The loss of the inducible *Aspergillus carbonarius* MFS transporter MfsA leads to ochratoxin A overproduction

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Abstract

Ochratoxin A (OTA), a nephrotoxic compound produced by certain *Aspergillus* and *Penicillium* species, is one of the most abundant mycotoxins in food commodities. *Aspergillus carbonarius* is the main source of OTA in wine, grape juice and dried vine fruits. Although many studies have focused the attention on OTA production by *A. carbonarius*, little is known about the genes related with OTA production and transport. We have found a transporter that belongs to the major facilitator superfamily (MfsA) which is highly expressed with a 102-fold induction in an ochratoxigenic *A. carbonarius* strain compared to a low OTA producer strain. The encoding *mfsA* gene shows similarity to the multidrug efflux transporter *flu1* from *Candida albicans*. A high number of putative transcription factor binding sites involved in the response to stress were identified within the promoter of *mfsA*. Phenotypical analysis of Δ*mfs*A deletion mutants revealed that the loss of *mfsA* leads to a slight growth reduction and increased OTA production. We therefore hypothesize that MfsA could be a stress response transporter whose disruption could cause an increase of oxidative stress together with a stimulation of mycotoxin production.

1. Introduction

Aspergillus carbonarius is an ochratoxin producing fungus that has been considered to be the major responsible of ochratoxin A (OTA) contamination in grapes and wine (Battilani et al., 2006; Perrone et al., 2007). OTA has a diverse range of toxicological effects, including nephrotoxicity, teratogenicity, immunotoxicity, neurotoxicity and hepatotoxicity (Gagliano et al., 2006; Petzinger, Ziegler, 2000; Pfohl-Leszkowicz, Manderville, 2007; Sava et al., 2007; Wangikar et al., 2005). Therefore, OTA was classified in 1993 as a possible human carcinogen (group 2B) by the International Agency for Research on Cancer (IARC, 1993).

Fungi have evolved mechanisms that enable them to survive and adapt to different natural environments. The ability to produce toxic compounds as a defense strategy together with the capacity to resist the effects of toxicants, are key elements of survival. In this sense, some fungi have transport mechanisms to protect themselves against fungicides, natural antimicrobial compounds or plant defense compounds and to secret toxins and virulence factors. The two major families of transporter proteins are the ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) transporters. ABC transporters contain an ATP-binding cassette, which generates energy for substrate translocation by hydrolysis of ATP. MFS transporters, by contrast, lack ABC domains and translocation is made in response to a chemiosmotic ion gradient (Pao et al., 1998). It has been reported that these efflux pumps contribute to secretion of mycotoxins, such as cercosporin in *Cercospora kikuchii* and *Cercospora nicotianae*, with the involvement of the MFS transporters CFP and CTB4, and the ABC transporter ATR1 (Amnuaykanjanasin, Daub, 2009; Callahan et al.,

1999; Choquer et al., 2007). Additionally, two mycotoxin transporters, Tri12 and ZRA1, have been described in *Fusarium graminearum*. Tri12 is a MFS transporter which allows trichothecene secretion (Menke et al., 2012) and ZRA1 is an ABC transporter responsible of zearalenone transport (Lee et al., 2011). Transporters are also well known for their role in resistance to chemical fungicides, herbicides and insecticides, developing not only resistance to a specific compound but also to structurally and functionally unrelated compounds, a phenomenon known as multidrug resistance (MDR). In fungi, various ABC (e.g., ATrB, ATrC, ATrD and MgATr) and major facilitator superfamily transporters (MgMfs1) are implicated in MDR, protecting fungi against cytotoxic agents and antibiotic production (Andrade et al., 2000a; Andrade et al., 2000b; Roohparvar et al., 2007; Schouten et al., 2008; Stergiopoulos et al., 2002; Zwiers et al., 2003).

In a previous study we used a suppression subtractive hybridization approach to identify differentially expressed genes in two closely related strains of *A. carbonarius* that differ in their OTA-producing capabilities. Among them we identified four ESTs corresponding to MFS transporters putatively related to OTA production (named MfsA, MfsB, MfsC and MfsD with GenBank accession numbers GW327952, GW327970, GW328001 and GW328022, respectively) (Crespo-Sempere et al., 2010). The aim of this study was to further investigate whether these MFS transporters have a role in the production or transport of OTA. For that purpose, gene expression analysis was conducted. In addition, the *mfsA* gene was cloned and characterized. We have obtained a *mfsA* null mutant (Δ*mfsA*) in the ochratoxigenic *A. carbonarius* W04-40 strain by targeted

gene replacement and its phenotype has been evaluated. Finally we discuss the possible relationship between *mfsA* and OTA production.

2. Materials and methods

2.1. Fungal strains, media, and culture conditions

The *A. carbonarius* strains W04-40 and W04-46 were isolated from a Spanish vineyard by Martínez-Culebras and Ramon (2007) and deposited in the Institute of Agrochemistry and Food Technology of the Spanish National Research Council (IATA-CSIC). While the strain W04-40 is a high OTA producer, the strain W04-46 produces low amount of OTA (Crespo-Sempere et al., 2010). The *Agrobacterium tumefaciens* AGL-1 strain was kindly provided by L. Peña (Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain).

