

Full Length Research Paper

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

Y. Couoh-Uicab¹, I. Islas-Flores^{1*}, N. Kantún-Moreno², L.-H. Zwiers³, M. Tzec-Simá², S. Peraza-Echeverría², L. Brito-Argáez¹, L. Peraza-Echeverría², R. Grijalva-Arango², A. James², C. Rodríguez-García², and B. Canto-Cánchez²

¹Unidad 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.

²Unidad de Biotecnología, Centro de Investigación Científica de Yucatán A.C., Calle 43 No. 130, Colonia Chuburná de Hidalgo, C.P. 97200, Mérida, Yucatán, México.

³CBS-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. *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 *M. fijiensis*. Here, we reported the full cloning of *MfAtr4* a putative ortholog of *MgAtr4*, an ABC transporter of the related *Mycosphaerella graminicola* with a function in virulence. Similarities and differences with its presumed ortholog *MgAtr4* are described, and the putative function of *MfAtr4* are discussed. Analysis of *MfAtr4* gene expression in field banana samples exhibiting visible symptoms of black Sigatoka disease indicated a higher expression of *MfAtr4* during the first symptomatic stages in comparison to the late necrotrophic phases, suggesting a role for *MfAtr4* in the early stages of pathogenic development of *M. fijiensis*.

Key words: ABC transporters, virulence factors, *MgAtr4* ortholog, *Mycosphaerella fijiensis*, black Sigatoka, *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 Driessens, 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 Driessens, 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).

*Corresponding author. E-mail: islasign@cicy.mx. Tel: +52 (999) 9 42 83 30. Fax: +52 (999) 9 81 39 00.

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 TMD₂-NBD₂ 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 (TMD₆-NBD)₂ topology, while members of the ABC-G PDR subfamily exhibit the reverse topology (NBD-TMD₆)₂. The TMDs 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 pulicaris* (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 (Qo 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 *MfAtr4* 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).

MfAtr4 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 *MgAtrs* 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), AAF05069 (*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 degenerancies), and AFYHPATE (reverse 1; 2728 degenerancies), TFSTAEVLV (forward 2; 2180 degenerancies) and FAHMCAA (reverse 2; 136 degenerancies). 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 µM 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 DOC photodocumentation 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?db=Mycfi1&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-MfAtr4-5', ORF-MfAtr4-3', 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 (PROMEGA) 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.

Primer name	Type	Sequence (5'-3')	Observation
dAtr4-F1	Degenerate	CARGARGTIGAYAACRCACTTYCC	dAtr4-F1 + dAtr4-R1
dAtr4-R1	Degenerate	CIGTIGCIGGRTGRTARAAGC	Expected the amplification of a <i>MfAtr4</i> fragment
dAtr4-F2	Degenerate	GTITTYMGIMGIGGICAYGTICC	dAtr4-F2 + dAtr4-R2
dAtr4-R2	Degenerate	ATIGCIGCIATRCACATRTGIGC	Expected the amplification of a <i>MfAtr4</i> fragment
ORF-MfAtr4-5'	Specific	GCCACCATGTCGTCAACGGACAAGGAC	ORF-MfAtr4-5' + ORF-MfAtr4-3' Amplification of complete <i>MfAtr4</i> ORF (from ATG to TGA)
ORF-MfAtr4-3'	Specific	CTAAATGATCTGGGCATTCCCTATTG	
IFAtr4	Specific	TACGGCTACACATACGATCATG	IFAtr4 + IRAtr4, primers flanking the putative intron
IRAtr4	Specific	AAGGAAAGCACAGATAGACCAAG	
MfAtr4267F	Specific	GGTCTTCTCTACGATCGTGCAG	Specific primers to amplify a 267 bp fragment of <i>M. fijiensis</i> <i>MfAtr4</i> gene
MfAtr4267R	Specific	GAAGGTCGATGCATAGATCAAGAAG	
MfAct247F	Specific	CATCACCAATTGGCAACGAGC	Specific primers to amplify a 247 bp fragment of <i>M. fijiensis</i> actin gene
MfAct247R	Specific	GATCTTGACCTTCATGCTGG	
Mac267F	Specific	CTGCTGGTATCCATGAGACC	Specific primers to amplify a 267 bp fragment of <i>M. acuminata</i> actin gene
Mac267R	Specific	CCTTGGAGATCCACATCTGC	

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 acuminata* cv Grande Naine.

Banana plants (*Musa acuminata* 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. acuminata* 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.

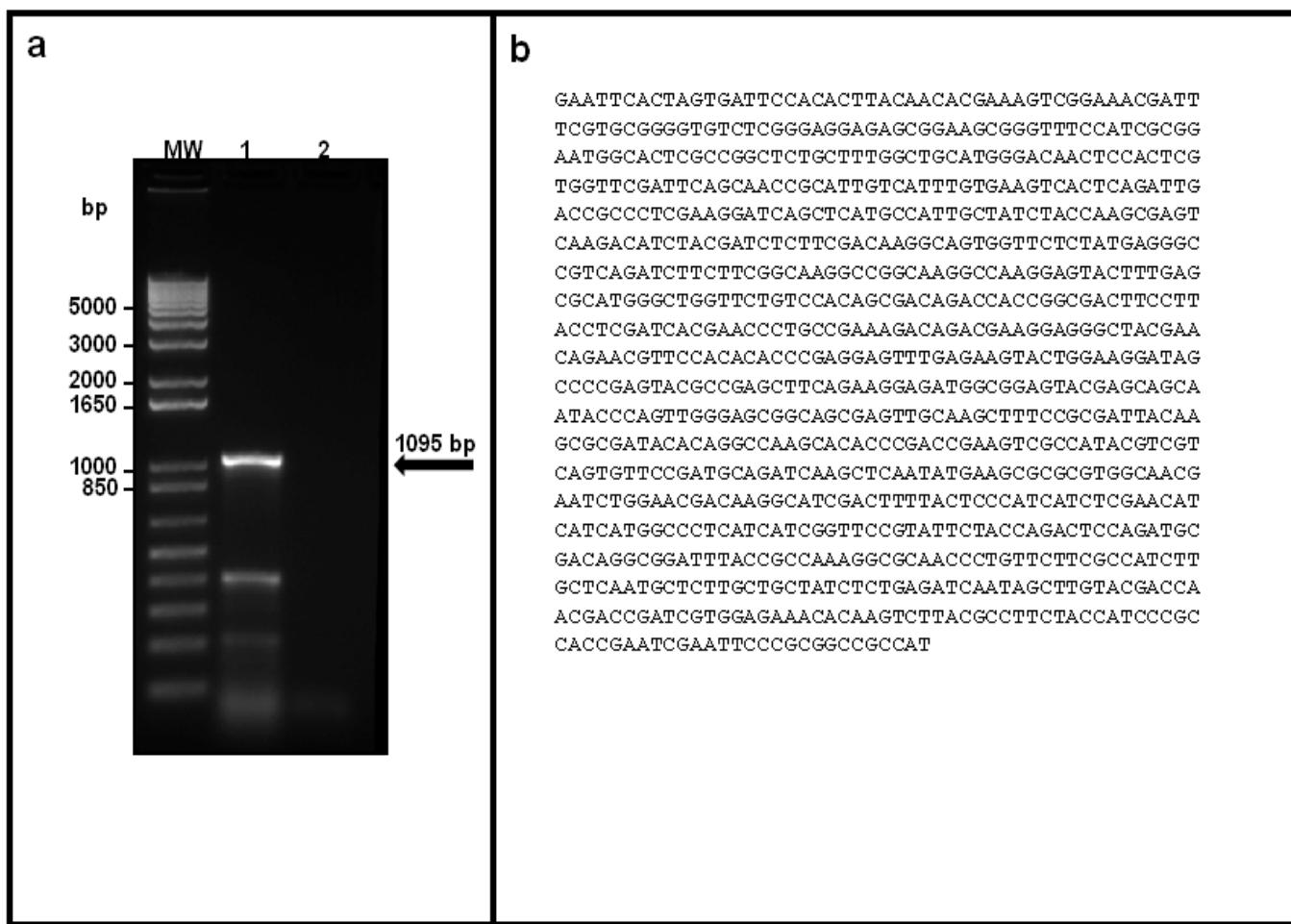


Figure 1. *MfAtr4* genomic fragment amplified by degenerated PCR. (A) PCR product separated on a 1% agarose gel. The arrow indicates the DNA band with the expected size, which was purified from gel and cloning for sequencing. (B) The nucleotide sequence obtained for two independent clones.

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.

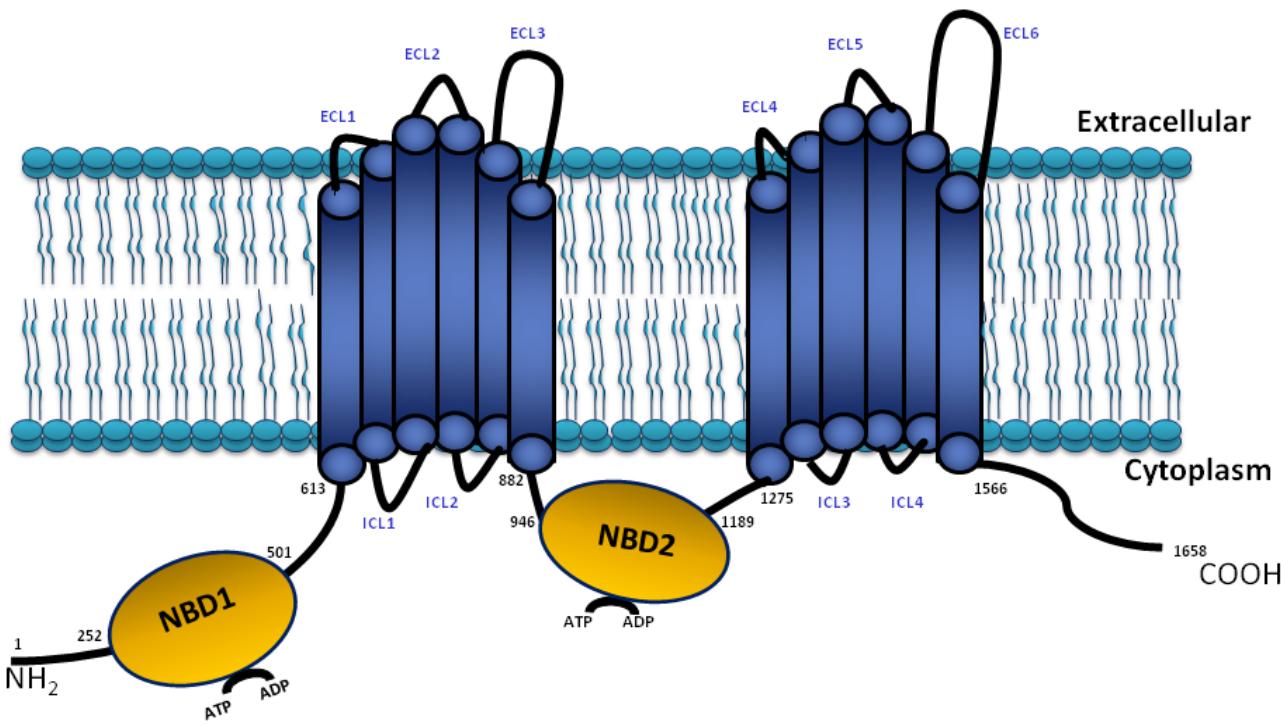


Figure 2. Two-dimensional topological model of MfAtr4. The model shows the 12 transmembrane helices, the two NBDs, the six extracellular loops (ECL 1–6) and the four intracellular loops (ICL 1–4), with amino and carboxyl terminal ends and NBD motifs oriented toward cytoplasm. SOSUI website was used to 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 (5 amino acids), ECL2 (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 (GRPGSGCST and LAAWDNSTRGQLD) are degenerated when compared to the canonical motifs (Walker A: GXXGXGKS/T, Walker B: ϕϕϕD, where ϕ 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

GVSGGERKRVSIAEMA (canonical sequence is LSGGGQ). The Walker A motif of the C-terminal NBD of MfAtr4 (GTSGAGKT) contains the canonical lysine; the Walker B sequence is LLFLDEPTSGLD and the second signature ABC sequence LNVEQRKLLTIGVELAA (Figure 3).

