungus affects leaf tissues causing a reduction of photosynthetic area, which leads to premature fruit ripening and loss of production. The methods being used to control M. fijiensis (chemical control and cultural practices) have failed or are ineffi-
cient (Romero and Suton, 1998; Amil et al., 2007; Orozco et al., 2008). Rapid acquisition of resistance to strobilurin (QoI respiration inhibitors) and benzimidazole (interfering with mitosis) fungicides has occurred (Sierotzki et al., 2000; Albertini et al., 1999; Cañaz-Gutiérrez et al., 2006). In both cases, the resistance is the result from a single change at the nucleotide level of target genes. Very little is still known about M. fijiensis pathogenicity or virulence factors. However, we hypothesized that ABC transporters are involved in the virulence of this fungus. Therefore, we set out an in silico strategy to identify putative virulence related ABC transporters in this important pathogen on the basis of homology (Igarashi et al., 2004; Piehler et al., 2008; Seret et al., 2009; Sturm et al., 2009). The closest related fungus with the same infection strategy as M. fijiensis in which a virulence-related ABC transporter has been identified is M. graminicola, a hemibiotrophic pathogen of wheat. Seven ABC transporters denominated MgAtr1 to MgAtr7 have been described in this fungus. Besides MgAtr7 which is involved in the maintenance of iron homeostasis (Zwiers et al., 2007), most of them play a role in providing protection against toxic compounds. A role in pathogenicity has only been attributed to MgAtr4. The expression of MgAtr4 occurs concomitantly with the development of necrotic lesions in infected wheat leaves and MgAtr4 disruption mutants displayed reduced intercellular growth and an impaired capacity to colonize substomatal cavities (Stergiopoulos et al., 2003). Here, we reported the full cloning of the putative MgAtr4 homolog in M. fijiensis, the sequence characterization of MgAtr4 and the analysis of its expression in naturally infected banana leaves with different degrees of black Sigatoka disease. This study is a first step in improving our understanding of the pathogenicity of M. fijiensis on banana.

MATERIALS AND METHODS

Biological material

M. fijiensis strain C1233 was grown on modified solid V8 medium according to Mourichon et al. (1987). Briefly, 200 ml V8 juice were added to 2 g/L CaCO3 and 2% agar-agar, autoclaved, and placed on Petri dishes. Individual plates were inoculated with 16 mm2 mycelium, and left to grow at 26 ± 2 °C, with a 12 h light/12 h dark photoperiod. Liquid V8 culture medium was prepared by the same procedure but without agar. The liquid medium was inoculated with 0.5 ml of M. fijiensis mortar and pestle disaggregated mycelium (1 g mycelium from an active culture disaggregated in 5 ml sterile water), using the same temperature and light conditions stated previously. For DNA extraction, mycelium was harvested after 15 days of culture, filtered through two pieces of fine cheesecloth, weighed, and distributed in portions of 0.3 g mycelium and immediately snap-frozen in liquid nitrogen and stored at -80°C until DNA extraction.

DNA extraction

Genomic DNA extraction was carried out according to Johanson (1997). DNA concentration in samples was determined using a spectrophotometer (Genesys 10 UV).

MgAtr4 cloning

To improve the chance to obtain an ortholog of MgAtr4 from M. fijiensis a two- step strategy was followed. First, the MgAtr4 protein (AAK15314) was analyzed for the presence of particular specific motifs by comparison with the other known ABC-G transporters from M. graminicola; MgAtr1 (CAB46279), MgAtr2 (CAB46280), MgAtr3 (AAK62341), MgAtr5 (AAK62340) and MgAtr7 (EF062310); this strategy successfully identified amino acids characteristic for MgAtr4 (Supplementary 1). Furthermore, to prevent the selection of motifs unique for M. graminicola, MgAtr4 and the other MgAtr5s were aligned with ABC transporter proteins from fungal plant and human pathogen species: CAC40023 (G. pulicaris; Sordariomycete), T30541 (M. grisea; Sordariomycete), CAD10327 (Aspergillus fumigatus; Eurotiomycete), CAF32148 (A. fumigatus; Eurotiomycete), CAC42218 (Emericella nidulans; Eurotiomycete), CAC41639 (Botryotinia fuckeliana; Leotiomycete), AAFO5069 (Candida glabrata; Saccharomycotina), O74676 (C. glabrata; Saccharomycotina), P43071 (Candida albicans; Saccharomycotina), BAC67160 (Botryotinia fuckeliana; Leotiomycete), AAN28699 (Trichophyton rubrum; Eurotiomycete), AAK62810 (Venturia inaequalis; Dothideomycete) and CAA93140 (E. nidulans; Eurotiomycete); a phylogenetic tree was made using MEGA 4.0 (Figure 7). In a second step, a third alignment was developed with sequences of ABC transporters clustering in the same clade with MgAtr4 protein (AAN28699, AAK62810, BAC67160 and CAA93140).

Motifs identified in the first multi-alignment were manually searched in the last one. Degenerated primers were designed on motifs (mentioned from amino to carboxyl ends) EVDKHFP (forward 1; 320 degeneracies), and AFYHPATE (reverse 1; 2728 degenerencies), TFSTAEVLV (forward 2; 2180 degenerencies) and FAHMCIA (reverse 2; 136 degenerencies). Nucleotide sequences of primers are given in Table 1. Amplification was performed by standard polymerase chain reaction (PCR) in 25 µL final volume containing 2 µL of each one of the degenerated primers, 0.2 µM of each dNTP, 0.2 mM MgCl2, 25 ng of M. fijiensis genomic DNA and 1 µL (10 U) Taq DNA polymerase (Invitrogen). PCR cycle conditions were: 4 min of 95°C; followed by 30 cycles of 95°C for 30 s, 60°C for 40 s, and 72°C for 1.2 min; and a final elongation at 72°C for 10 min. The PCR products were analyzed on 1% agarose gel electrophoresis and photographs were taken in a UV-Gel documentation system (Bio Rad). The 1 Kb DNA ladder (Invitrogen) was used as reference for size. The amplicon was cloned in the pGEM-Teasy vector (Promega) according to the manufacturer instructions, transferred into E. coli and then sequenced.