A. carbonarius was grown on Petri dishes containing Malt Extract Agar (MEA) medium in the dark at 28 °C for 6 days to achieve conidia production. Conidia were collected with a sterile solution of 0.005% (v/v) Tween 80 (J.T. Baker, Holland) and were adjusted to 10^6 conidia/mL using a haemocytometer. 100 µL of the conidial suspension was homogeneously spread on Petri dishes containing Czapeck Yeast Extract Agar (CYA) medium and sub-cultured in the dark at 28 °C. To study the expression of *mfsA* under oxidative stress, hydrogen peroxide (Sigma-Aldrich, USA) was amended to *A. carbonarius* W04-40 cultures on CYA plates.

2.2. Genomic DNA extraction

The rapid DNA extraction protocol described by Cenis (1992) was followed with minor modifications. Cultures were grown for 1 day at 28 ºC in 500 µl of Czapeck's Yeast medium. Mycelium was recovered after 10 min of

centrifugation at 17500 X *g* and 300 µl of extraction buffer (200 mM Tris-HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was added. The mycelium was disrupted with five 2.8 mm stainless steel beads (Precellys, Bertin Technologies, France) for 2 minutes in a cell disruptor (Mini BeadBeater-8, Biospec, USA). After centrifugation at 17500 X *g* for 10 min, 150 µL of 3 M sodium acetate (pH 5.2) was added to the supernatant, which was further incubated at -20ºC for 10 minutes and centrifuged (17500 X *g*, 10 min). The DNA-containing supernatant was transferred to a new tube and nucleic acids were precipitated by adding 1 volume of isopropyl alcohol. After 5 minutes of incubation at room temperature, the DNA suspension was centrifuged (17500 X *g*, 10 min). The DNA pellet was washed with 70**%** ethanol to remove residual salts. Finally, the pellet was air-dried and the DNA was resuspended in 50 µl of TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA).

2.3. RNA isolation and cDNA synthesis

Mycelia were collected from cultures, frozen in liquid nitrogen and stored at −80°C before nucleic acid extraction. RNA was isolated from 1 g of mycelium previously grounded to a fine powder with a mortar and pestle with liquid nitrogen. Pulverized mycelium was added to a pre-heated mixture of 10 mL of extraction buffer (100 mM Tris–HCl, pH 8.0, 100 mM LiCl, 10 mM EDTA, 1% (w/v) sodium-n-laurylsarcosine (SDS), 1% (w/v) polyvinyl-pyrrolidone 40, 1% β-mercaptoethanol) and 5 mL of Tris-equilibrated phenol. After homogenization with a Polytron PT 45/80 (Kinematica AG; Switzerland) for 1 min, the extract was incubated at 65 °C for 15 min and cooled before adding 5 mL of chloroform:isoamyl alcohol (24:1, v/v). The homogenate was centrifuged at 3900 X *g* for 20 min at 4 °C and the aqueous phase was re-extracted with 10

mL of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v). Nucleic acids were precipitated by adding 2 volumes of cold ethanol and centrifuged immediately at 27,200 X *g* for 15 min. The resulting pellet was dissolved in 900 µL of TES (10 mM Tris–HCl, 5 mM EDTA, 0.1% SDS, pH 7.5) and RNA was precipitated overnight at − 20 °C by adding 300 µL of 12 M LiCl. After centrifugation at 27,200 X *g* for 60 min, the precipitated was re-extracted with 250 µL of 3 M sodium acetate (pH 6.0) to remove residual polysaccharides and, finally, dissolved in 200 µL of water. RNA concentration was measured spectrophotometrically and verified by ethidium-bromide staining of the gel. Total RNA was treated with DNase (TURBO DNase, Ambion, USA) to remove contaminating genomic DNA. Single-strand cDNA was synthesized from 10 µg of total RNA using SuperScript III reverse transcription kit and an oligo(dT), according to the manufacturer's instruction (Invitrogen, USA).

2.4. Quantification of relative gene expression by real-time RT-PCR

Gene-specific primer sets (mfsAf/r, mfsBf/r, mfsCf/r and mfsDf/r) were designed for gene expression analysis of *mfsA*, *mfsB*, *mfsC* and *mfsD* with the OLIGO Primer Analysis Software V.5, amplifying PCR fragments between 138 and 257 bp in length (Table 1). Real-time RT-PCR reactions were performed in a LightCycler 480 System (Roche) using SYBR Green to monitor cDNA amplification. The ribosomal 18S RNA gene was used as a reference gene (primers S18f and S18r, Table 1). To calculate the normalized relative gene expression levels (fold induction), data were analyzed using the Relative Expression Software Tool (REST) and the mathematical model based on mean cross point differences between the sample and the control group (Pfaffl et al., 2002). REST was also used for a randomization test with a pair-wise

175 reallocation to assess the statistical significance of the differences in expression
176 between the control and treated samples (significance at $p \leq 0.05$).

177 **2.5. Characterization of the *mfsA* gene**

178 The complete DNA sequence of *mfsA* gene and its flanking regions were
179 obtained using the Universal Genome Walker kit (Clontech, USA) according to
180 the manufacturer's instruction. The gene-specific primers used for PCR
181 amplification and sequencing are given in Table 1 (from mfsA.1 to mfsA.10 and
182 AP1-AP2). PCR products were purified with the UltraClean PCR Clean-up DNA
183 Purification kit (MoBio, USA) and directly sequenced using the BigDye
184 Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, UK) in an Applied
185 Biosystems automatic DNA sequencer model 373A.

186 A computational structural search was carried out for the predicted MfsA
187 protein using the Pfam database (Finn et al., 2008) (http://pfam.sanger.ac.uk/).
188 The search for putative binding sites of transcription factors in the promoter
189 region of *mfsA* was done using the program MatInspector (version 2.7 of the
190 Genomatix Software Suite). Additionally, to predict the location of helical
191 transmembrane and loop regions a consensus predictor, TOPCONS, was used
192 (Bernsel et al., 2009).