MfAtr4 classification

MfAtr4 has the predicted NBD-TMS₆-NBD-TMS₆ topology (Figure 2; Table 3). This topology is characteristic for the ABC-G transporter sub-family, in contrast to the reverse (TMS₆-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

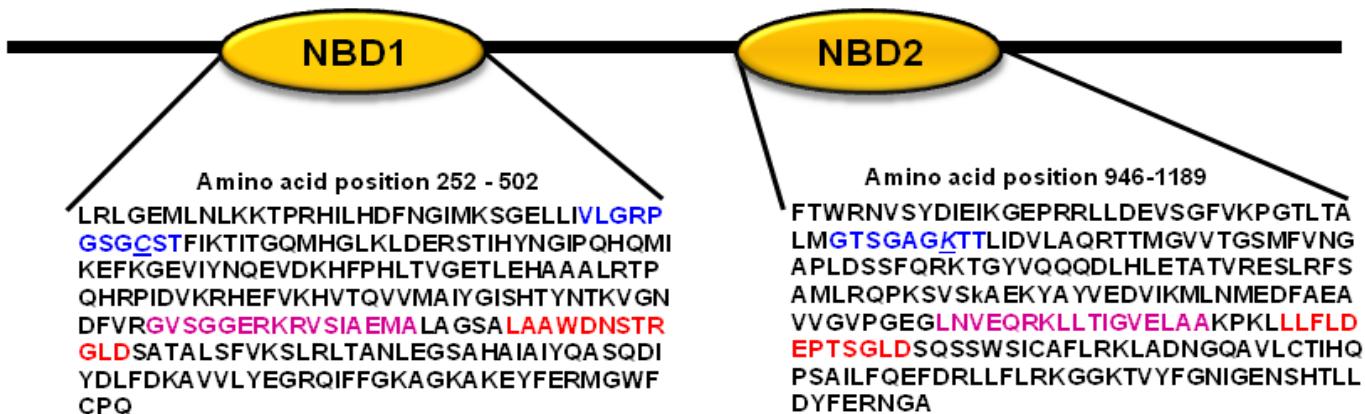


Figure 3. Prosite program identification of the two NBD domains in MfAtr4. NBDs were located 252 to 502 and 846-1189 residues downstream to the amino terminal end. Sequences of Walker A, Walker B and the signature sequence in each NBD domain are indicated with blue, red and pink letters respectively. The cysteine amino acid in the Walker A in NBD1 and the equivalent position of lysine in NBD2 are indicated with cursive underlined letters.

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

Comparison of:	% Identity	% similarity
Complete amino acid sequences	63.2	74.4
From NBD1 to TMS12	73.1	84.5
NBD1	78.9	91.6
TMD1	75.7	88.4
NBD2	91.8	96.3
TMD2	74.6	86.1

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 fungal ABCs

All ABC transporters which cluster with MfAtr4 belonged to fungi in the Pezizomycotina subphylum. This group is separated from the ABC-G proteins in the Saccha-

romycotina 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).

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. pulicaris* 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).

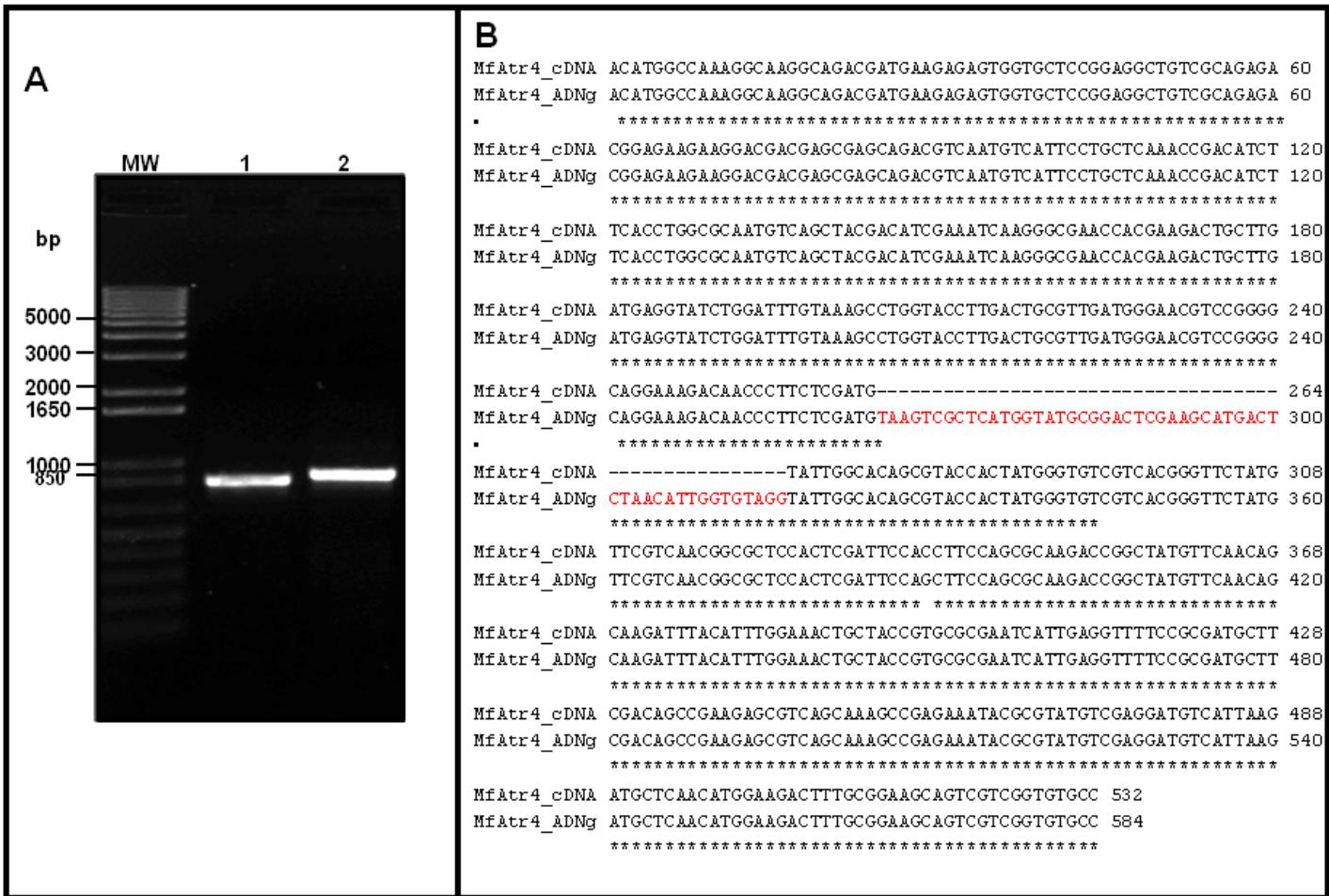


Figure 4. Presence of intronic sequence in *MfAtr4*. (A) Amplification of a fragment of *MfAtr4* with primers flanking the expected intron. Lane 1, amplicon obtained by using *M. fijiensis* cDNA as template; lane 2, using *M. fijiensis* gDNA as template; Mw, molecular markers. (B) Comparison of the nucleotide 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.

Parameter	MfAtr4	MgAtr4
Class	PDR (ABC-G family)	PDR (ABC-G family)
CDS size (ATG-TGA)	4977	4908
Peptide (number of amino acids)	1658	1635
Introns	One	None
Topology	(NBD-TMS ₆) ₂	(NBD-TMS ₆) ₂
Function	Exporter	Exporter
Role in virulence	Not determined	Yes
Walker A-1	VLGRPGSGCST	VLGRPGSGCST
Q-loop 1	VGETL	VGQTL
Signature-1	VSGGERKRVSIAEMA	VSGGERKRVSIAEMA
Walker B-1	LAAWDNSTRGLD	LAAWDNSTRGLD
Walker A-2	GTSGAGKTT	GTSGAGKTT
Q-loop 2	VQQD	VQQD
Signature-2	LNVEQRKLLTIGVELAA	LNVEQRKLLTIGVELAA
Walker B-2	LLFLDEPTSGLD	LLFLDEPTSGLD
Symmetry	Asymmetric	Asymmetric