During our research, the full genome sequence of M. fijiensis (JGI, http://genome.jgi-psf.org/cgi-bin/runAlignment?dbs=MycoFungi&advanced=1/) became publicly available and we benefitted from this by using the cloned sequence as query to retrieve the full genomic DNA sequence. Specific primers (ORF-MgAtr4-5’, ORF-MgAtr4-3’, Table 1) were designed on the basis of the downloaded genomic sequence. The complete ORF was amplified by long distance-PCR using similar PCR mixture as above, but using 5 U GoTaq DNA polymerase (Promega). PCR was performed as above, but extension step was for 5.2 min at 72°C each cycle. The PCR product was ligated into pGEM®-T Easy Vector (PGEMEGA) and sequenced.

Determination of intron exon boundaries

RNA from M. fijiensis was obtained according to Islas-Flores (2006)
**Table 1.** List of primers used in this study.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Type</th>
<th>Sequence (5´- 3´)</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>dAtr4-F1</td>
<td>Degenerate</td>
<td>CARGARTGIGAYARCAYTTYCC</td>
<td>dAtr4-F1 + dAtr4-R1</td>
</tr>
<tr>
<td>dAtr4-R1</td>
<td>Degenerate</td>
<td>CIGTIGCIGGRTGRTARAAIGC</td>
<td>Expected the amplification of a MfAtr4 fragment</td>
</tr>
<tr>
<td>dAtr4-F2</td>
<td>Degenerate</td>
<td>GTITTYMGIGGCGIGAYTICCC</td>
<td>dAtr4-F2 + dAtr4-R2</td>
</tr>
<tr>
<td>dAtr4-R2</td>
<td>Degenerate</td>
<td>ATIGCIGCIATRACATRTGIGC</td>
<td>Expected the amplification of a MfAtr4 fragment</td>
</tr>
<tr>
<td>ORF-MfAtr4-5´</td>
<td>Specific</td>
<td>GCCACCATGCCTCAACGGACAAGGAC</td>
<td>ORF-MfAtr4-5´ + ORF-MfAtr4-3´ amplification of complete MfAtr4 ORF (from ATG to TGA)</td>
</tr>
<tr>
<td>ORF-MfAtr4-3´</td>
<td>Specific</td>
<td>CTAAATGATCTGGGCATTCCTCCTATTC</td>
<td></td>
</tr>
<tr>
<td>IFAtr4</td>
<td>Specific</td>
<td>TACGGCTACACATACGATCATG</td>
<td>IFAtr4 + IRAtr4, primers flanking the putative intron</td>
</tr>
<tr>
<td>IRAtr4</td>
<td>Specific</td>
<td>AAGGAAAGCACAGATAGACCAAG</td>
<td></td>
</tr>
<tr>
<td>MfAtr4267F</td>
<td>Specific</td>
<td>GGCTTCCTCTAGCATCGTGCAG</td>
<td>Specific primers to amplify a 267 bp fragment of M. fijiensis MfAtr4 gene</td>
</tr>
<tr>
<td>MfAtr4267R</td>
<td>Specific</td>
<td>GAAGGTCGATGCAATGAAGGAC</td>
<td></td>
</tr>
<tr>
<td>MfAct247F</td>
<td>Specific</td>
<td>CATCACCATTGGCAACGGAC</td>
<td>Specific primers to amplify a 247 bp fragment of M. fijiensis actin gene</td>
</tr>
<tr>
<td>MfAct247R</td>
<td>Specific</td>
<td>GATCTTGACCTTCATGCTG</td>
<td></td>
</tr>
<tr>
<td>Mac267F</td>
<td>Specific</td>
<td>CTGCTGGTATCCATGAGACC</td>
<td>Specific primers to amplify a 267 bp fragment of M. acuminate actin gene</td>
</tr>
<tr>
<td>Mac267R</td>
<td>Specific</td>
<td>CCTTGGGAGATCCACATCTG</td>
<td></td>
</tr>
</tbody>
</table>

and cDNA synthesis was conducted using SuperScript III (Invitrogen) according to supplier's instructions. Primers IFAtr4 and IRAtr4 (Table 1) flanking the putative intron were used to amplify a fragment of MfAtr4, using M. fijiensis gDNA and cDNA as templates. Resulting PCR amplified cDNA or DNA product was ligated into pGEM®–T Easy Vector (PROMEGA) and then sequenced.

**Software and websites for bioinformatics analysis**

Tools to analyze protein structure were used directly in the ExPASy Server (Expert Protein Analysis System), proteomics server of the Swiss Institute of Bioinformatics (SIB) (http://www.expasy.org). The Prosite (Bairoch 1991) was used to determine the Nucleotide Binding Domains (NBDs), TMHMM and SOSUI program (http://www.expasy.org) were used to predict the Transmembrane Domains (TMD’s). Topology prediction was carried out in the PredictProtein website (http://www.predictprotein.org). Fungal ABC PDRs were retrieved by multiple blastp searches against the National Center for Biotechnology Information website and using the M. graminicola ABC transporters (ATRs) as queries.

Phylogenetic analysis was performed with the program package MEGA4 (Tamura et al., 2007) using neighbor-joining algorithm and bootstrapping with 500 replicates. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. In the last phylogenetic analysis, all virulence-associated ABC proteins identified so far were included, independently of the ABC family to which they belong; accession numbers are indicated in the figures. The percent amino acid identity and amino acid similarity between MfAtr4 and MgAtr4 sequences were calculated by pair-wise analyses using the Matrix Global Alignment Tool (MatGAT) v.2.01 (Campanella et al., 2003), and comparing complete sequences or particular domains.

**RT-PCR and MfAtr4 expression analysis at different stages of the interaction M. fijiensis-Musa acuminate cv Grande Naine.**

Banana plants (Musa acuminate cv. Grande Naine) naturally infected with M. fijiensis were collected in an experimental banana plantation located at Uxmal, Yucatan, Mexico.