193 **2.6. Construction of the *mfsA* gene replacement plasmid**

194 Amplified fragments around 1100 bp from the promoter and terminator
195 regions were cloned into the plasmid vector pRF-HU2 (Frandsen et al., 2008), a
196 binary vector designed to be used with the USER friendly cloning technique
197 (New England Biolabs), as described previously (Crespo-Sempere et al., 2011).
198 The specific primers for the promoter and terminator regions included 9 bp long
199 2- deoxyuridine containing overhangs, msfA.A, mfsA.B, mfsA.C and mfsA.D

(Table 1), which ensured directionality in the cloning reaction. The promoter and terminator regions were amplified with EcoTaq DNA Polymerase (Ecogen, Spain). Cycling conditions consisted of an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 56 °C for 1 min and 72 °C for 2 min and a final elongation step at 72 °C for 10 min. Both DNA inserts and the treated vector were mixed together and treated with the USER (uracil-specific excision reagent) enzyme (New England Biolabs, USA) to obtain plasmid pRFHU2-MFSA. An aliquot of the mixture was used to transform chemical competent *E. coli* DH5α cells. Kanamycin resistant transformants were screened by PCR. Proper fusion was confirmed by DNA sequencing. Then, plasmid pRFHU2-MFSA was introduced into electrocompetent *A. tumefaciens* AGL-1 cells.

2.7. Fungal transformation

Transformation of *A. carbonarius* was done as described previously (Crespo-Sempere et al., 2011) using *A. tumefaciens* AGL-1 cells carrying the plasmid pRFHU2-MFSA. Equal volumes of IMAS-induced bacterial culture (de Groot et al., 1998) and conidial suspension of *A. carbonarius* (10^6 conidia/mL) were mixed and spread onto nitrocellulose membrane filters, which were placed on agar plates containing the co-cultivation medium (same as IMAS, but containing 5 mM instead of 10 mM of glucose). After co-cultivation at 26 °C for 40 h, the membranes were transferred to CYA plates containing Hyg B (100 µg/ml) as the selection agent for fungal transformants, and cefotaxime (200 µg/mL) to inhibit the growth of *A. tumefaciens* cells. Hygromcycin resistant colonies appeared after 3 to 4 days of incubation at 28 °C.

2.8. Confirmation of *mfs*A deletion

Deletion of *mfs*A was confirmed by PCR analyses of the transformants. The insertion of the selection marker was checked with the primer pair Hmbr1 and Hmbf1, while the homologous recombination was checked with the primer pair mfsA.E-mfsA.F (Table 1). Additionally, real-time genomic PCR analyses were performed in order to determinate the number of T-DNA molecules that have been integrated in the genome of the transformants, following basically the procedure established by Solomon et al. (2008). The primer pair mfsA.G-mfsA.H (Table 1) was designed within the T-DNA in the promoter region of the target genes, close to the selection marker. qPCR reactions were performed in a LightCycler 480 System (Roche, USA) using SYBR Green to monitor DNA amplification. qPCR efficiency (E) for each pair of primers was calculated from the slopes of the standard curve using the LightCycler software (Rasmussen, 2001). The number of T-DNA copies that have been integrated in the genome was calculated based on the efficiency (E) and the Crossing point (Cp) value of transformant versus the wild type strain, and normalized in comparison to a reference gene that is present with the same copy number in both wild type and transformant. This can be whatever gene except *mfsA*. We chose the pyruvate carboxylase (*pyc*) gene (GenBank accession Nº GW328015) using primers pyc-F (5'-.GCAGGCCAAGAAGTGTGGTG-3') and pyc-R (5'-TGCTGGGGTTCAGCATGTC-3'). The number of T-DNA copies that have been integrated in the genome of the transformant was calculated according to the following equation based on Pfaffl (2001) and Rasmussen (2001):

$$\text{copy number} = \frac{(E_{\text{target gene}})^{\Delta Cp_{\text{target gene}}\,(\text{wild type} - \text{transformant})}}{}$$

$$\frac{}{(E_{\text{reference gene}})^{\Delta Cp_{\text{reference gene}}\,(\text{wild type} - \text{transformant})}}$$

Three technical replicates were done for each knockout mutant candidate, and PCR reaction quality was checked by analyzing the dissociation and amplification curves.

2.9. Determination of vegetative growth

For growth assessment, CYA plates were inoculated centrally with 5 µl of conidia suspensions (10^6 conidia/mL) from the wild-type strain of *A. carbonarius* W04-40 and the Δ*mfsA* knockout transformant. Two perpendicular diameters of the growing colonies were measured daily over four days until the colony reached the edge of the Petri dish. The assay was performed in triplicate.