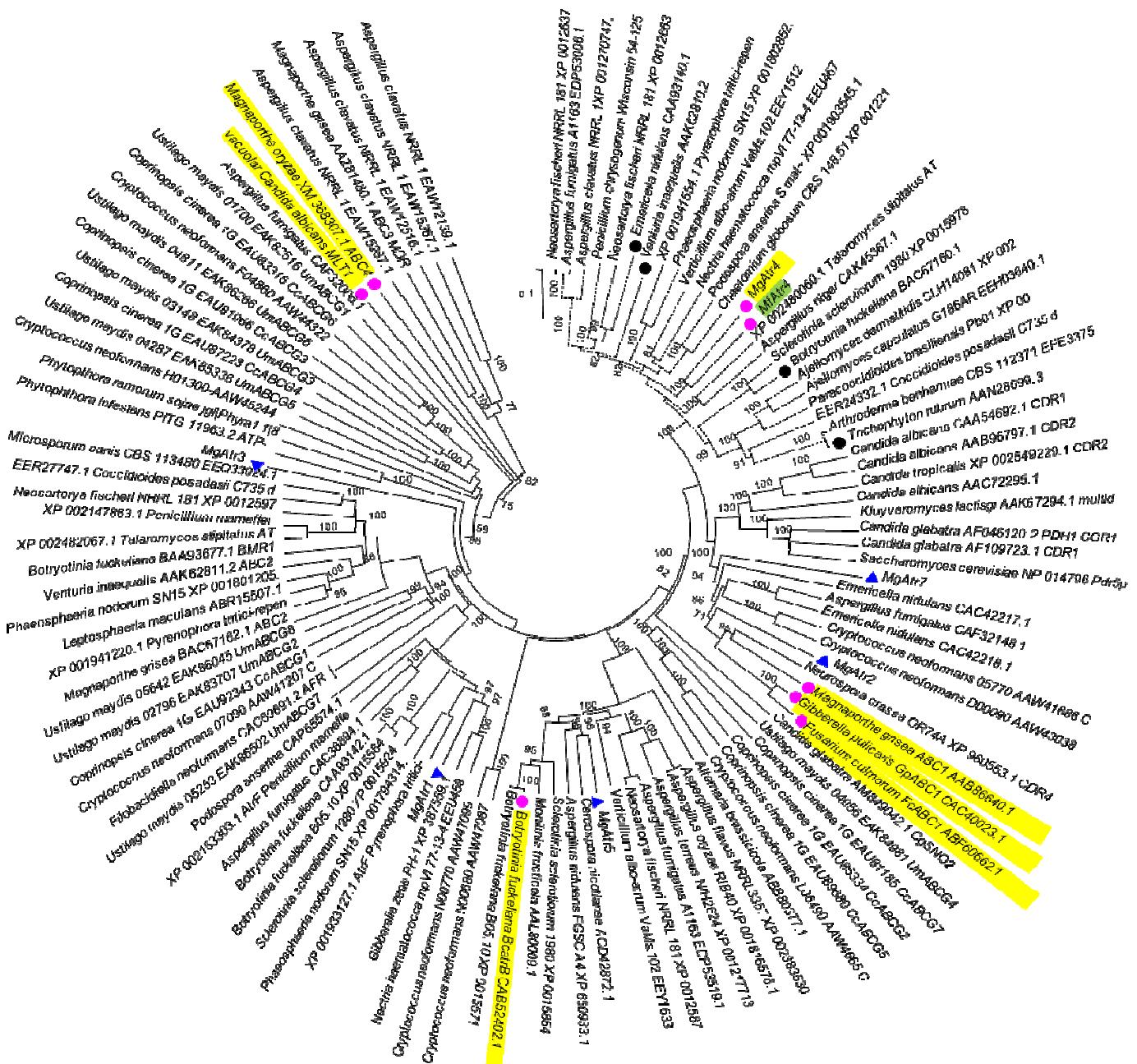


Figure 5. Phylogenetic tree of ABC transporters related to MfAtr4. The evolutionary history was inferred using the Neighbor-Joining method with the MEGA program version 4. Numbers on the branches indicate the percentage of 500 bootstrap replications (only >50% are shown). GenBank accession numbers are given for each sequence, except for *M. graminicola* Atrs (MgAtr1-7) and MfAtr4. MgAtrs are highlighted with blue triangles. Virulence-associated ABC transporters are highlighted with pink circles and yellow labeled (MfAtr4 is highlighted in green). The clade clustering the MfAtr4 is indicated with dotted branches. The ABC transporters clustering with MgAtr4 in Figure 7 (tree which helped to design the degenerated primers to amplify the first fragment of MfAtr4) are highlighted with black circles.

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

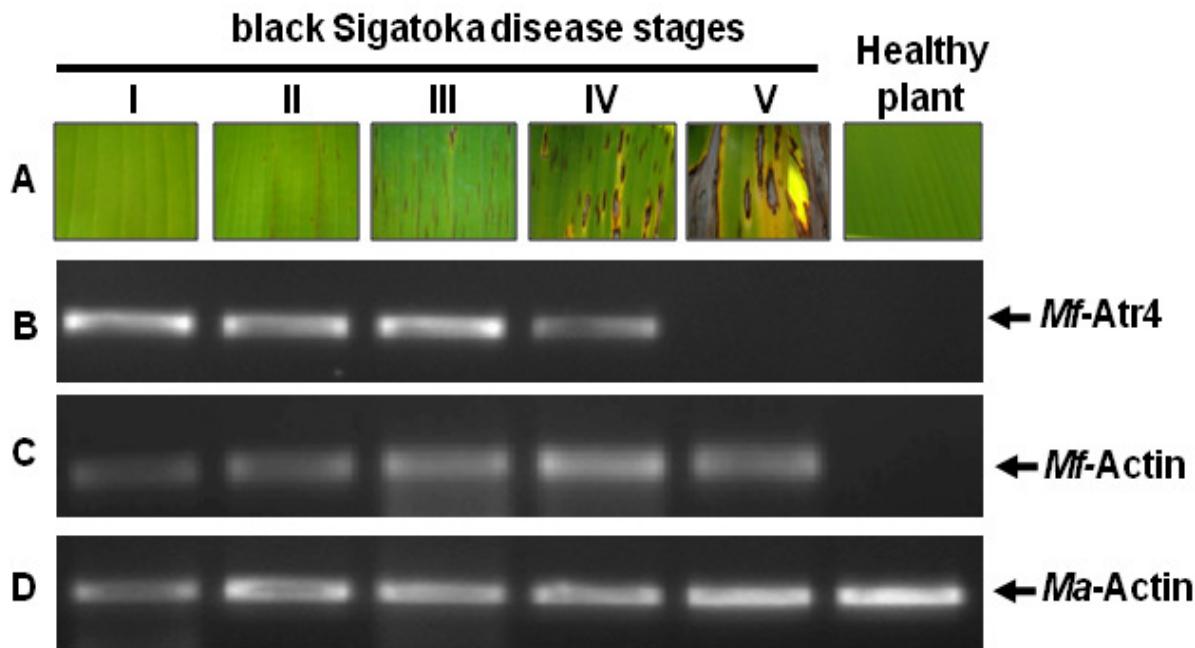


Figure 6. Analysis of expression of *MfAtr4* in field samples of *Musa acuminate* cv. Grande Naine with black-Sigatoka disease, at different stages. The photographs show the material used for this analysis (A). Reverse transcription–polymerase chain reactions (RT-PCR) of *MfAtr4* (B). RT-PCR of *M. fijiensis* 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.

et al., 2007). 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 (Kovalchuc 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 identified 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 tritici-repentis*, *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-phorbosyl-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. Punctual 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 neigh-

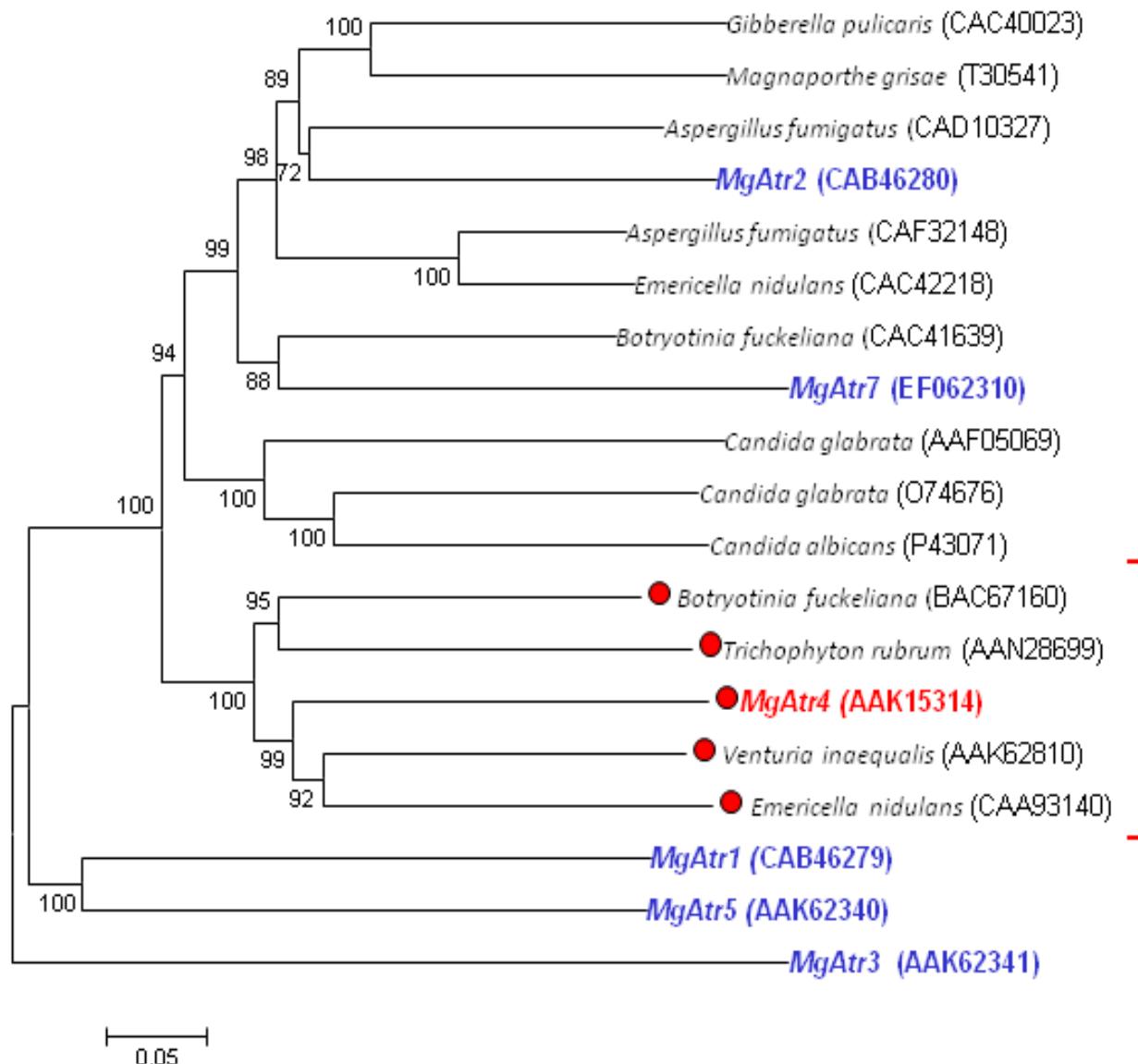


Figure 7. Neighbor-joining phylogenetic tree of MgAtrs and other fungal ABC (PDR) transporters. MgAtrs are highlighted with blue written names, except MgAtr4 which is in red. The sub-clade clustering MgAtr4 is indicated with a red bracket. Red circles indicate ABC transporters selected for a next alignment to search and exclude *M. graminicola* specific motifs in MgAtr4. GenBank accession numbers are given in parentheses after the name of each fungus species. The tree was constructed using the MEGA program version 4. Numbers on the branches indicate the percentage of 500 bootstrap replications.

borhood 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 Driessen, 2010). 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 Chatoo, 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 *MfAtr4* gene in field samples of black Sigatoka infected-banana leaves (Figure 6, panel B). The expression of *MfAtr4* was higher at early necrotrophic stages in comparison with later stages of the fungal infection. As *MgAtr4* in *M. graminicola*-wheat pathosystem, *MfAtr4* expression was undetectable in the late necrotrophic phase of *M. fijiensis*. The positive expression of the fungal reference gene (Mf-actin) indicated that the absence of *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 *MfAtr4* during the early-middle stages of the disease progress, although a role of *MfAtr4* during the biotrophic phase of *M. fijiensis* cannot be ruled out. Further exploration of *MfAtr4* expression during the biotrophic stages of black Sigatoka disease is therefore necessary.