The plant materials were cotton-cleaned using 70% ethanol, leaf areas showing stages I, II, III, IV and V of Sigatoka disease were excised with sterile knife and immediately stored in liquid nitrogen and then transported to the laboratory. Different stages of the disease were selected according to Fouré (1985).

Total RNA was obtained using the Concert® reagent (Invitrogen) according to the instructions of the manufacturer (0.25 g leave tissues/1.5 ml reagent).

Total RNA samples (5 μg/10 µL) were independently DNase I (Sigma) treated for 30 min at room temperature. RNA samples were ethanol precipitated, air dried by 5 min and resuspended in distilled sterile RNAse-free water (10 µL). Of each RNA sample, 2 μg was used as template for cDNA synthesis using the SuperScript III RT-PCR kit (Invitrogen), according to instructions of the manufacturer. Subsequently, 500 ng of cDNA was used independently for RT-PCR, with primers to amplify fragments of the M. fijiensis genes, MfAtr4 (amplicon 267 bp) and actin (247 bp), and the M. acuminate actin (267 bp) (sequences of primers in Table 1).

As negative control, uninfected banana were included. The result was a representative of at least three independent experiments.
RESULTS

Cloning and in silico characterization of MfAtr4

The degenerated PCR amplification yielded few unspecific bands and also a ~1095 bp amplicon (Figure 1a) that was purified and cloned. Two clones were picked and 940 bp sequenced; both clones yielded an identical sequence (Figure 1b). The BlastX analysis using the sequence of the 940 bp DNA fragment as query against the NCBI database gave highest hit with MgAtr4 (E = 2e^-124), showing amino acid 73% identity and 82% amino acid similarity. Upon the availability of the whole genome sequence of M. fijiensis, the 940 bp nucleotide sequence was also used to query the whole genome sequence of M. fijiensis by BLASTN, which resulted in one hit with a gene with local 98% homology with the query. This gene was annotated as MfAtr4. Pair-wise comparison of the deduced full amino acid sequences of MfAtr4 and MgAtr4 results in 73% identity and 82% similarity. Furthermore, in silico PCR with these degenerate primers and annotated ABC-G genes in the M. fijiensis genome predicted only short amplicons (51-104 nt; data not shown), thus validating our approach.

MfAtr4 was amplified from the deduced translational start to translational stop (ATG to TAG), and this resulted in an amplicon of 4977 nucleotides that was fully sequenced twice in two independent clones to rule out possible PCR or sequencing errors. Comparison of the MfAtr4 nucleotide sequence obtained in this study (M. fijiensis strain C1233) to the sequence from the M. fijiensis genome portal (isolate CIRAD86) indicated 99.1% identity. Most changes were silent. A comparison of protein level between the MfAtr4 from isolate C1233 and from CIRAD86 indicated that the predicted proteins exhibited a 99.8% similarity and a 99.7% identity. In general, changes were conservative, that is- glutamine to histidine at the C-terminal end, alanine to valine in NBD1, isoleucine to alanine in TMS2 and lysine to arginine in NBD2-TMS7 linkage.

Figure 1. MfAtr4 genomic fragment amplified by degenerated PCR. (A) PCR product separated on a 1% agarose gel. The arrow indicates the DNA band with the expected size, which was purified from gel and cloning for sequencing. (B) The nucleotide sequence obtained for two independent clones.
Figure 2. Two-dimensional topological model of MfAtr4. The model shows the 12 transmembrane helices, the two NBDs, the six extracellular loops (ECL 1–6) and the four intracellular loops (ICL 1–4), with amino and carboxyl terminal ends and NBD motifs oriented toward cytoplasm. SOSUI website was used to deduce structure and PredictProtein website for topology. Number of first and last amino acids in each NBD and TMD are indicated.

Features of MfAtr4

The predicted MfAtr4 structure consisted of two hydrophilic nucleotide binding domains (NBDs) located at the cytoplasmic surface, and two transmembrane domains (TMDs). Within each of the putative TMD (amino acid residues 613 to 880 and 1279 to 1566), six membrane-spanning segments (TMS) were predicted. The amino and carboxyl ends of the protein are oriented toward the cytoplasm (Figure 2). Four small intracellular loops were predicted (ICLs), ICL1 (25 amino acids), ICL2 (8 amino acids), ICL3 (32 amino acids) and ICL4 (13 amino acid) and all them inside of the cell. On the extracellular side, MfAtr4 has four small extracellular loops (ECLs), ECL1 (11 amino acids), ECL4 (10 amino acids), ECL5 (5 amino acids), and two large ECLs (ECL3, between TMS5/6, and ECL6, between TMS11/12) of 77 and 91 amino acid residues, respectively.

The amino terminal Walker A and Walker B motifs of MfAtr4 (GRPGSG\(\text{CST}\) and LAAWDNSTRGLD) are degenerated when compared to the canonical motifs (Walker A: GXXGXG\(\text{K/S/T}\), Walker B: \(\phi\phi\phi\phi\text{D}\), where \(\phi\) is any hydrophobic amino acid), (Walker et al., 1982). The conserved lysine in the Walker A motif is replaced in MfAtr4 by a cysteine amino acid (Figure 3). Walker motifs are flanking the ABC signature motif of MfAtr4, sequence GVSGGERKRVSSAEMA (canonical sequence is LSGGQ). The Walker A motif of the C-terminal NBD of MfAtr4 (GTSGAGKT) contains the canonical lysine; the Walker B sequence is LLFDEPTSGGLD and the second signature ABC sequence LNVEQRKLTIGVELAA (Figure 3).

MfAtr4 classification

MfAtr4 has the predicted NBD-TMS\(_{6}\)-NBD-TMS\(_{6}\) topology (Figure 2; Table 3). This topology is characteristic for the ABC-G transporter sub-family, in contrast to the reverse (TMS\(_{6}\)-NBD\(_{2}\)) topology observed in the ABC-A, ABC-B (MDR), ABC-C (MRP) and ABC-D sub-families (Table 3; Kovalchuk and Driessen, 2010). The predicted topology of MfAtr4 corresponds to the topology of eukaryotic-type exporters (Igarashi et al., 2004; Cannon et al., 2009; Coleman and Mylonakis, 2009).