2.10. Extraction and detection of OTA

OTA was extracted using a variation of a simple method described previously (Bragulat et al., 2001). The isolates were grown on CYA and incubated at 28 °C for four days (Pitt, Hocking, 1997). For each day, three agar plugs (diameter 6 mm) from three independent plates were removed from the inner, middle and outer part of the colonies and placed in a vial containing 500 µL of methanol. All extractions were done from different Petri dishes. After 60 min, the extracts were shaken and filtered (Millex® SLHV 013NK, Millipore, Bedford, MA, USA) into another vial and stored at 4 °C until chromatographic analysis. Separation, detection and quantification of OTA were performed by injecting 20 µl of extract from each vial into an HPLC system consisting of a Dionex model P680A pump (Sunnyvale, USA) connected to a Dionex model RF-2000 programmable fluorescence detector and to a Dionex PDA-100 photodiode array detector. For determination of OTA, a C18 reversed-phase

column (150×4.6 mm i.d., 5 µm particle size Kromasil C18 (Análisis Vínicos S.L., Spain), connected to a precolumn Kromasil C18 (10×4.6 mm i.d., 5 µm particle sizes, Análisis Vínicos S.L.) were used. For chromatographic separation of OTA, the mobile phase was acetonitrile: water: acetic acid, (57:41:2 v/v/v) under isocratic elution during 10 min, at a flow rate of 1 mL/min. OTA was determined by fluorescence detection at an excitation wavelength of 330 nm and an emission wavelength of 460 nm. The ochratoxin standard was obtained from *A. ochraceus* (Sigma-Aldrich, USA).

2.11 Statistical analyses

All comparisons were analyzed by One way ANOVA followed by the Least significant different test (LSD), using Statgraphics Centurion Version XVI. Significance was defined as $p<0.05$.

3. Results and Discussion

3.1 Gene expression analysis of MFS transporters

In a previous work in which a suppression subtractive hybridization approach was performed with two strains of *A. carbonarius*, antagonistic in their OTA-production ability, four MFS transporters were identified as putative differentially expressed genes (Crespo-Sempere et al., 2010). To confirm whether these MFS transporters have distinct gene expression patterns in the high OTA (W04-40) and low (W04-46) OTA producer strains, total RNAs from three replicates were extracted and analyzed by real-time qRT-PCR. As observed in Fig. 1, *mfsA* was the gene with the highest relative expression in the *A. carbonarius* strain W04-40 when compared to the non-producer W04-46 strain. While *mfsA* showed a 102-fold induction in the OTA producer strain, the

expression levels of *mfsB*, *mfsC* and *mfsD* were similar in both strains, with relative gene expression ratios of 0.88, 1.028 and 0.786, respectively. Therefore, *mfsA* was chosen for further analysis.

3.2 Sequence analysis and characterization of *mfsA*

A genome walking strategy was used to obtain the complete nucleotide sequence of *mfsA* as well as the upstream and downstream flanking regions from *A. carbonarius* W04-40 strain (GenBank accession N^{o} KF297338). A total of 4162 bp of genomic DNA was obtained, which included 1189 and 1303 bp corresponding to the adjacent regions of *mfsA*. The first 864 nucleotides within the upstream region showed homology with the last nucleotides of a zinc finger, $Zn(II)_2Cys_6$, transcription factor in *Aspergillus niger* (XP_001388476) while the following 325 bp correspond to the *mfsA* promoter. Regarding the downstream region, the last 303 nucleotides of the sequenced region showed homology to a haloacid dehalogenase-like hydrolase (XP_001388478) from *A. niger*. Thus, *mfsA* is flanked by a transcriptional factor upstream and a haloacid dehalogenase-like hydrolase downstream.

The *mfsA* gene consists of 1670 bp including three introns of 65, 66 and 54 bp, so that it encodes a protein of 495 amino acids. The identification of intron boundaries was performed based on the analysis of transcripts and in the fact that the splice site sequence in filamentous fungi tends to be 5'-GT...AG-3' (Kupfer et al., 2004). In addition, protein homologues were identified by a BLAST search on the NCBI database. MfsA is highly homologous (84%) to a multidrug transporter from *A. niger* (XP_001388477.2), which shows similarity to the multidrug efflux transporter FLU1 from *Candida albicans* that confers resistance to fluconazole (Calabrese et al., 2000).

The putative transcription factor binding sites present in the *mfsA* promoter were analyzed using the software Matinspector in the Genomatix database (Cartharius et al., 2005). MatInspector analysis showed that the promoter of *mfsA* contains putative consensus binding motifs of transcriptional factors involved in the stress response to the presence of reactive oxygen species or fungicides and nutrient starvation (GCN4, HAC1, HAP1, MSN2, REB1, RDS2, SKN7 and XBP1) (Figure 2 and Table 2). Transcription factor binding sites involved in the regulation of catabolism reactions (ARO80, DAL82, GAL4), amino acid biosynthesis (LEU3), mating (MATALPHA2), activation of persoxisomal proteins (OAF1), morphogenesis and virulence (PHD1) and nutrient signaling (RPH1) were also detected. Although validation of these putative binding sites would require further experimental evidence, it is interesting to mention the high number of putative transcription factor binding sites identified that are involved in the response to stress.

Conserved Pfam domains (http://pfam.sanger.ac.uk/) were localized in the MfsA protein, confirming that this protein belongs to the Major Facilitator Superfamily and, in particular, to the MFS_1 Family (PF07690). The majority of the proteins (59%) within the MFS_1 family have a single conserved domain, as is the case of MfsA, but some of the proteins belonging to this family have also other domains corresponding to acyltransferases, sugar transferases or AMP-binding enzymes. Interestingly, the MFS_1 domain was found together with domains of proteins involved in the biosynthesis of mycotoxins, such as AflR, a transcriptional factor that regulates aflatoxin biosynthesis (Ehrlich et al., 1999) and Tri12, a transporter of trichothecenes (Alexander et al., 1999), in *Penicillium*

marneffei and *A. niger*, respectively, although it has not been described that these species produce aflatoxins or trichothecenes.