Taking together the analysis presented here, it is suggested that *MfAtr4* could play a role in *M. fijiensis* pathogenesis, similar to the role previously described for ABC4 and *MgAtr4* of *M. grisea* and *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), *MfAtr4* could be involved in the efflux of banana defense toxic compounds, example, preformed phytoprotectants as banana phytoanticipins (Cruz-Cruz et al., 2010) or inducible banana phytoalexins (Lazzaro et al., 2004). Research is currently being conducted to analyze the role of *MfAtr4* in *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 *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.

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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 1 -----

M.graminicola_Atr7_EF062310 1 -----

 MNASNSSLIASDPDGICEKILDVILEYALHKFGDTKQKLALGRPKFLETIAGFVVQGRCI

M.graminicola_Atr4_AAK15314 1 -----

M.graminicola_Atr1_CAB46279 1 -----

M.graminicola_Atr5_AAK62340 1 -----

M.graminicola_Atr3_AAK62341 1 -----

 consensus 1

M.graminicola_Atr2_CAB46280 1 -----

M.graminicola_Atr7_EF062310 61
 QMCLPAFPFKSSNKIDKVLTLPDKAEEELALGRLNTMCAKVQAIHAPGAALTIISDGLVY

M.graminicola_Atr4_AAK15314 1 -----

M.graminicola_Atr1_CAB46279 1 -----

M.graminicola_Atr5_AAK62340 1 -----

M.graminicola_Atr3_AAK62341 1 -----

 consensus 61

M.graminicola_Atr2_CAB46280 1 -----

M.graminicola_Atr7_EF062310 121
 NDLLSISDKDTWAYGEALRSMAIAHEFQHIRFARIRDLIKFPGSEVLNEITYVASATNFR

M.graminicola_Atr4_AAK15314 1 -----

M.graminicola_Atr1_CAB46279 1 -----

M.graminicola_Atr5_AAK62340 1 -----

M.graminicola_Atr3_AAK62341 1 -----

 consensus 121

M.graminicola_Atr2_CAB46280 1 -----

M.graminicola_Atr7_EF062310 181
 RSLLNEFGKDDIDIDHEIATSEDTKMTLYGYRRFLESDLKHIFPLGSDRSANSYKRNVKF

M.graminicola_Atr4_AAK15314 1 -----

M.graminicola_Atr1_CAB46279 1 -----

M.graminicola_Atr5_AAK62340 1 -----

M.graminicola_Atr3_AAK62341 1 -----

 consensus 181

M.graminicola_Atr2_CAB46280 1 -----

M.graminicola_Atr7_EF062310 241
 LAKEIIIRGYAFAGAVRAAFPEHLRLSIHQSTGEHKISISL|HTKTGFTT|WHCSVARLA

M.graminicola_Atr4_AAK15314 1 ---MATGGASTPIVGDYNPERVEQLFDIPRPEQGN--
 IY|EAENAART|SGAQESQLS

M.graminicola_Atr1_CAB46279 1 -----MWGYSVDERK|QREDTNGP|TNQWHNAQR

M.graminicola_Atr5_AAK62340 1 -----MT|QR-----SSSWGVTG

M.graminicola_Atr3_AAK62341 1 -----

 consensus 241 | ps

M.graminicola_Atr2_CAB46280 1 -----M|ARMASESKIDDFG--DEKSNRHSPSN|DTLMKEKNE|SPG|V|SP

M.graminicola_Atr7_EF062310 301
 D|TWVSAQKADFELQD|M|MRMICENGGRPSYFQE|EASEQHFVPTQAFGLGGAKMDGSH|LG

M.graminicola_Atr4_AAK15314 55 G-----S|PG|INTADHADDSYLTANGREAAESIN|DDK|KSSGFNSDD|QPIN

M.graminicola_Atr1_CAB46279 30
 T|GTHPTTEEVEGEGEG|WGENDVGGFTTRQAMEDYEALRKDLTQLSKTRSRT|QHSLKRT

M.graminicola_Atr5_AAK62340 15 T-----EEVYRTLSRTFSNRPKEE

M.graminicola_Atr3_AAK62341 1 -----MAT|S|SR

 consensus 301 g t m de h d ks s ds

*M.graminicola*_Atr2_CAB46280 44 S TSINE NKEVNCLARRFSEISGWNEIPSNPLEA K -----TEL L
*M.graminicola*_Atr7_EF062310 361 ATELSLYANETLSQSTSEDFATLKEQVVVANTH V PTTEQLINTFLRHDYPPL K
*M.graminicola*_Atr4_AAK15314 101 AEERNIL RLATNS SRRSMNN NEDPEALQRT TLDGL
*M.graminicola*_Atr2_CAB46279 90 TTGQTAKSGRKSLT SRQATH SEAAEQDVEA PQEEVE-----ESKKDDD
*M.graminicola*_Atr5_AAK62340 35 TYPESTSS-----SDDSTKKAADW LTEELK-----QTQQQNE
*M.graminicola*_Atr3_AAK62341 9 ASRSRF DWDAIFLPDLSP PTTVAFEGKLKPKN MVP-----
 consensus 361 s t t r etls t et g davg dp s

*M.graminicola*_Atr2_CAB46280 89 PN KART V SMLKFSKQD-
 GRTPMRTG AFRN SAH F LATDY KDVGNIW LDSI
*M.graminicola*_Atr7_EF062310 421 DS EARS L NFEGLIEANRASN SKQIV SYR S EAFWQPSDY K FWNQP AIIDT
*M.graminicola*_Atr4_AAK15314 149 PR DLYK L LTLKLVNDE--
 DIKI RS LAFK H S S SALNL P VSSM SAPLR-
*M.graminicola*_Atr1_CAB46279 135
 DEDDFELDRFMREGHFEKRS DGTSD RV VYK T K I STTS VR LPDA GT GP
*M.graminicola*_Atr5_AAK62340 68 N-----DGAKD KL ITWT DIK I ADAA A---ENV SQ N-
*M.graminicola*_Atr3_AAK62341 48 -----VPTVASAVLSTITL PWRVGQRL RR HETPRKPI S-
 consensus 421 fe wvk krvgv frdsvhg g t fqktv lia f

*M.graminicola*_Atr2_CAB46280 148 -----
 VKKLFGMESKR RID QD E LES V P F TLT ETH
*M.graminicola*_Atr7_EF062310 481 -----AQKLTASRNVKRS KKCD I H Q SIA ELDQ
*M.graminicola*_Atr4_AAK15314 206 -----
 GEMFSMAKKPHKQ RS D MKS L I SLT QMH
*M.graminicola*_Atr1_CAB46279 195
 DLFKIIICRFVPALAKRTGETRTL NG T C D M F AISNNRET
*M.graminicola*_Atr5_AAK62340 104 ---IPKK KEGRQKPP--
 LKT VDKSH C KP A TS ILANRRL
*M.graminicola*_Atr3_AAK62341 84 -----LF P V ASAEP AE T Q RA VR---
 consensus 481 i k rrsil feglvr GEmlIVlgrp gsgCstl Lkltg g

dAtr4-F1

*M.graminicola*_Atr2_CAB46280 200
 FQVDKNSYMN FQ AGE SVF S I TA V I Q E T T RFI G
*M.graminicola*_Atr7_EF062310 533
 LR GNTTYMN Q M GHV EF QA T HQ KE E A CESIP
*M.graminicola*_Atr4_AAK15314 258
 LTMDEKTT H N DQKQ I EFQ VI Q V K H EH AL MSQQRL
*M.graminicola*_Atr1_CAB46279 255 YAEVTG-
 DVS G ADKQK MY V Q D AT N W FI LMN KTKKETG
*M.graminicola*_Atr5_AAK62340 158 YAEIDG-DVK GSMDH-
 KQAQQY QI M T EELF T MD T MKV YNV S
*M.graminicola*_Atr3_AAK62341 112 -----VSYP V FCSAD PHV E L R RL AVRSPSA
 consensus 541 f v s m yqgipa mhk frgeavy n eddvhfp LtVgqtlefaarar pr pg

M_graminicola_Atr2_CAB46280 260 DME [] -
 NVYAEHM [] VV [] Y [] S [] R [] YL [] T [] S [] S [] A [] LQA
M_graminicola_Atr7_EF062310 593 GVN [] -
 DTYVTHV [] AYI [] LR [] IAD [] A [] L [] V [] AVARS [] Q []
M_graminicola_Atr4_AAK15314 318 GTS [] -
 QSAVEYLQVV [] VY [] LS [] Y [] MA [] A [] S [] LAA
M_graminicola_Atr1_CAB46279 314 N[P]-----
 VIAEA [] K [] P [] KY [] L [] D [] TLASKST [] V []
M_graminicola_Atr5_AAK62340 216
 NFSSAKELQQAQ [] F [] KSM [] E [] DD [] EY [] L [] TMAARAT [] V []
M_graminicola_Atr3_AAK62341 153 EDDA-----VEL [] RL [] R[ESIR] [] L [] AV [] I [] R [] L [] V [] L [] S [] AN [] L []
 consensus 601 v r mrdvl mamfgi ht nTkvGndfvRGvSGGErkRvsiaEall g avvc

M_graminicola_Atr2_CAB46280 319
 [] S [] N [] VE [] CKSV [] LGCELG [] SAAM [] AI [] PRAA [] Q [] I [] Q [] Y []
M_graminicola_Atr7_EF062310 652
 [] S [] A [] D [] VQT [] TS [] IA [] T [] IA [] PQSV [] N [] S [] Q []
M_graminicola_Atr4_AAK15314 377
 [] S [] T [] IKA [] LN [] LV [] SAHA [] AI [] SQA [] D [] A [] E []
M_graminicola_Atr1_CAB46279 367
 [] AS [] DYARS [] VMT [] VSNR [] TL [] GEG [] VM [] L [] D [] E [] YS []
M_graminicola_Atr5_AAK62340 276
 [] AS [] EYTRCV [] AMT [] VL [] LSSI [] GNG [] L [] D [] KE [] Y []
M_graminicola_Atr3_AAK62341 205
 F [] DL [] A [] HCVQC [] SV [] AYRL [] IFAS [] SA [] SDT [] AT [] QLVA [] RD [] HQV [] G []
 consensus 661 wDnsTRGLD atAlef vkslRlmadl gst mvlyqA qaiYelfDkvivlyeGr iff