Comparative analysis with MgAtr4

Comparison of the deduced MfAtr4 protein with MgAtr4 showed 63.2% identity and 74.4% similarity on amino acid level. Major differences between MfAtr4 and MgAtr4...
proteins are at the N-(cytosolic stretch of amino acids before NBD1) and C-(cytosolic stretch of amino acids after TMS12) terminal ends. Both proteins were 73.1% identical and 84.5% similar when comparing from NBD1 to TMS12 (Table 2). In contrast to MgAtr4 that lacks introns, MfAtr4 is predicted to contain an intron of 52 nucleotides (Figure 4; Table 3), which splits the gene in two exons of 2979 and 1998 nucleotides. Amplification of a fragment of MfAtr4 on gDNA and cDNA with primers flanking the putative intron resulted in amplicons with different sizes (Figure 4a). Sequencing of the CDS fragment corroborates the occurrence of the 52 nucleotides intron at the predicted position (Figure 4b). Comparison of the sizes of MfAtr4 PCR products amplified with different combinations of primer pairs, and using M. fijiensis gDNA and cDNA, excluded the presence of other introns in this gene (data not shown).

**Phylogenetic relationship between MfAtr4 and other ABCs involved in virulence**

Phylogenetic analysis of multiple ABC-G proteins from several fungi indicated that MgAtr4 and MfAtr4 clustered together and in a different clade than the other ABC transporters with a proven function in pathogenicity. Virulence-associated ABC transporters fall in three different PDR-(ABC-G) subgroups and one non-ABC-G group. One ABC-G clade contains MgAtr4 and MfAtr4, the second ABC-G cluster consists of ABC1 from M. grisea, ABC1 from G. pullicaris and ABC1 from F. culmorum and the third cluster contain B. fuckeliana BcAtrB. The non ABC-G group includes C. albicans MLT1 (a MRP ABC transporter) and M. grisea ABC4 (a MDR ABC transporter) and cluster separate from all the other virulence-associated ABC-G members (Figure 5).

**Table 2.** Pair-wise comparison of MfAtr4 and MgAtr4.

<table>
<thead>
<tr>
<th>Comparison of:</th>
<th>% Identity</th>
<th>% similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete amino acid sequences</td>
<td>63.2</td>
<td>74.4</td>
</tr>
<tr>
<td>From NBD1 to TMS12</td>
<td>73.1</td>
<td>84.5</td>
</tr>
<tr>
<td>NBD1</td>
<td>78.9</td>
<td>91.6</td>
</tr>
<tr>
<td>TMD1</td>
<td>75.7</td>
<td>88.4</td>
</tr>
<tr>
<td>NBD2</td>
<td>91.8</td>
<td>96.3</td>
</tr>
<tr>
<td>TMD2</td>
<td>74.6</td>
<td>86.1</td>
</tr>
</tbody>
</table>

**Phylogenetic relationship between MfAtr4 and other fungal ABCs**

All ABC transporters which cluster with MfAtr4 belonged to fungi in the Pezizomycotina subphylum’s (S. cerevisiae, C. albicans, K. lactis) and the Basidiomycetes phylum (Ustilago maydis, Cryptococcus neoformans, Coprinopsis cinerea). As expected, MfAtr4 clustered in the same clade as the ABC transporters, initially used in the design of the degenerated primers (Figure 5).
Figure 4. Presence of intronic sequence in MfAtr4. (A) Amplification of a fragment of MfAtr4 with primers flanking the expected intron. Lane 1, amplicon obtained by using M. fijiensis cDNA as template; lane 2, using M. fijiensis gDNA as template; Mw, molecular markers. (B) Comparison of the nucleotide sequences obtained in each case. Red letters show nucleotides in gDNA which are absent in the cDNA.

Table 3. Comparative analysis of MfAtr4 and MgAtr4 features.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MfAtr4</th>
<th>MgAtr4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class</td>
<td>PDR (ABC-G family)</td>
<td>PDR (ABC-G family)</td>
</tr>
<tr>
<td>CDS size (ATG-TGA)</td>
<td>4977</td>
<td>4908</td>
</tr>
<tr>
<td>Peptide (number of amino acids)</td>
<td>1658</td>
<td>1635</td>
</tr>
<tr>
<td>Introns</td>
<td>One</td>
<td>None</td>
</tr>
<tr>
<td>Topology</td>
<td>(NBD-TMS6)2</td>
<td>(NBD-TMS6)2</td>
</tr>
<tr>
<td>Function</td>
<td>Exporter</td>
<td>Exporter</td>
</tr>
<tr>
<td>Role in virulence</td>
<td>Not determined</td>
<td>Yes</td>
</tr>
<tr>
<td>Walker A-1</td>
<td>VLGRPGSCGST</td>
<td>VLGRPGSCGST</td>
</tr>
<tr>
<td>Q-loop 1</td>
<td>VGTL</td>
<td>VGTL</td>
</tr>
<tr>
<td>Signature-1</td>
<td>VSGGERKRSIAEMA</td>
<td>VSGGERKRSIAEMA</td>
</tr>
<tr>
<td>Walker B-1</td>
<td>LAAWDSTRGLD</td>
<td>LAAWDSTRGLD</td>
</tr>
<tr>
<td>Walker A-2</td>
<td>GTSGAGKTT</td>
<td>GTSGAGKTT</td>
</tr>
<tr>
<td>Q-loop 2</td>
<td>VQQQD</td>
<td>VQQQD</td>
</tr>
<tr>
<td>Signature-2</td>
<td>LNVERORLTTGVELAA</td>
<td>LNVERORLTTGVELAA</td>
</tr>
<tr>
<td>Walker B-2</td>
<td>LLFLDEPTSGLD</td>
<td>LLFLDEPTSGLD</td>
</tr>
<tr>
<td>Symmetry</td>
<td>Asymmetric</td>
<td>Asymmetric</td>
</tr>
</tbody>
</table>
Analysis of expression of *MfAtr4* in black Sigatoka-infected banana leaves

Symptomatic plant material showing visual stages I, II, III, IV and V of the Sigatoka disease was selected in the field and then each stage individually harvested for the analysis (Figure 6, panel A). The actin genes from *M. fijiensis* and *M. acuminata* were used as reference genes (Figure 6, panels C, D). *Mf*-actin expression was lower at stages I and II than in later stages (Figure 6, panel C), which is congruent with the fungal biomass increment in the banana tissues with the disease progress (Arzanlou,...
Figure 6. Analysis of expression of *MfAtr4* in field samples of *Musa acuminate* cv. Grande Naine with black-Sigatoka disease, at different stages. The photographs show the material used for this analysis (A). Reverse transcription–polymerase chain reactions (RT-PCR) of *MfAtr4* (B). RT-PCR of *M. fijiensis* actin, as reference fungal gene (C). RT-PCR of *M. acuminata* actin, as reference plant gene (D). cDNA prepared from healthy banana leaves was included as negative control.