The deduced MfsA protein was analyzed also by bioinformatic tools using the five TOPCONS algorithms (SCAMPI-seq, SCAMPI-msa, PRODIV, PRO and OCTOPUS) which predicted that MfsA has twelve transmembrane α-helices (Bernsel et al., 2009) (Figure 3). This data is congruent with the fact that almost all MFS proteins have a uniform topology of 12 transmembrane α-helices connected by hydrophilic loops, with both their N- and C-termini located in the cytoplasm (Pao et al., 1998; Saier, 2003).

3.3 Deletion of the *mfsA* gene

To study whether *mfs*A is involved in the production or transport of OTA, the gene was deleted in the *A. carbonarius* W04-40 strain by replacing it with a hygromycin resistance cassette. The gene replacement plasmid was constructed with the 1.1 kb upstream and downstream fragments of the *mfs*A gene, which were cloned in the vector pRF-HU2 (Frandsen et al., 2008) flanking the hygromycin resistance marker. The resulting plasmid (pRFHU2-MFSA) was used to obtain the *mfsA* knockout (Δ*mfsA*) mutant through *A. tumefaciens*-mediated transformation (ATMT) (Figure 4A and B). T-DNA integration was confirmed by PCR analyses based on the expected genomic patterns using locus specific primers (Figure 4B and C). While the expected size for the PCR product amplified with primers mfsA.E and mfsA.F in the wild type strain is 1345 bp, the size in the transformants with homologous T-DNA insertion is 2551 bp. Two out of eleven analyzed transformants had integrated the T-DNA at the *mfsA* locus by homologous recombination, which gives a homologous recombination frequency of 18.2%. We then determined the

number of the T-DNA copies that had been integrated in the *A. carbonarius* genome by qPCR. If a Δ*mfsA* mutant had one or several extra copies of the T-DNA integrated elsewhere in the genome, then the phenotype of the strain could not be unambiguously attributed to the lack of the *mfsA* gene. Both null mutants (Δ*mfsA*.4 and Δ*mfsA*.5) harbor only a single copy of the T-DNA, the one that is replacing the original *mfsA* gene, while an ectopic transformant, included as a control, harbors nine copies of the T-DNA (Table 3). Further studies were carried out with Δ*mfsA*.4 and Δ*mfsA*.5 null mutants as independent microbiological duplicates.

3.4 Phenotypical analysis of the Δ*mfsA* deletion mutants of *A. carbonarius*

The loss of the *mfsA* gene in the *A. carbonarius* W04-40 strain leads to a slightly debilitated strain, which shows a slower growth (Figure 5). Δ*mfsA*.4 and Δ*mfsA*.5 transformants showed a colony diameter 5 to 10% smaller than the wild type. This growth reduction has been observed previously in other *A. carbonarius* transformants and could reflect a loss of fitness probably due to the expression of the hygromycin resistance gene (Crespo-Sempere et al., 2011). However, there were no significant differences in sporulation among the strains (Figure 6).

In contrary to what we could expect, Δ*mfsA*.4 and Δ*mfsA*.5 null mutants showed a high increase in total OTA production compared to the wild type strain (Figure 7).As mentioned in Section 3.1, the *mfsA* gene is induced in the high OTA producer *A. carbonarius* strain compared to the low OTA producer (Figure 1). However, Δ*mfsA* null mutants have a much higher mycotoxin production than the parental strain. Since OTA is still detected when *mfsA* is disrupted, it could be argued that either *mfsA* does not transport OTA or there are other OTA transport mechanisms apart from *mfsA*. The similar distribution of OTA between mycelia and extracellular medium in the parental wt strain and the

401 Δ*mfsA*.4 and Δ*mfsA*.5 null mutants (Figure 7) further supports a marginal role of
402 MfsA in the transport of OTA. Similar results were obtained when a MFS
403 transporter, *aflT*, located within the aflatoxin gene cluster in *A. parasiticus*, was
404 disrupted (Chang et al., 2004). The Δ*aflT* deleted mutants produced and
405 secreted aflatoxins at levels similar to the wild type strain. Recently, a
406 mechanism of aflatoxin secretion alternative to those conducted by MFS
407 transporters has been proposed (Roze et al., 2011). Chanda et al. (2009)
408 showed the existence of multifunctional vesicles, named aflatoxisomes, where
409 aflatoxin is biosynthesized and exported to the outside of the cell in a similar
410 way to the exocytosis process. In addition, these authors claim that despite the
411 fact that the involvement of *aflT* in the aflatoxin transport is not clear, the
412 possibility of a mixed mechanism for aflatoxin secretion with MFS transporters
413 and exocytosis, could not be discharged. Therefore, OTA could be secreted
414 similarly by *A. carbonarius*, although the role of *mfsA* is not clear.
415 As mentioned before MfsA showed a high level of homology with the
416 protein FLU1, a multidrug efflux transporter which has been associated with the
417 transport of toxic substances and the resistance to fungicides in *C. albicans*
418 (Calabrese et al., 2000). Additionally, a high number of putative transcription
419 factor binding sites related to response to stress have been identified within the
420 *mfsA* promoter. These facts led us to investigate the expression of *mfsA* under
421 a stress situation. When *A. carbonarius* W04-40 was incubated on petri dishes
422 amended with hydrogen peroxide, the expression of *mfsA* reached a 50-fold
423 induction when 5000 μM of hydrogen peroxide was added to the media (Figure
424 8). This result points to a possible role of MfsA in detoxification. If this would be
425 the case, the deletion of the *mfsA* gene would lead to an accumulation of the

toxic compound and, consequently, an increase in oxidative stress which could stimulate mycotoxin production, as has been demonstrated in previous studies for several fungi (Reverberi et al., 2012; Reverberi et al., 2010). This hypothesis would be congruent with the results obtained in this study, although further analysis would be needed to elucidate which substances are transported by MfsA. While no direct relation has been found between *mfsA* and the production of OTA, *mfsA* might indirectly influence mycotoxin production because of the close relationship between oxidative stress generated by toxic substances and mycotoxin production.