M_graminicola_Atr2_CAB46280 379
 [] NIHAG [] T [] Q [] D [] P [] M [] AV [] VVKP [] W [] KV [] S [] E [] AA [] KA []
M_graminicola_Atr7_EF062310 712
 [] E [] E [] EPKP [] A [] RI [] KDFVG [] I [] A [] FVV [] QK []
M_graminicola_Atr4_AAK15314 437
 [] K [] V [] K [] M [] Y [] S [] G [] N [] A [] QL [] E [] A [] G [] EKY [] HD []
M_graminicola_Atr1_CAB46279 427
 [] KE [] RQ [] I [] YEAE [] E [] A [] D [] V [] KF [] K [] HKA [] KG [] EALEK [] FRE []
M_graminicola_Atr5_AAK62340 336
 [] M [] Q [] PFM [] V [] TDGANV [] G [] V [] T [] RI [] DE [] F [] NA [] EVRA [] YQK []
M_graminicola_Atr3_AAK62341 265 PTAVRPCGD [] AQTQPAP-----
 P [] A [] YFDHLHARIAPRASSP [] AH [] MAD [] FRR []
 consensus 721 Gpas ak yfeDlzf cpdrqtadfltsvtsp errvrkgvedr prtpddf awkkS

M_graminicola_Atr2_CAB46280 439 [] TR [] K [] Q [] E [] ER--RF [] FKGEA--
 YQQ [] VDSRKAQ [] A [] H [] F [] YLQ [] CQV []
M_graminicola_Atr7_EF062310 772 QQFKH [] QD [] DKFNE--SN [] IGGPS--
 LEE [] RN [] RRSLQE [] Q [] SR [] F [] L [] LPS [] IDL []
M_graminicola_Atr4_AAK15314 497
 [] E [] T [] QKE [] QGHTKEGST [] SATSSGTSKLSAASKNDNQA [] HA [] P [] VV [] VPM [] KLN []
M_graminicola_Atr1_CAB46279 487 [] N [] KVLE [] T [] EN---
 YLKETDYNDARE [] ED [] VQDG [] S [] RVSN [] S [] V [] FQR [] LA []
M_graminicola_Atr5_AAK62340 396 -NIKARMEQEY [] SD---TEEAKTCTQT--
 [] CE [] VQAE [] H [] LPK [] L [] FYT [] QTS []
M_graminicola_Atr3_AAK62341 316 AH [] ADTAVCLAT [] LA-----EEQASSGSSNQSPRHQKRHAMNRK [] VAL
 consensus 781 pqyqkliiddi dy p g f darn k ks r kspytis qv vc

M. graminicola Atr2 CAB46280 495
 LW|GYRR|CA|PEL|Y|LFM|S|V|TL|KLT|ASFYQ|AVI|GTMM|FG
M. graminicola Atr7 EF062310 828
 W|GFR|R|KR|MGI|S|IF|S|SIV|V|G|NDNAALYS|VL|Y|MLA|FA
M. graminicola Atr4 AAK15314 557
 TK|SW|R|I|AQTF|PM|F|M|I|F|S|PA|SAFTA|AV|A|I|LS
M. graminicola Atr1 CAB46279 544
 K|EAOWL|TT|W|KLF|ISNG|V|L|GESFD|S|AFT|A|FLGWL
M. graminicola Atr5 AAK62340 450
 I|QYL|ATFFIKQ|STV|SQ|A|I|A|ANSS|LFIK|A|L|Y|LV
M. graminicola Atr3 AAK62341 360
 LQ|QMLLA|K|RFA|TV|W|LFLV|GCT|LACFQ|QRTGA|AQA|LYTCV|GI|VS
 consensus 841 v R yq lwgDkg lytslimnvilaliiGsfynlp t g y rGgvlfslmna

← dAtr4-R1

M. graminicola Atr2 CAB46280 555
 SSL|LT|H|ER|L|S|V|SMI|Y|SNC|NTV|VN|
M. graminicola Atr7 EF062310 888
 SAL|LV|A|QAR|C|F|SMLC|N|TTA|GSS|P|H|
M. graminicola Atr4 AAK15314 617
 IS|NS|D|KS|T|GIV|V|L|F|VAVC|N|V|SG|
M. graminicola Atr1 CAB46279 604
 QT|LMKAVSG|AV|KR|ED|R|S|VT|RVV|VIL|Q|L|GIIMF|NMTI|
M. graminicola Atr5 AAK62340 510
 MN|VTDS|FSA|LA|RGF|Y|FCV|Q|TA|||Q|TLLS|PM|WL|G|KP|
M. graminicola Atr3 AAK62341 420
 QV|VAKTTLG|LLR|VFA|R|ER|FWT|Q|AV|TM|ESMR|F|ACV|L|A|H|
 consensus 901 ailEil ly qRpivekh yafyhPaaeaiA iimDlplkivnviif lvlyfmtglrr

M. graminicola Atr2 CAB46280 615
 EPG|F|F|L|I|LT|MM|T|SC|LS|TP|ALIL|W|LF|FA|VD--
M. graminicola Atr7 EF062310 948
 PGH|V|LVFT|AC|LT|MY|C|LA|APASVFS|FA|TR--
M. graminicola Atr4 AAK15314 677
 EPAQ|L|FLIA|VS|FV|M|TL|TK|IS|AL|VMV|FVV|TK--
M. graminicola Atr1 CAB46279 664
 SASQ|YMLF|VY|LLT|LY|MF|S|PE|T|VRF|IALNL|YV|RPQL
M. graminicola Atr5 AAK62340 570
 AA|TYWAIL|A|SMAIT|F|M|G|GCA|F|A|SKV|FAVS|IM|YML|KP--
M. graminicola Atr3 AAK62341 480
 AP|LY|LAVLLL|YMCNVLMN|A|GCMC|SF|S|IRTATFVFVFYMTA|YIVSQA--
 consensus 961 t gaFfvfivfittl msamfR iaalsrtldqAmr sglillalviytGfvip

M. graminicola Atr2 CAB46280 673 -|LG|CR|LNY|D|V|S|I|SG|NYA|KS-DD|S--
 |GTL|A|SQV|N
M. graminicola Atr7 EF062310 1006 -|R|LR|LNYL|VG|S|I|HD|SP|S----
 EY|H|EA|N|IQARERI|A
M. graminicola Atr4 AAK15314 735 -|K|G|RW|IF|I|VA|HA|EEF|S---QF|T---TQFGGGETF|S
M. graminicola Atr1 CAB46279 724 LTKY|G|W|S|S|VIT|AG|TMA|AP|SQ|Q|G-
 |P|Y|GCA--
M. graminicola Atr5 AAK62340 628 -N|H|V|W|D|G|G|SNQVIP|AN--
 NN|N|G|A|S|F|ACTG-
M. graminicola Atr3 AAK62341 538 -
 TQPS|LK|T|YL|L|S|M|EHIT|D|QSSVLSFP|TR|LPVNALQSGVT|G
 consensus 1021 ymrpWfrWiyinPlaya=realmiNEF grsi C qlvPsgpay d aaqg vc

dAtr4-F2

M._graminicola_Atr2_CAB46280 727
AV [REDACTED] RDY [REDACTED] E [REDACTED] I NSAYS [REDACTED] YR [REDACTED] K [REDACTED] I [REDACTED] F [REDACTED] MVG [REDACTED] LTV [REDACTED] V [REDACTED] S [REDACTED] N [REDACTED] RAKKS [REDACTED]
M._graminicola_Atr7_EF062310 1061
TS [REDACTED] ST [REDACTED] AEAID [REDACTED] DV [REDACTED] VN [REDACTED] G [REDACTED] HA [REDACTED] L [REDACTED] L [REDACTED] M [REDACTED] LMI [REDACTED] GCS [REDACTED] L [REDACTED] YV [REDACTED] EQKPK [REDACTED]
M._graminicola_Atr4_AAK15314 787
VV [REDACTED] V [REDACTED] ELT [REDACTED] T [REDACTED] D [REDACTED] I [REDACTED] EMYG [REDACTED] YY [REDACTED] V [REDACTED] FFA [REDACTED] MVI [REDACTED] FV [REDACTED] V [REDACTED] LN [REDACTED] STFSTA [REDACTED]
M._graminicola_Atr1_CAB46279 779
LA [REDACTED] DVNAQS [REDACTED] D [REDACTED] S [REDACTED] TQ [REDACTED] N [REDACTED] SR [REDACTED] NL [REDACTED] VVI [REDACTED] IV [REDACTED] YILVTVI [REDACTED] TV [REDACTED] FAGGG [REDACTED]
M._graminicola_Atr5_AAK62340 684 VR [REDACTED] PR [REDACTED] STI [REDACTED] T [REDACTED] EQ [REDACTED] DS [REDACTED]
LS [REDACTED] SP [REDACTED] NV [REDACTED] V [REDACTED] W [REDACTED] WWL [REDACTED] VALTIYF [REDACTED] SNW [REDACTED] QVSGNS [REDACTED]
M._graminicola_Atr3_AAK62341 597
LATSAADRTFL [REDACTED] A [REDACTED] GT [REDACTED] QLD [REDACTED] D [REDACTED] DTHDKV [REDACTED] TILN [REDACTED] LVG [REDACTED] F [REDACTED] AN [REDACTED] ALA [REDACTED] ILDWDSPAS [REDACTED]
consensus 1081 v ga ag veGeaYlas f Y shlwRnfgillAfmvflvvylvate is g

M._graminicola_Atr2_CAB46280 787 [REDACTED] L [REDACTED] QK [REDACTED] R [REDACTED] AALKEKS [REDACTED] MSEVN--RVS [REDACTED] MCR-----QMSNV [REDACTED] AT [REDACTED] ILR--
M._graminicola_Atr7_EF062310 1121 [REDACTED] T [REDACTED] L [REDACTED] Q [REDACTED] G [REDACTED] ---RNRP [REDACTED] SVGN [REDACTED] NI [REDACTED] TT [REDACTED] LMA-----
EPTC [REDACTED] GRV [REDACTED] TFRPE [REDACTED]
M._graminicola_Atr4_AAK15314 847 [REDACTED] R [REDACTED] HV [REDACTED] ---AYM [REDACTED] N [REDACTED] D [REDACTED] KPGKEDG [REDACTED] AAAAEKG-----
PEKGD [REDACTED] GG [REDACTED] SA [REDACTED] P [REDACTED]
M._graminicola_Atr1_CAB46279 839
GA [REDACTED] KKSKKAKKQVKHAKADEEKG [REDACTED] IA [REDACTED] DS [REDACTED] SSSKKNASLGDAPNED [REDACTED] DEA [REDACTED] DKLTK [REDACTED]
M._graminicola_Atr5_AAK62340 743 GF [REDACTED] IP [REDACTED] -EKAKKA AHLMN [REDACTED] AQPA [REDACTED] MS [REDACTED] KKTAEDK-----E [REDACTED] DGNVDSQLIR [REDACTED]
M._graminicola_Atr3_AAK62341 657 S [REDACTED] M [REDACTED] SVPKN [REDACTED] QPPQQT [REDACTED] RTACMTAFVPTPPTSEF [REDACTED] QSP-----AAWTR [REDACTED]
consensus 1141 evlfv rgrip k qdee g e ssv r kea dvt i k