RT-PCR revealed the expression of *MfAtr4* in banana infected material and its probable temporal regulation during the infection process (Figure 6, panel B). Compared to the *Mf-actin* expression, the *MfAtr4* expression was highest in the initial infection stages and decreased with the progress of the necrotrophic phase (stages III and later). The seemingly complete absence of *MfAtr4* expression during the necrotrophic stage V was very striking and could definitely not be attributed to the absence of fungal biomass. Panel D shows the expression of the *M. acuminata* actin as reference gene.

**DISCUSSION**

**Cloning**

The degenerated primers enabled us to get a fragment of *MfAtr4* in the first attempt. Therefore, these primers could be suitable to clone MgAtr4 homologs from closely related fungi with no available genomes and other Dothideomycetes, particularly in the order capnodiales to which *M. graminicola* and *M. fijiensis* belong. In addition, this strategy for designing primers could be extrapolated to clone other particular members or subfamilies in the ABC transporter family, or in other large gene families (kinases and permeases).

Comparison at nucleotide level of *MfAtr4* as cloned in this study and *MfAtr4* from the *M. fijiensis* genomic portal showed an identity of 99.7% and a similarity of 99.8%. This suggested a low degree of polymorphism in *MfAtr4*. Single nucleotide polymorphisms with similar degree occur in PDR5, an important ABC transporter implicated in pleiotropic drug resistance in *S. cerevisiae* (Guan et al., 2010), and also in *Candida glabrata* CDR1 (Haque et al., 2007), an ortholog of ScPDR5. In this ABC protein, the polymorphism, although low, is supposed to be significant for azole resistance. Some reports show that virulence-associated ABC transporters can contribute to resistance against fungicides and other cytotoxic xenobiotics (Gupta and Chattoo, 2008; Schoonbeek et al., 2001; Zwiers et al., 2003), but occurrence and contribution of polymorphism to tolerance to natural substrates or xenobiotics in these or other classes of fungal ABC transporters remains to be determined.

**MfAtr4 classification**

The predicted *MfAtr4* topology (NBD-TMS6-NBD-TMS6), the presence of a cysteine residue in the N-terminal Walker A motif instead of a lysine residue, and the specific LNVEQ motif in the C-terminal ABC signature are all characteristics of a full-sized ABC-G (PDR) type transporter sensu stricto (Seret et al., 2009; Figure 3; Table 3). Many members of the ABC-G (PDR) family are involved in the prevention of the intracellular accumulation of toxicants (Cannon et al., 2009; Coleman and Mylonakis, 2009). Except for one, all the virulence-associated ABC transporters identified so far in fungal phytopathogens are members of the PDR family of ABC transporters. The only exception is ABC4 of *M. grisea* that belongs to the ABC-B (MDR) family (Gupta and Chattoo, 2008; Coleman and Mylonakis, 2009).
Features of MfAtr4

The ABC signature in the N-terminal NBD of MfAtr4 is canonical while the signature in the C-terminal NBD is degenerated; an asymmetric organization that is quite common in fungal ABC transporters (Rai et al., 2006; Preeti et al., 2006; Ernst et al., 2008; Cannon et al., 2009). The conserved lysine in the N-terminal Walker A motif is replaced in MfAtr4 by a cysteine amino acid (Figure 3).

This seems to be a feature characteristic for most of the fungal ABC-G transporters (Preeti et al., 2006), but the functional relevance of the change of the lysine by the cysteine amino acid is unknown.

Phylogenetic relationship between MfAtr4 and other fungal ABCs

MfAtr4 clusters in a different clade than other PDR virulence associated ABC transporters. This suggests that fungal ABC transporters with roles in pathogenicity might have diversified in different times. Virulence-associated ABC1 transporter members are apparently ancient since they cluster with Cryptococcus neoformans (a Basidiomycete fungus) PDRs, suggesting these PDRs existed before the diversification of the major fungal lineages Ascomycetes and Basidiomycetes. MgAtr2 and MgAtr7 fall in this clade (Figure 5). Similar to other ABC families (ABC-B, ABC-C subfamilies) that are all present as multigene families in the genome of eukaryotic fungal species (Kovalchuk and Driessen, 2010), the fungal ABC-G (PDR) family might have become expanded by a series of gene duplications (Lupski, 2007; Seret et al., 2009).

PDR transporters have taken a massive expansion in fungal genomes, especially in species belonging to the Pezizomycotina group, and several groups of these proteins are specific for this subphylum (Kovalchuk and Driessen, 2010). This seems to be the case of the clade containing the MfAtr4 and the MgAtr4. All ABC transporter proteins in this clade are PDRs from fungi belonging to the Pezizomycotina group, belonging to the classes Dothideomycetes, Leotiomycetes, Eurotiomycetes and Sordariomycetes, thus suggested that these PDRs evolved after the divergence of the main fungal lineages.

MfAtr4 and MgAtr4 fall in a different clade than MgAtr1, MgAtr2, MgAtr3, MgAtr5, and MgAtr7, the other ABC-G transporters identify in M. graminicola. Each of these PDR members clustered separately from each other (Figure 5). They are paralogous among themselves, but according to the phylogenetic tree, with putative orthologues in other fungi. Because of the complexity of the PDR family, this is common in fungi (Cannon et al., 2009; Kovalchuk and Driessen, 2010).