4. Conclusions

The *A. carbonarius mfsA* gene, encoding a Major Superfamily Family transporter, is up-regulated in an high OTA producing strain in comparison to a low OTA producing strain. Deletion of this gene leads to OTA overproduction, which suggests that MfsA forms part of the defense against toxic compounds, as manifested by its induction in the presence of hydrogen peroxide.

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656

657 **Figure captions**

658

659 Figure 1. Relative gene expression abundance for MFS transporters genes in
660 W04-40 (high OTA producer) versus W04-46 (low OTA producer). Error bar
661 represents standard deviation. (*) significance $p < 0.005$.
662 Figure 2. Potential binding sites of regulatory elements predicted by
663 MatInspector (Genomatix Software version 2.7) within the promoter of *mfsA*
664 (325 bp).
665 Figure 3. Prediction of the location of helical transmembrane and loop regions
666 by the consensus predictor TOPCONS.
667 Figure 4. Deletion of the *mfsA* gene in *A. carbonarius*. (A) Physical map of the
668 plasmid pRFHU2-MFSA. LB = Left Border, HR2 = Homologous flanking region

669 2, PTrpC = *trpC* promoter from *Aspergillus nidulans*, HygR = hygromycin
670 phosphotransferase, TtrpC = *trpC* terminator from *A. nidulans*, HR1 =
671 Homologous flanking region 1, RB = Right Border, oriV = origin of replication in
672 *E. coli*, KanR = kanamycin resistance, TrfA = replication initiation gene (broad-
673 host-range). (B) Diagram of the replacement of *mfsA* with the hygR selectable
674 marker from pRFHU2-MFSA by homologous recombination to generate the
675 Δ*mfsA* null mutants. (C) Expected amplification band patterns with primer pairs
676 E-F and Hmbr1-Hmbf1 for the wild-type and Δ*mfsA* null mutants.
677 Figure 5. Mycelial growth of Δ*mfs*A mutants and wild type strain of *A.*
678 *carbonarius* (W04-40) inoculated in CYA plates without selection marker. Error
679 bars indicate standard deviations. For each day identical letters indicate
680 homogeneous groups (ANOVA; LSD, $p < 0.05$). Numbers indicate the relative
681 growth in comparsion with the wild type strain.
682 Figure 6. Conidiation in the Δ*mfsA* mutants and wild type strain of *A.*
683 *carbonarius* (W04-40) inoculated in CYA plates. Error bars indicate standard
684 deviations. For each day identical letters indicate homogeneous groups
685 (ANOVA; LSD, $p < 0.05$).
686
687 Figure 7. OTA production in the Δ*mfsA* mutants and wild type strain of *A.*
688 *carbonarius* (W04-40) inoculated in CYA plates. Error bars indicate standard
689 deviations. For each day identical letters indicate homogeneous groups for total
690 (agar+mycelium) OTA production (ANOVA; LSD, $p < 0.05$). Numbers indicate
691 the percentage of OTA found in the agar.
692 Figure 8. Relative expression *mfsA* in *A. carbonarius* W04-40 grown in the
693 presence of different concentrations of hydrogen peroxide with respect to

694 expression level in the same medium without hydrogen peroxide. Error bars
695 indicate standard errors.

696

697

698 **Table legends**

699 Table 1. List of primers used in this study.

700 Table 2. Potential binding sites of regulatory elements predicted by
701 MatInspector (Genomatix Software version 2.7) within the promoter of *mfsA*
702 (325 bp).

703 Table 3. Estimation of the number of T-DNA copies that have been integrated in
704 the genome of the mutants.

705

706

Table 1. List of primers used in this study.