M._graminicola_Atr2_CAB46280 835 A [REDACTED] S [REDACTED] RN [REDACTED] C [REDACTED] KI [REDACTED] EE--
[REDACTED] N [REDACTED] D [REDACTED] V [REDACTED] T [REDACTED] V [REDACTED]
M._graminicola_Atr7_EF062310 1170 E [REDACTED] H [REDACTED] D [REDACTED] SF [REDACTED] G [REDACTED] SS--
K [REDACTED] QG [REDACTED] D [REDACTED] IR [REDACTED] V [REDACTED] D [REDACTED] V [REDACTED]
M._graminicola_Atr4_AAK15314 895 D [REDACTED] I [REDACTED] R [REDACTED] D [REDACTED] EI [REDACTED] EP--
[REDACTED] H [REDACTED] S [REDACTED] T [REDACTED] T [REDACTED]
M._graminicola_Atr1_CAB46279 899 SE [REDACTED] I [REDACTED] K [REDACTED] E [REDACTED] TVPYM [REDACTED] E--
K [REDACTED] NK [REDACTED] N [REDACTED] YA [REDACTED] VMV [REDACTED] A [REDACTED] NT [REDACTED] Q [REDACTED]
M._graminicola_Atr5_AAK62340 791 N [REDACTED] KGLT [REDACTED] TVK [REDACTED] PT [REDACTED] D--
V [REDACTED] D [REDACTED] K [REDACTED] M [REDACTED] G [REDACTED] S [REDACTED] K [REDACTED]
M._graminicola_Atr3_AAK62341 699
GV [REDACTED] RL [REDACTED] FCR [REDACTED] R [REDACTED] A [REDACTED] ER [REDACTED] QQS [REDACTED] E [REDACTED] T [REDACTED] V [REDACTED] ES [REDACTED] Q [REDACTED] L [REDACTED] V [REDACTED] A [REDACTED] NL [REDACTED] SG [REDACTED] E [REDACTED]
consensus 1201 qtsvftwrdrv ydi tkgge rrlld V GwvkpGltAIMG SGAGKTTLLdvLaqR

M._graminicola_Atr2_CAB46280 893
[REDACTED] E [REDACTED] ERDN [REDACTED] K [REDACTED] V [REDACTED] A [REDACTED] N [REDACTED] AS [REDACTED] Q [REDACTED]
M._graminicola_Atr7_EF062310 1228
SV [REDACTED] N [REDACTED] L [REDACTED] RGPD [REDACTED] R [REDACTED] Q [REDACTED] A [REDACTED] AS [REDACTED] N [REDACTED] T [REDACTED] PND [REDACTED]
M._graminicola_Atr4_AAK15314 953
T [REDACTED] N [REDACTED] F [REDACTED] N [REDACTED] A [REDACTED] LDD [REDACTED] K [REDACTED] V [REDACTED] E [REDACTED] S [REDACTED] R [REDACTED] M [REDACTED] T [REDACTED] KQ [REDACTED]
M._graminicola_Atr1_CAB46279 957
S [REDACTED] E [REDACTED] F [REDACTED] LGRE [REDACTED] N [REDACTED] FCL [REDACTED] G [REDACTED] DG [REDACTED] A [REDACTED] I [REDACTED] E [REDACTED] I [REDACTED] DAS [REDACTED] E [REDACTED]
M._graminicola_Atr5_AAK62340 849
E [REDACTED] T [REDACTED] K [REDACTED] S [REDACTED] D [REDACTED] VPI [REDACTED] SA [REDACTED] CE [REDACTED] L [REDACTED] EPLA [REDACTED] E [REDACTED] D [REDACTED] P [REDACTED] ED [REDACTED]
M._graminicola_Atr3_AAK62341 759 HS [REDACTED] RG [REDACTED] TVSACTS---
RGLPPTI [REDACTED] AE [REDACTED] V [REDACTED] EPK [REDACTED] ML [REDACTED] C [REDACTED] KH [REDACTED] FA [REDACTED]
consensus 1261 tmGvvsG mlvdgrpl sfqr tGy qQqDIHlatsTvREal FSAILRQprsVsrdE

M. graminicola Atr2 CAB46280 953 LD K D Q E Y A V --
 M. *graminicola* Atr7 EF062310 1288 I E A D E A Y S V --
 M. *graminicola* Atr4 AAK15314 1013 YE E K M N E F A E V --
 M. *graminicola* Atr1 CAB46279 1017 T D L N M Q I I S S G S
 M. *graminicola* Atr5 AAK62340 909 LK T D H I E N T L I T T Y A --
 M. *graminicola* Atr3 AAK62341 816 R W H L P I T P I Q N I V S S E S A R D T A --
 consensus 1321 layVdevl ILem d davvgvpgeg LnveqRKrlTigVELaaKP IIIFLDEPTsG

M. graminicola Atr2 CAB46280 1011
 M. *graminicola* Atr7 EF062310 1346
 M. *graminicola* Atr4 AAK15314 1071
 M. *graminicola* Atr1 CAB46279 1071
 M. *graminicola* Atr5 AAK62340 967
 consensus 1381 Ldsqtawalcr IrKLad GQAilctIHQPSamlfqqFdrlLfLakGgktvYFGevG na

M. graminicola Atr2 CAB46280 1071 K I L S T E N G H P D A I G A A P G S R - T D I H Q R A -
 PE Y A T Q R
M. graminicola Atr7 EF062310 1406 S A I Q D K A R G G D E I D T - N T P R N I - D G T A A D D T -
 E E R Q A V K A
M. graminicola Atr4 AAK15314 1131 R T L L E S N A R D G E E E Y || G D D S S - - -
 V G T N D K E A R R G T A G D
M. graminicola Atr1 CAB46279 1131 K D T K S D R V D P H K V F I T A A K P H K R K D G K K I N Q E V E - - -
 Q Q A K D V
M. graminicola Atr5 AAK62340 1027 Q T K G Y D A K N A H I D S G T L S - - - K D K N R L D - - -
 P E H S A M
M. graminicola Atr3 AAK62341 934 C R P A E I A Q T C S S V G R D V T T Q I G S S P A H - - - H E T S D A A S - - -
 E
 consensus 1441 k v d Y f r n g h p C p d n p a E w m l e v v g a t r d W v W d S a a e

M. graminicola Atr2 CAB46280 1128
 D H K D N G A V R Q P M V E S D N E S F N A G S L R E V V V A L Q I Y A I S C
M. graminicola Atr7 EF062310 1462 A R M K P - - S I T P I A I D A D R - - -
 P Y A G T L G H R G F S H L W S V A C
M. graminicola Atr4 AAK15314 1184
 R T H S Q G T L L R G E E L D R R Q R R P L R P R I R H A I R R P T Q D G H S P R V P T I L A Y A E L P V R E N G S L
M. graminicola Atr1 CAB46279 1186
 E E D G L K Q T R I H V T S Q K N K D D E K E T M L C T E R T F R Q D L Y G F V S
M. graminicola Atr5 AAK62340 1078 T T E D --
 R I V S D A A K P P G T L D D G R E T L T I K L V T N I N N I S L F I N N D I T D N M H
M. graminicola Atr3 AAK62341 983
 A D M Q A S L K P T Y S E T S R A E S M R G H P C Q V Y I H Q I T L I N S T A L E G F S R I N H
 consensus 1501 ldhl g lts is ad e r faasfwsqrell r y wrtpsyiy kf l

M. graminicola Atr2 CAB46280 1188
 TLAG [REDACTED] YKAPLTH GLT QMLSI MIF L SNVS I M H VLQ S [REDACTED] R
M. graminicola Atr7 EF062310 1516
 [REDACTED] FS [REDACTED] VS [REDACTED] KMP SI GT QL V LLL TNFC MM HA R E A [REDACTED] L
M. graminicola Atr4 AAK15314 1244
 HRRWSLHR [REDACTED] LLLLRLDATL G [REDACTED] VIYS MLT [REDACTED] STLV [REDACTED] IQ L V Q S [REDACTED] V R
M. graminicola Atr1 CAB46279 1246
 [REDACTED] IVG [REDACTED] N T QL TQ D [REDACTED] M TA LII PPTIVNAVVK Y NMA WQ [REDACTED] Y
M. graminicola Atr5 AAK62340 1136
 [REDACTED] GS [REDACTED] N T QI SV DL L [REDACTED] NF FVAPGVIA LQ L LER D [REDACTED] KK
M. graminicola Atr3 AAK62341 1043 AL VT RIVLPHAH HSIRD VH VL WQTTM PAFILTTVEYR HAS [REDACTED]
 RLSL [REDACTED] SAT
 consensus 1561 v alfigf fwkmgniq mqnrmfafmiltifs vvqqimphfvtrq lyeaRErps

M. graminicola Atr2 CAB46280 1248
 [REDACTED] T [REDACTED] QV L [REDACTED] N L L WNAFAGL F L Y [REDACTED] YANASPTDSVAE G F M I A
M. graminicola Atr7 EF062310 1576
 [REDACTED] V QT [REDACTED] IL D [REDACTED] M WNSLM [REDACTED] V A W [REDACTED] QQNAIDAGQTGE AILM F I A
M. graminicola Atr4 AAK15314 1304 A [REDACTED] K L IANM [REDACTED] QIIAGI VYATF VGIQ-----S E QVL M LCIV
M. graminicola Atr1 CAB46279 1306 RI GYF [REDACTED] VTAQV A PAIIG [REDACTED] YWVLW W T PT-----
 D [REDACTED] STS [REDACTED] FMT [REDACTED]
M. graminicola Atr5 AAK62340 1196 M H S [REDACTED] VTGL S [REDACTED] LVVC Y V F T FPA-----
 A SSA A FVM F
M. graminicola Atr3 AAK62341 1102 ST [REDACTED] NT LAV AML [REDACTED] CF C TAGFV LPLFSL [REDACTED] PLV-----
 LSQVT FA AVFL
 consensus 1621 kvYsw aflsnivvEiPynilaavlfflcyypvgl s rggyvflmilv

dAtr4-R2 ←
M. graminicola Atr2 CAB46280 1308 L TS [REDACTED] TNLMIG DS ETS [REDACTED] A L LC [REDACTED] N I AS KTM K [REDACTED]
 LFL R S [REDACTED]
M. graminicola Atr7 EF062310 1636 N AG [REDACTED] TSMAV L ST ES [REDACTED] TNL [REDACTED] LS I C AT QA [REDACTED]
 IF R S L
M. graminicola Atr4 AAK15314 1357 L VYAS AHMCIA A D QT A V F A I N MQ SA [REDACTED]
 IF R S [REDACTED]
M. graminicola Atr1 CAB46279 1359
 [REDACTED] L QASWG WIT FS SFTVIS VLP F V FSL N VR YAS V YW Y N S
M. graminicola Atr5 AAK62340 1249
 YEFIYTGIG FVA YASN LF FL NP II LAL C V YAQ QP YWF YLN [REDACTED]
M. graminicola Atr3 AAK62341 1154
 VQ CV LA A ATLS YERISTLFN PSITVFA L C ISV RSE [REDACTED] NWL YLN Y
 consensus 1681 fflf stfaqlmia mpsa tagni tf fsm liF Gvl pp lpgFwrlfmY v Pf