Intron in MfAtr4

ABC transporters grouping in the same clade as MgAtr4 (Figure 7) have no introns. However, this is not a characteristic feature of genes present in the clade clustering with MfAtr4 (in Figure 5). Fifty percent of the PDRs in this clade contain 4 to 6 introns, but remarkably the Dothideomycetes PDRs in this clade (Venturia inaequalis, Pyrenophora tritic-repens, Phaeosphaeria nodorum, Alternaria brassicicola) have no introns, thereby suggesting that the intron is a recent gain in MfAtr4. Occurrence of intron gain in fungal individual genes or gene families has been previously reported. Nielsen et al. (2004) analyzed in silico a set of orthologous 1-phos-phoribosyl-5-pyrophosphate (PRPP) synthetase genes and found a significant higher number of introns in N. crassa (six introns) and in M. grisea (fourteen introns) as compared to the PRPPs of other fungi. Nielsen et al. (2004) suggested that intron gain is a significant driving force that might be involved in the evolution of genes in fungi. Haugen et al. (2004) aligned Ascomycete and Basidiomycete S788 intron family and inferred that S788 gained access to Basidiomycete by lateral transferring and vertical inheritance. Puntal deletion events in S788 introns (example, by unequal crossing over, or by stepwise deletion) drive to genetic changes. In Aspergillus, intron gain is the outcome of the error-prone repair of DNA mediated by the capture of DNA fragments during non-homologous end joining of double strand breaks; intron gain or loss is the dynamics of evolution that cause changes in the rates of mutations, thus, introducing variants (mutation bias) or transmitting variants which may further be fixed or eliminated by selection (Zhang et al., 2010; Farlow et al., 2011).

As mentioned above, the PDR ABC transporter family is rapidly evolving in this kingdom, particularly by gene duplication (Coleman and Mylonakis, 2009). In addition to gene duplication, intron gain may be contributing to the evolution of individual genes; such seems to be the case of MfAtr4. Except MgAtr4, all PDR-ABC transporter encoding genes in M. graminicola contain introns, 19 introns in Mgatr7 (Zwiers et al., 2007), supporting a potential important role of introns in fungal PDR gene evolution.

Is MfAtr4 an ortholog of MgAtr4?

ABC transporter orthologs can be identified by neighborhood and similarity searches (Seret et al., 2009). Eukaryotic ABC transporters have no substrate binding component as prokaryotes, but ligand recognition and specificity are mediated by the TMS (Igarashi et al., 2004). The active pocket has to allocate a variety of structurally different compounds because most ABC transporters can have multiple substrates. Congruent with their function, these structural components are the most divergent regions in ABC transporters. When TMDs are used as BLAST queries, generally this retrieves only proteins belonging to the same subfamily (Kovalchuk and
The first MfAtr4 fragment (obtained in this study) contained the MTS1 and MTS2; when this fragment was used as query to Blast the GenBank it retrieved as first hit the MgAtr4.

Considering that MgAtr4 and MfAtr4 cluster in the same clade in the phylogenetic tree (Figure 5) and the high overall similarity between them (Tables 2 and 3), we hypothesized that MfAtr4 is the ortholog of MgAtr4. Although, *M. graminicola* belongs to the same genus as *M. fijiensis*, it was phylogenetically more distant to *M. fijiensis* than other *Mycosphaerella* species. Closer phylogenetic relatives of *M. fijiensis* are *M. musicola* and *M. eumusae*, *M. africana*, *M. keniensis*, *M. marksii*, among many others (Carlier et al., 2000; Goodwin et al., 2001). Therefore, orthologs of MfAtr4 may exist in other *Mycosphaerella* species.

**Analysis of expression of MfAtr4 in black Sigatoka-infected banana leaves**

It was previously demonstrated that ABC4 from *M. grisea* (Gupta and Chatoor, 2008) and MgAtr4 from *M. graminicola* (Stergiopoulos et al., 2003), are involved in fungal virulence of these plant pathogens. Disruption or deletion of these genes reduced the ability of mutant strains to colonize the hosts. Molecular analysis of the full infection process using an artificial infection assay of wheat with *M. graminicola* showed no expression of MgAtr4 during biotrophic phase. MgAtr4 expression was observed from days 12 to 18 post-inoculation, while at day 22 no expression of MgAtr4 was detectable; these times corresponded to the early/middle and late necrotrophic phase respectively (Stergiopoulos et al., 2003).
A similar pattern of expression was found in this study for \textit{MfAtr4} gene in field samples of black Sigatoka infected-banana leaves (Figure 6, panel B). The expression of \textit{MfAtr4} was higher at early necrotrophic stages in comparison with later stages of the fungal infection. As \textit{MgAtr4} in \textit{M. graminicola}-wheat pathosystem, \textit{MfAtr4} expression was undetectable in the late necrotrophic phase of \textit{M. fijiensis}. The positive expression of the fungal reference gene (\textit{Mf-actin}) indicated that the absence of \textit{MfAtr4} transcripts at this stage was not due to the absence of fungal biomass, but could be explained by assuming a regulation dependent on the disease progress. This suggests a role of \textit{MfAtr4} during the early-middle stages of the disease progress, although a role of \textit{MfAtr4} during the biotrophic phase of \textit{M. fijiensis} cannot be ruled out. Further exploration of \textit{MfAtr4} expression during the biotrophic stages of black Sigatoka disease is therefore necessary.

Taking together the analysis presented here, it is suggested that \textit{MfAtr4} could play a role in \textit{M. fijiensis} pathogenesis, similar to the role previously described for ABC4 and \textit{MgAtr4} of \textit{M. grisea} and \textit{M. graminicola}, respectively. A number of reports have proposed that virulence-associated ABC transporters may be primarily involved in protection against exogenous compounds (Urban et al., 1999; Del Sorbo et al., 2000; Fleibner et al., 2002; Stefanato et al., 2009). Therefore, although its role in the efflux of fungal secondary metabolites or virulence factors cannot be discarded (Cruz-Cruz et al., 2009; Chuc-Uc et al., 2011), \textit{MfAtr4} could be involved in the efflux of banana defense toxic compounds, example, preformed pytoalexin-like compounds as well as phytoalexins (Cruz-Cruz et al., 2010) or in inducible banana phytoalexins (Lazzaro et al., 2004). Research is currently being conducted to analyze the role of \textit{MfAtr4} in \textit{M. fijiensis} virulence and its probable role in detoxification of banana toxicants.