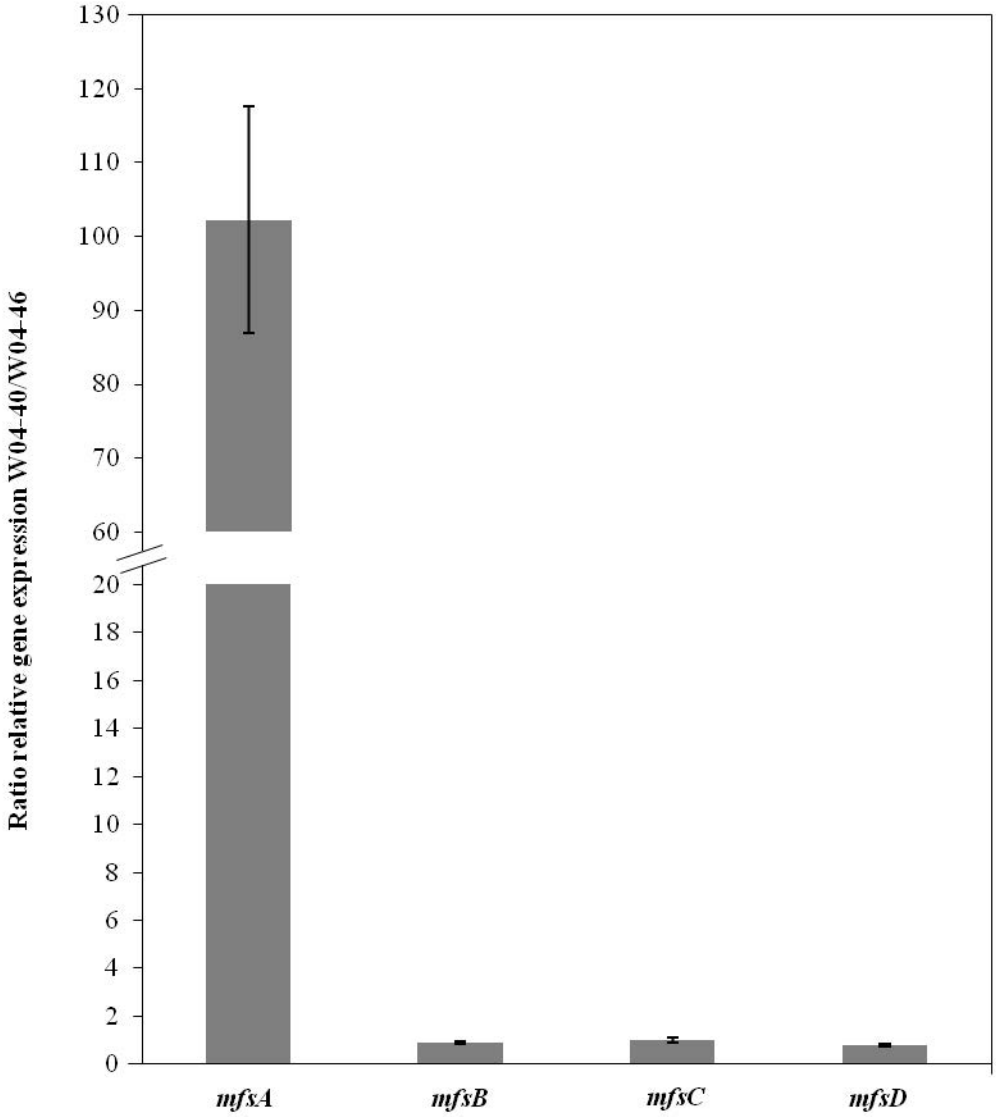
	Oligo sequence (5’-3’)
mfsAf	GGCCATCATTAATTGGCATCG
mfsAr	CCAGGATAGATCGCAGCAGCAAAGT
mfsBf	AAGCCCTCTCAACCGGAGC
mfsBr	TATGCTTTGTGCGCATGGTC
mfsCf	ACGCCTTCACCGGATTCTTATC
mfsCr	TCCGGTTGAGTGTTTACGGTATG
mfsDf	CGAGGTGGATCTGTTGTTCG
mfsDr	TCATGCTGCTCGTCCTTGC
S18f	GCAAATTACCCAATCCCGACAC
S18r	GAATTACCGCGGCTGCTG
mfsA.1	CACTTTGCTGCGATCTATCCTG
mfsA.2	AGCAGCCATGCTGTATGGTGTAG
mfsA.3	GAATGCGATGCCAATTAATGATG
mfsA.4	GGTCCATCCGAAGAGAAATAAGC
mfsA.5	GCAGATCTCAGACAGCACAACG
mfsA.6	GCTGGATAACTTCAGAACCTCAACG
mfsA.7	CATCTCGAGGATTATTCCCAGATTC
mfsA.8	CATATTAGCTCGGCCAAGTCACTG
mfsA.9	TGTGGGGCAGGGATATTGTC
mfsA.10	TGCGTATCCGGAACTCTCCTG
AP1	GTAATACGACTCACTATAGGGC
AP2	ACTATAGGGCACGCGTGGT
mfsA.A	GGTCTTAAUGAGAATCTCAGCATGCGTAGTCTG
mfsA.B	GGCATTAAUGAATTCTTGCGCAATCTGCTG
mfsA.C	GGACTTAAUCGCAACATTGGGGTAGACTG
mfsA.D	GGGTTTAAUGCCGTTTTCAATACAAAATTTGTG
mfsA.E	CGTCGGCCGTCTATTCAGC
mfsA.F	CACCGTGATGCCCCAGTCT
mfsA.G	CGAGTACCAGGCTGTGTGGAC
mfsA.H	CTTGGTAACCACTGGGCTGTG
Hmbf1	CTGTCGAGAAGTTTCTGATCG
Hmbr1	CTGATAGAGTTGGTCAAGACC

Table 2. Potential binding sites of regulatory elements predicted by MatInspector (Genomatix Software version 2.7) within the promoter of *mfsA* (325 bp).

Factor	Funtional Features	Reference
ARO80	Activates catabolism of aromatic amino acids in response to aromatic amino acids that act as inducers	(Lee, Hahn, 2013)
DAL82	Activates allantoin catabolic genes	(Scott et al., 2000)
GAL4	Involved in catabolism of galactose	(MacPherson et al., 2006)
GCN4	Involved in stress and nitrogen starvation response	(Ecker et al., 2010; Rodrigues-Pousada et al., 2010)
HAC1	Regulates the unfolded protein response which is a protective event against endoplasmatic reticulum stress	(Kimata et al., 2006)
HAP1	Regulates expression of genes required for respiration and for controlling oxidative damage	(Lan et al., 2004)
LEU3	Regulates branched amino acid biosynthesis	(Zhou et al., 1987)
MATALPHA2	Regulates mating in yeasts	(Johnson, Herskowitz, 1985)
MSN2	Transcriptional activator for genes in multi stress response	(Sadeh et al., 2011)
OAF1	Activates peroxisomal proteins in the presence of a fatty acid such as oleate	(Karpichev, Small, 1998)
PHD1	Regulates morfogenesis and virulence in ascomycetes	(Sheppard et al., 2005)
RDS2	Mediates ketoquenazole resistance and regulates gluconeogenesis	(Akache, Turcotte, 2002; Soontorngun et al., 2007)
REB1	Involved in termination of rRNA transcription and regulates G1 phase under nitrogen starvation	(Rodríguez-Sánchez et al., 2011)
RPH1	Involved in nutrient signaling	(Orzechowski Westholm et al., 2012)
SKN7	Contributes to the stress response	(Fassler, West, 2011)
XBP1	Contributes to the unfolded protein response against endoplasmatic reticulum stress	(Yoshida et al., 2001)

Table 3. Estimation of the number of T-DNA copies that have been integrated in the genome of the mutants.