M. graminicola Atr2 CAB46280 1367 IT M G MDQTT H AN FVT QPDGGEGG T GT D S [REDACTED]
 AN TV GKGY
M. graminicola Atr7 EF062310 1695 VS V VG ANTR H LDE LH SA P---STN ST LA [REDACTED] L EG LQE--
 -
M. graminicola Atr4 AAK15314 1416 WVASMA M HDRQ T SDT IST QP Q----
 GQ Q LE GAA LQN--
M. graminicola Atr1 CAB46279 1419 WW V A T DGIP Q ET TAH DA ----P AS AGAFA SAG--
 GYLL--
M. graminicola Atr5 AAK62340 1309 N M S VFTTWNP T KTS AV DT N--
 A QE LAGFL MGRTSNLL--
M. graminicola Atr3 AAK62341 1214 W MS TNSIHGFN Q GN TDITV LP----
 QQA SLLSTLHDP IAG--
 consensus 1741 tylvgllsatl v CadtEl f p gqtCgtymapyiqg a gyv n

M. graminicola Atr2 CAB46280 1426 ILN[D][D]K[S]FNT[H]F[KL]VS[D]-
PA[■■■■■]T[YILV]VAGA[A[■■■■■]LF
M. graminicola Atr7 EF062310 1747 -VPDSNP[Q]VF[TGSQT]IF[KSVSAQ-
YG[■■■■■]N[■■■■■]AY[AF]LVAT[■■■■■]LA
M. graminicola Atr4 AAK15314 1469 ---[D][T][D][G]SIRVADTF[SGVG]S-
W[N]F[L]Y[F]LMA[F]FF
M. graminicola Atr1 CAB46279 1471 ---QDNTN[M]PLS[■■■■■]QY[AQL]N[N-
A[K]FV[F]WFLVYFFIYT[■■■■■]
M. graminicola Atr5 AAK62340 1364 ---
[Q]TSG[EV]QYR[ADY]YG[N]LTKK[G]A[■■■■■]VLFA[■■■■■]SY[CVYL]MK---
M. graminicola Atr3 AAK62341 1268 ----TSEV[H]PLT[■■■■■]DRYSESGLGS-
YAQ[KY]VFS[FCV]V[LILL]AQQFR[R]
consensus 1801 p ata C yCistgn fl v is ysdrWrldGivwwyvfnvgmvvilyw rv

M. graminicola Atr2 CAB46280 1485 PKG[KKKAEKKEKT]-----
M. graminicola Atr7 EF062310 1805 PKK[RSK]-----
M. graminicola Atr4 AAK15314 1525
RSH[KKTSKKSKKS]DKKGAAVAGTEKDDKKVKKTDTSSEGNTPAASVDPEKDAEA
M. graminicola Atr1 CAB46279 1527 KGWTFGFPLFGAL[KGVELIKPFKKGE]KKEQSEE-----
M. graminicola Atr5 AAK62340 1417 -----LRTKASKKAE-----
M. graminicola Atr3 AAK62341 1322 -----
consensus 1861 sk g d

M. graminicola Atr2 CAB46280 -----
M. graminicola Atr7 EF062310 -----
M. graminicola Atr4 AAK15314 1585 QRSGSTTSAKAVKRQTSRTAGLRTVSEMGQSVLTTNKGRSRANERNAHY
M. graminicola Atr1 CAB46279 -----
M. graminicola Atr5 AAK62340 -----
M. graminicola Atr3 AAK62341 -----
consensus 1921

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	1	-----MDDR[■■■■■]EHDDY[■■■■■]SGAMYET[VRI]WSPQS
Botryotinia_fuckeliana_BAC6716	1	-----MERL[■■■■■]MSWRNK[■■■■■]CMGLSWG[■■■■■]QHW[P]
Venturia_inaequalis_AAK62810	1	--MAYAGTKGDG[I]RTASGHETYAPDGLGTPQRE--EEIAPS[D]SRATSERYAPI[G]
Mycosphaerella_graminicola_Atr4	1	--MATGGSASTP[■■■■■]GDYNPERVEQLFDIPRPEQGN[■■■■■]VEAENAART[■■■■■]SGAQESQLSGS[P]
Emericella_nidulans_CAC42218	1	MSSFLGTGTFNTS[■■■■■]SPSQAVESRG[■■■■■]ENHGNAIT[■■■■■]ETEL[■■■■■]NESHA[S]GEKLASS[■■■■■]NSILS
Trichophyton_rubrum_AAN28699	29	RPELVRIAS-----VFS[■■■■■]IDSHP--DV
Botryotinia_fuckeliana_BAC6716	28	TPQILTYIN-----G[KCKFWHV--QV
Venturia_inaequalis_AAK62810	57	HLYRRNSS[■■■■■]TEKG-SDEDFTMATRSKSFTEN---MDTDDDKDN[■■■■■]L---S[■■■■■]
Mycosphaerella_graminicola_Atr4	59	GINTADHA[■■■■■]DSYLTANGREAAESINDDKSSGFNSDDQPIN[■■■■■]ERNI[R]LA[■■■■■]NSRRSM
Emericella_nidulans_CAC42218	61	STETAREK-----ERDYELDAEEEV[■■■■■]RLAQQL
Trichophyton_rubrum_AAN28699	49	APT[■■■■■]EDGGQ[■■■■■]NRD[■■■■■]LA[■■■■■]VKIG[■■■■■]L[■■■■■]TKE[■■■■■]F[■■■■■]ARMFTHVMEK-[■■■■■]KRN[■■■■■]T[■■■■■]V
Botryotinia_fuckeliana_BAC6716	48	FIWTQTN-----CTCRM[■■■■■]MRVDE-N[■■■■■]VIQR[■■■■■]
Venturia_inaequalis_AAK62810	103	SQHQTRSST[■■■■■]R[■■■■■]ND[■■■■■]IS[■■■■■]LKED[■■■■■]F[■■■■■]SHKD[■■■■■]L[■■■■■]YLRLFMRDLQA-D[■■■■■]RET[■■■■■]KK[■■■■■]
Mycosphaerella_graminicola_Atr4	119	NNTNEDPEA[Q]TG[■■■■■]LD[■■■■■]LGMD[■■■■■]F[■■■■■]NS[■■■■■]R[■■■■■]L[■■■■■]LKLT[■■■■■]K[■■■■■]VND-[■■■■■]DIKIK[■■■■■]S[■■■■■]L
Emericella_nidulans_CAC42218	89	THQSTKYSTHNIENPFLE[■■■■■]VG-E[■■■■■]STLN[■■■■■]HS[■■■■■]KAKN[■■■■■]MKNILLA[■■■■■]SSRDP[■■■■■]RYLPRQ[■■■■■]V

Trichophyton_rubrum_AAN28699 107 M R T L V Q Y D F L P F A F P G L C G K G R N E V H D N A I R E M
 Botryotinia_fuckeliana_BAC6716 76 V K K C I N V K N G L L M L F K F I G K G - E T N D N V L M
 Venturia_inaequalis_AAK62810 161 M R S S A L Q L S S D F V L F L R L F S S S K S H Q I D K V L
 Mycosphaerella_graminicola_Atr4 177 A K D H S L N L P S M L S L I G M F S M A K K H Q R S L M
 Emericella_nidulans_CAC42218 148 S T S H Y P T D Y K D F N S V L Q I G G I V R S M M G H G K Q K I E R N L V A M V

dAtr4-F1

Trichophyton_rubrum_AAN28699 167 A I C H Q K K E I V S H T F K L A S A D E H
 Botryotinia_fuckeliana_BAC6716 134 S M Y D K A Q E T L Q I V
 Venturia_inaequalis_AAK62810 220 T C T T V K G V P K I K V
 Mycosphaerella_graminicola_Atr4 237 L S T Q M H T E K T T D I Q V I
 Emericella_nidulans_CAC42218 208 T I A M N I F E K Q L N Q P A R Q A I T A T V Q

Trichophyton_rubrum_AAN28699 227 A I S K V L L K D F S T L A R M S F D Y
 Botryotinia_fuckeliana_BAC6716 194 E S V Q Q L V E T T I S A W A K M T K I Y
 Venturia_inaequalis_AAK62810 280 V S N L H E T E F S S Q V A K F
 Mycosphaerella_graminicola_Atr4 297 H L M S Q Q P L T Q S A V E Y L T Q Y
 Emericella_nidulans_CAC42218 268 S D K L A C R N L P V E Q Y A V M R D M L I R

Trichophyton_rubrum_AAN28699 286 I S A I C C T K I G S Q V G I T Q C L
 Botryotinia_fuckeliana_BAC6716 254 I S A A T S M T N S C L
 Venturia_inaequalis_AAK62810 339 V A A K V E T I S D T S
 Mycosphaerella_graminicola_Atr4 356 A A T I L N D V A
 Emericella_nidulans_CAC42218 327 A T S A Q C N C R T N L M K Y S A T M

Trichophyton_rubrum_AAN28699 346 I I V F T R I E P A M K I A K
 Botryotinia_fuckeliana_BAC6716 314 Q Q V Y C D Q E S S K S K A
 Venturia_inaequalis_AAK62810 399 R V S F A S K F E Q K S
 Mycosphaerella_graminicola_Atr4 416 L E F K A S V K F S V A Q L
 Emericella_nidulans_CAC42218 387 S A V V T R T D D F I F E A L S A I V