**ACKNOWLEDGEMENTS**

We are grateful to the Joint Genome Institute for the facilities provided to gain access to the sequence of the \textit{M. fijiensis} genome, and to CONACyT for the economical support to project No. 45788Z and for the Ph. D., scholarship No. 204766 to Y. Couoh-Uicab.

**REFERENCES**


Supplementary material 1. ClustalX alignment of amino acid sequences of *Mycosphaerella graminicola* Atrs. Identical amino acids are shaded in black and conservative substitutions are shaded in grey.

M._graminicola_Atr2_CAB46280
M._graminicola_Atr7_EF062310
M._graminicola_Atr4_AAK15314
M._graminicola_Atr1_CAB46279
M._graminicola_Atr5_AAK62340
M._graminicola_Atr3_AAK62341

**consensus**

M._graminicola_Atr2_CAB46280
M._graminicola_Atr7_EF062310
M._graminicola_Atr4_AAK15314
M._graminicola_Atr1_CAB46279
M._graminicola_Atr5_AAK62340
M._graminicola_Atr3_AAK62341

**consensus**

M._graminicola_Atr2_CAB46280
M._graminicola_Atr7_EF062310
M._graminicola_Atr4_AAK15314
M._graminicola_Atr1_CAB46279
M._graminicola_Atr5_AAK62340
M._graminicola_Atr3_AAK62341

**consensus**

M._graminicola_Atr2_CAB46280
M._graminicola_Atr7_EF062310
M._graminicola_Atr4_AAK15314
M._graminicola_Atr1_CAB46279
M._graminicola_Atr5_AAK62340
M._graminicola_Atr3_AAK62341

**consensus**

M._graminicola_Atr2_CAB46280
M._graminicola_Atr7_EF062310
M._graminicola_Atr4_AAK15314
M._graminicola_Atr1_CAB46279
M._graminicola_Atr5_AAK62340
M._graminicola_Atr3_AAK62341

**consensus**
**Supplementary material 2.** ClustalX alignment of amino acid sequences of fungal ABC transporters from the subclade clustering the MgAtr4 in supplement 2. The arrows indicate the amino acids used to design the degenerated primers after manual reviewing of the alignment to discard *M. graminicola* specific motifs in MgAtr4. Beginning and end of the arrows indicate the first and last amino acid used for each primer.

Trichophyton_rubrum_AAN28699
Botryotinia_fuckeliana_BAC6716
Venturia_inaequalis_AAK62810
Mycosphaerella_graminicola_Atr4
Emericella_nidulans_CAC42218

Trichophyton_rubrum_AAN28699
1 __________________________M
Botryotinia_fuckeliana_BAC6716
1 __________________________MER
Venturia_inaequalis_AAK62810
1 ————G
Mycosphaerella_graminicola_Atr4
1 ————T
Emericella_nidulans_CAC42218
1 MSS

Trichophyton_rubrum_AAN28699
29 RPELVRA-
Botryotinia_fuckeliana_BAC6716
28 TPQILTY-
Venturia_inaequalis_AAK62810
57 HLYRRNS-
Mycosphaerella_graminicola_Atr4
59 GINTADHA-
Emericella_nidulans_CAC42218
61 STETARE-

Trichophyton_rubrum_AAN28699
49 APT
Botryotinia_fuckeliana_BAC6716
48 FIW
Venturia_inaequalis_AAK62810
103 SQHQTRSST
Mycosphaerella_graminicola_Atr4
119 NNTNEDEAA
Emericella_nidulans_CAC42218
89 SHOTKYSTHIEN

Trichophyton_rubrum_AAN28699
107 MFRNL
Botryotinia_fuckeliana_BAC6716
76 VFNK
Venturia_inaequalis_AAK62810
161 VFNK
Mycosphaerella_graminicola_Atr4
177 AFK
Emericella_nidulans_CAC42218
148 SFNL

Trichophyton_rubrum_AAN28699
167 LGRP
Botryotinia_fuckeliana_BAC6716
134 LGRP
Venturia_inaequalis_AAK62810
220 LGRP
Mycosphaerella_graminicola_Atr4
237 LGRP
Emericella_nidulans_CAC42218
208 LGRP