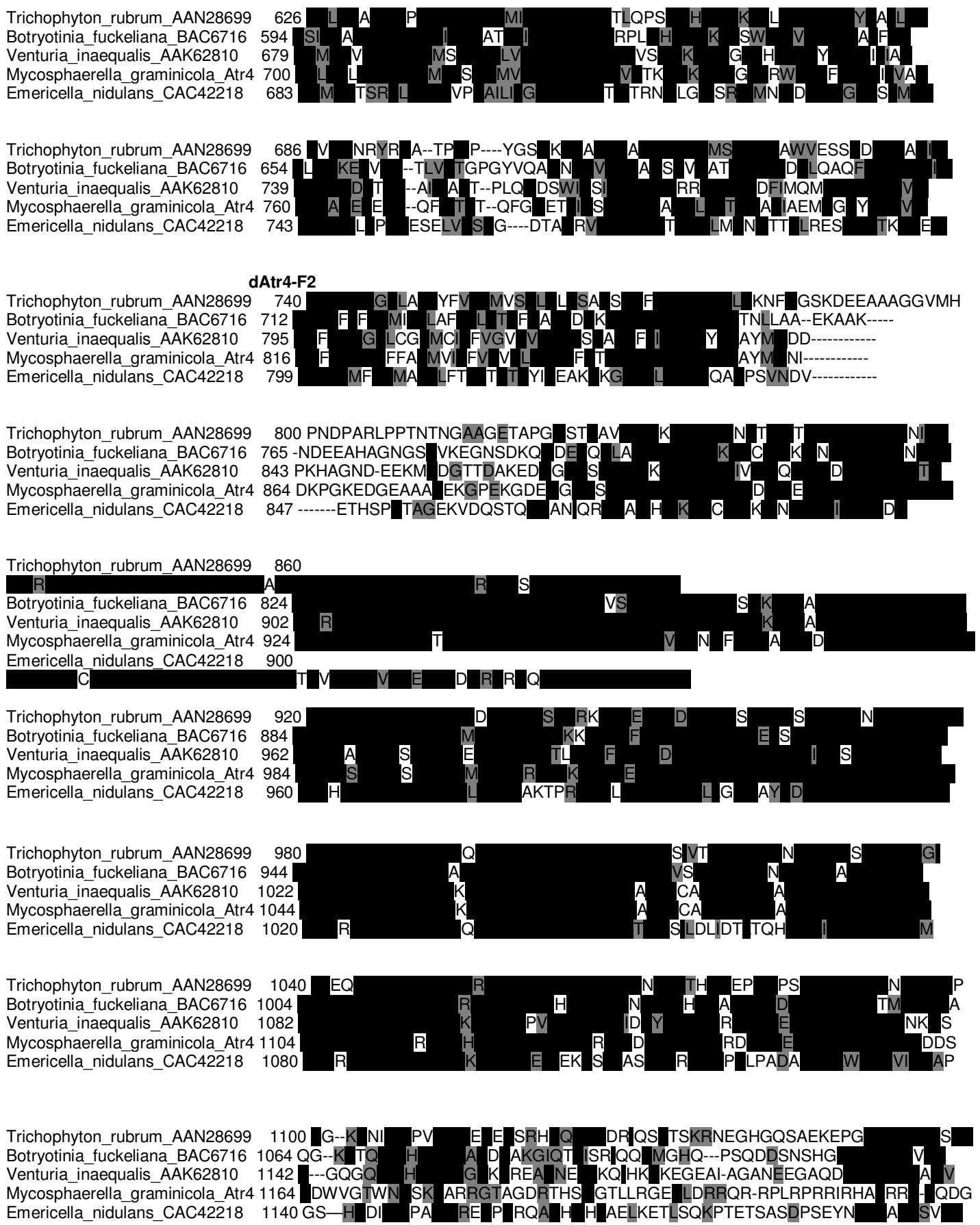
Trichophyton_rubrum_AAN28699 406 N A V R Q Q N N K L L A N M D R F A Y P E E G H E -- R E T H G Q - A
 Botryotinia_fuckeliana_BAC6716 374 P N K E F D K I F R M M S M K S E M R K E -- Q F K A S R K G - M
 Venturia_inaequalis_AAK62810 459 M K Q E D R N E M S I Q K E D T I G E G -- Q Q Q K R N - A
 Mycosphaerella_graminicola_Atr4 476 D A G D D H D P E T I Q K Q G T K G S T P S A S S G T S S A A S K N D N
 Emericella_nidulans_CAC42218 447 K G Q D A A A N D A Q M R E E Y N Q L G E S V N -- F I E S R R A - M

Trichophyton_rubrum_AAN28699 462 I T A S R V V V Y I A T N S M L F D Q T
 Botryotinia_fuckeliana_BAC6716 430 D L E T V I V T A R V N T I T V G A I N S N
 Venturia_inaequalis_AAK62810 515 S T M I K Y I N M L M F S S V G N A
 Mycosphaerella_graminicola_Atr4 536 A V V V N K S W I A Q F P M F N V I F N S P A
 Emericella_nidulans_CAC42218 503 S N Q V T M M E V H M I G F K A L L S Q L G N F V V D L D N D

Trichophyton_rubrum_AAN28699 522 I D G A S I I G M S T G D A V V N F
 Botryotinia_fuckeliana_BAC6716 490 A S Q G I S T S D Q A
 Venturia_inaequalis_AAK62810 575 A G S V M T S D N
 Mycosphaerella_graminicola_Atr4 596 S A T A R S S S D K
 Emericella_nidulans_CAC42218 563 G S Y S R L F G S A L V C L I L R L F L S L A D S L Q I L T A Q A R M

dAtr4-R1

Trichophyton_rubrum_AAN28699 566 A Y S L A I L L V I G E S A A K I I T I
 Botryotinia_fuckeliana_BAC6716 534 F L V V V A I T C S K A G A V N A Q I Y
 Venturia_inaequalis_AAK62810 619 A S V L V V G V F A S Q L Y V S Y I M F V A V
 Mycosphaerella_graminicola_Atr4 640 A M V L V V V C L V M S A Q L I A S T F V V
 Emericella_nidulans_CAC42218 623 F A S M L C M Y I T N T F T P M T N G A I L S T T M L



Trichophyton_rubrum_AAN28699 1158 YC|TT|T|S|IWG|LL|L|A|LQN|SSMA|SL|
 Botryotinia_fuckeliana_BAC6716 1119 LE|M|K|T|G|YS|LV|V|A|H|DASQ|D|I|
 Venturia_inaequalis_AAK62810 1198 KA|T|M|S|FA|WA|I|G|Q|NT|Q|V|L|A|IA|
 Mycosphaerella_graminicola_Atr4 1222 HSPR|PTILA|AEL|RENGS|HRRWS|HR|LLL|RDA|L|M|IY|L|
 Emericella_nidulans_CAC42218 1198 WECL|S|S|V|YS|AA|SILTS|Y|Q|QN|R|QM|M|

Trichophyton_rubrum_AAN28699 1218 |S|R|D|F|R|V|L|I|L|IAW|SLF|T|
 Botryotinia_fuckeliana_BAC6716 1179 |T|L|D|R|L|N|S|M|V|NF|V|P|F|CYL|A|
 Venturia_inaequalis_AAK62810 1258 |S|L|N|S|M|V|NF|V|P|F|CYL|A|
 Mycosphaerella_graminicola_Atr4 1279 |T|Q|L|S|M|V|NF|V|P|F|CYL|A|
 Emericella_nidulans_CAC42218 1258 GN|N|A|MT|L|L|WNTLMAVIMYFCWY|V|

dATr4-R2



Trichophyton_rubrum_AAN28699 1278 ---F|AH|L|GIL|YCV|Q|G|G|TM|G|MV|
 Botryotinia_fuckeliana_BAC6716 1239 ---YTKNGIPP|G|G|L|L|S|N|M|S|T|
 Venturia_inaequalis_AAK62810 1318 ---A|II|---V|V|I|M|Y|G|A|C|AAVV|ATS|
 Mycosphaerella_graminicola_Atr4 1339 ---V|IQ|---V|VM|CIVL|Y|C|M|Q|AVF|FMA|I|
 Emericella_nidulans_CAC42218 1318 GLYRNAEPTDSVH|GA|MF|ILA|L|T||GIET|G|N|Q|S|C|I|

Trichophyton_rubrum_AAN28699 1332 |L|K|N|R|W|I|T|GGL|A|S|S|E|K|AQN|LAI|D|S|A|A|
 Botryotinia_fuckeliana_BAC6716 1295 |F|P|Q|L|L|A|G|S|Q|DN|LAVMQ|A|D|G|
 Venturia_inaequalis_AAK62810 1371 |M|S|Q|I|W|SLV|M|R|E|SS|TSR|S|A|Q|Q|
 Mycosphaerella_graminicola_Atr4 1392 |M|P|S|W|ASM|AM|D|Q|T|DT|ST|Q|Q|G|
 Emericella_nidulans_CAC42218 1378 |C|LAG|DV|L|AML|GVS|TTAY|EQV|YLTLY|SNT|S|

Trichophyton_rubrum_AAN28699 1392
 |KLVEAG|P|K|Y|MS|SQ|Q|PL|SG|G|SE|HWSD|N|G|A|V|
 Botryotinia_fuckeliana_BAC6716 1355 S|S|ATAAG|S|Y|E|MAD|Q|S|N|S|A|S|TT|Y|FV|F|
 Venturia_inaequalis_AAK62810 1431 |AD|LQT|P|T|ND|TN|R|L|T|L|A|SNVK|DT|S|V|
 Mycosphaerella_graminicola_Atr4 1452 |M|P|LEGG|A|Y|D|AD|G|IRV|T|G|SWSN|L|V|VF|
 Emericella_nidulans_CAC42218 1438 E|MDP|ISQVG|Y|D|SE|TF|Q|ST|T|A|YSN|DDA|N|LM|A|A|

Trichophyton_rubrum_AAN28699 1452 |AT|A|I|S|GRPNRIISVITYHLSQF|TYC|-----RAFITGRK-----
 Botryotinia_fuckeliana_BAC6716 1414 |M|L|I|S|GKS|-----LKEKF|ALG|-----ALF|KN|-----
 Venturia_inaequalis_AAK62810 1490 |V|LT|F|K|WN|GTGK|-----SDGAKKA|FLG|-----K|L|KGANKGND|---
 Mycosphaerella_graminicola_Atr4 1512 |LGM|F|WFF|SH|KTSKK-SKKSGDKKGAAAAGTEKDDKKVK|TDTSSSSEGNT|
 Emericella_nidulans_CAC42218 1497 |AA|F|W|A|P|GK|N|-----

Trichophyton_rubrum_AAN28699 1498 -----EKCPRKREQ
 Botryotinia_fuckeliana_BAC6716 -----
 Venturia_inaequalis_AAK62810 1535 -----AKTEKGEQQANQH
 Mycosphaerella_graminicola_Atr4 1571 PAAASVDPEKDAEAQRSGSTTSAKAVKRQTSRTAGLRTVSEMGQSVLTTNKGRSRANER
 Emericella_nidulans_CAC42218 -----

Trichophyton_rubrum_AAN28699 1507 IGK|Y
 Botryotinia_fuckeliana_BAC6716 -----
 Venturia_inaequalis_AAK62810 1548 QRA|
 Mycosphaerella_graminicola_Atr4 1631 NAHVY
 Emericella_nidulans_CAC42218 -----