Trichophyton_rubrum_AAN28699
227 LVGGTLE-
Botryotinia_fuckeliana_BAC6716
194 LVGGTLE-
Venturia_inaequalis_AAK62810
280 LVGGTLE-
Mycosphaerella_graminicola_Atr4
297 LVGGTLE-
Emericella_nidulans_CAC42218
268 LVGGTLE-
<table>
<thead>
<tr>
<th>Sequence Name</th>
<th>Start Position</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trichophyton rubrum_AAN28699</td>
<td>286</td>
<td>GGERKVSIAEALSATPICDONSTRGLSDATALEFKLAKGSGYSSHQCLAIVAS</td>
</tr>
<tr>
<td>Botryotinia fuckeliana_BAC6716</td>
<td>254</td>
<td>GGERKVSIAEMALAGSPTDNARGLDALEFKSLRITANLEGSCGLAIVAS</td>
</tr>
<tr>
<td>Venturia inaequalis_AAK62810</td>
<td>329</td>
<td>GGERKVSIAEMALAGSPTDNARGLDALEFKSLRITANLEGSCGLAIVAS</td>
</tr>
<tr>
<td>Mycosphaerella graminicola_Atr</td>
<td>356</td>
<td>GGERKVSIAEALSATPICDONSTRGLSDATALEFKLAKGSGYSSHQCLAIVAS</td>
</tr>
<tr>
<td>Emericella nidulans_CAC42218</td>
<td>327</td>
<td>GGERKVSIAEALSATPICDONSTRGLSDATALEFKLAKGSGYSSHQCLAIVAS</td>
</tr>
<tr>
<td>Trichophyton rubrum_AAN28699</td>
<td>346</td>
<td>QAIAKDFDKTVLYEGRQIEGPIRIAKYFYEMGTYCEBQTTDFTSLTNPSRIRG</td>
</tr>
<tr>
<td>Botryotinia fuckeliana_BAC6716</td>
<td>314</td>
<td>QAIAKDFDKTVLYEGRQIEGPIRIAKYFYEMGTYCEBQTTDFTSLTNPSRIRG</td>
</tr>
<tr>
<td>Venturia inaequalis_AAK62810</td>
<td>399</td>
<td>QAIAKDFDKTVLYEGRQIEGPIRIAKYFYEMGTYCEBQTTDFTSLTNPSRIRG</td>
</tr>
<tr>
<td>Mycosphaerella graminicola_Atr</td>
<td>416</td>
<td>QAIAKDFDKTVLYEGRQIEGPIRIAKYFYEMGTYCEBQTTDFTSLTNPSRIRG</td>
</tr>
<tr>
<td>Emericella nidulans_CAC42218</td>
<td>387</td>
<td>QAIAKDFDKTVLYEGRQIEGPIRIAKYFYEMGTYCEBQTTDFTSLTNPSRIRG</td>
</tr>
<tr>
<td>Trichophyton rubrum_AAN28699</td>
<td>406</td>
<td>EGYEKVVRPAEVEPYKGSQKNKLLANMMPFAPEMPEEHL---KLRFTGAEAG</td>
</tr>
<tr>
<td>Botryotinia fuckeliana_BAC6716</td>
<td>374</td>
<td>EGYEKVVRPAEVEPYKGSQKNKLLANMMPFAPEMPEEHL---KLRFTGAEAG</td>
</tr>
<tr>
<td>Venturia inaequalis_AAK62810</td>
<td>459</td>
<td>EGYEKVVRPAEVEPYKGSQKNKLLANMMPFAPEMPEEHL---KLRFTGAEAG</td>
</tr>
<tr>
<td>Mycosphaerella graminicola_Atr</td>
<td>476</td>
<td>EGYEKVVRPAEVEPYKGSQKNKLLANMMPFAPEMPEEHL---KLRFTGAEAG</td>
</tr>
<tr>
<td>Emericella nidulans_CAC42218</td>
<td>447</td>
<td>EGYEKVVRPAEVEPYKGSQKNKLLANMMPFAPEMPEEHL---KLRFTGAEAG</td>
</tr>
<tr>
<td>Trichophyton rubrum_AAN28699</td>
<td>462</td>
<td>AKHTASKSPYRVSPPMKLCTVARYRLWQKSTTATSSQAINALLIGSFDFEPFT</td>
</tr>
<tr>
<td>Botryotinia fuckeliana_BAC6716</td>
<td>430</td>
<td>AKHTASKSPYRVSPPMKLCTVARYRLWQKSTTATSSQAINALLIGSFDFEPFT</td>
</tr>
<tr>
<td>Venturia inaequalis_AAK62810</td>
<td>515</td>
<td>AKHTASKSPYRVSPPMKLCTVARYRLWQKSTTATSSQAINALLIGSFDFEPFT</td>
</tr>
<tr>
<td>Mycosphaerella graminicola_Atr</td>
<td>536</td>
<td>AKHTASKSPYRVSPPMKLCTVARYRLWQKSTTATSSQAINALLIGSFDFEPFT</td>
</tr>
<tr>
<td>Emericella nidulans_CAC42218</td>
<td>503</td>
<td>AKHTASKSPYRVSPPMKLCTVARYRLWQKSTTATSSQAINALLIGSFDFEPFT</td>
</tr>
<tr>
<td>Trichophyton rubrum_AAN28699</td>
<td>522</td>
<td>TDFFAKSGVAFVFAVLLNLALSTIN-----------------GLDARPIVKRVLNAF</td>
</tr>
<tr>
<td>Botryotinia fuckeliana_BAC6716</td>
<td>490</td>
<td>TDFFAKSGVAFVFAVLLNLALSTIN-----------------GLDARPIVKRVLNAF</td>
</tr>
<tr>
<td>Venturia inaequalis_AAK62810</td>
<td>575</td>
<td>TDFFAKSGVAFVFAVLLNLALSTIN-----------------GLDARPIVKRVLNAF</td>
</tr>
<tr>
<td>Mycosphaerella graminicola_Atr</td>
<td>596</td>
<td>TDFFAKSGVAFVFAVLLNLALSTIN-----------------GLDARPIVKRVLNAF</td>
</tr>
<tr>
<td>Emericella nidulans_CAC42218</td>
<td>563</td>
<td>TDFFAKSGVAFVFAVLLNLALSTIN-----------------GLDARPIVKRVLNAF</td>
</tr>
<tr>
<td>dAtr4-R1</td>
<td>566</td>
<td>YHFEALAGIVADIPKFDLATNPNIIYFICGLESARKEPEPLLPIITLMSAER</td>
</tr>
<tr>
<td>Trichophyton rubrum_AAN28699</td>
<td>534</td>
<td>YHFEALAGIVADIPKFDLATNPNIIYFICGLESARKEPEPLLPIITLMSAER</td>
</tr>
<tr>
<td>Botryotinia fuckeliana_BAC6716</td>
<td>619</td>
<td>YHFEALAGIVADIPKFDLATNPNIIYFICGLESARKEPEPLLPIITLMSAER</td>
</tr>
<tr>
<td>Venturia inaequalis_AAK62810</td>
<td>540</td>
<td>YHFEALAGIVADIPKFDLATNPNIIYFICGLESARKEPEPLLPIITLMSAER</td>
</tr>
<tr>
<td>Mycosphaerella graminicola_Atr</td>
<td>623</td>
<td>YHFEALAGIVADIPKFDLATNPNIIYFICGLESARKEPEPLLPIITLMSAER</td>
</tr>
<tr>
<td>Emericella nidulans_CAC42218</td>
<td>566</td>
<td>YHFEALAGIVADIPKFDLATNPNIIYFICGLESARKEPEPLLPIITLMSAER</td>
</tr>
</tbody>
</table>