Efficiency		**Cp *mfs*A**				**Cp *pyC***				**Copy number**		
mfsA	***pyC***	**Wild type**	**Δ*mfs*A.4**	**Δ*mfs*A.5**	**Ectopic**	**Wild type**	**Δ*mfs*A.4**	**Δ*mfs*A.5**	**Ectopic**	**Δ*mfs*A.4**	**Δ*mfs*A.5**	**Ectopic**
2.065	2.047	21.08±0.13	20.91±0.18	21.00±0.28	18.01±0.30	20.83±0.23	20.82±0.17	20.92±0.11	20.80±0.18	1.12	1.13	9.065


Ratio relative gene expression W04-40/W04-46
130
120
110
100
90
80
70
60
20
18
16
14
12
10
8
6
4
2
0
mfsA
mfsB
mfsC
mfsD


1 ATCAAGCAGAGGACAGCACTTCCCTTGATCACACACGTAAGTCATGTGTTAGCACCCCCATAATGTGGTTACACACCTTTCCCGAGGAAGTGCGATCATC
>>>>>>>>>>>>>>>>>>>>
HAC1
<<<<<<<<<<<<<<<<<<
ARO80
>>>>>>>>>>>>>
MATALPHA2
<<<<<<<<<<<<<
GCN4
<<<<<<<<<<<<<<<<
RPH1
101 TGCAAACTCCCAGTCCAAGTTACCGGGGGGATCGGGATCGGGTAAGCCTATGCGAGCGGAGCCAAGTTTCCTCGACCCTCCGGCCCAGCCATCGCTTGCG
>>>>>>>>
PHD1
<<<<<<
REB1
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SKN7
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DAL82
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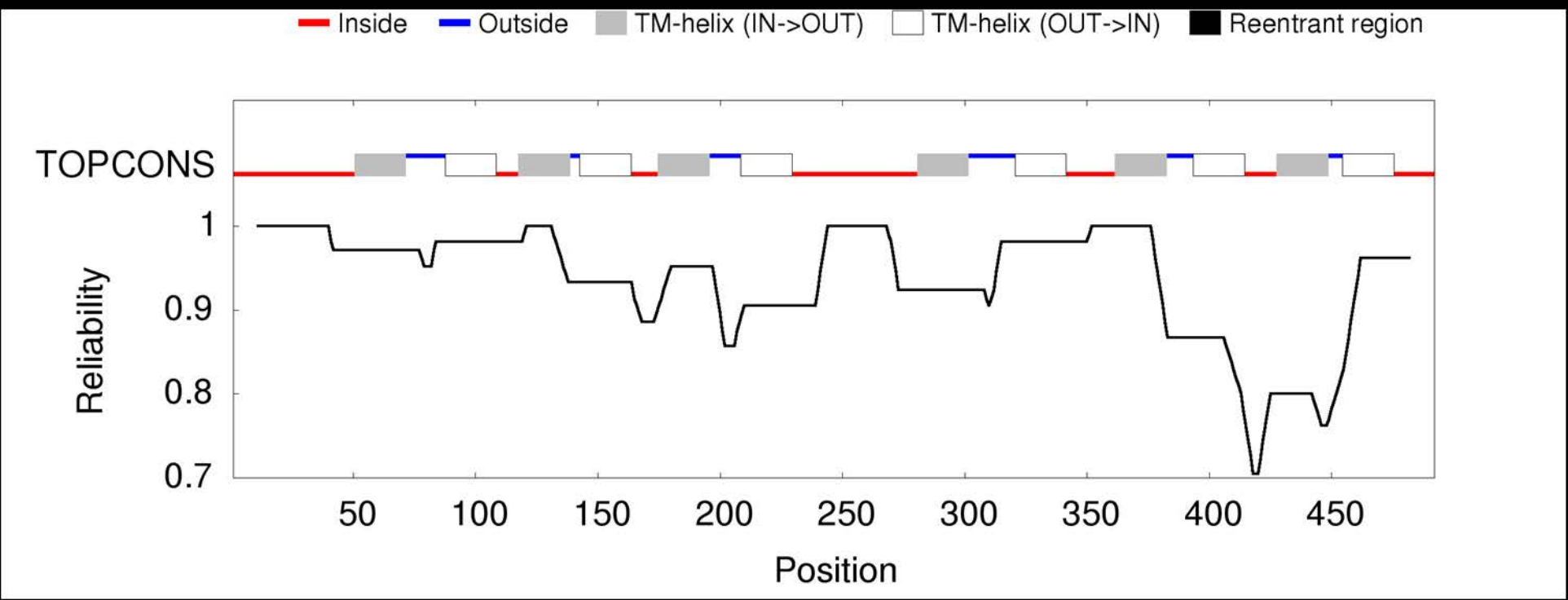
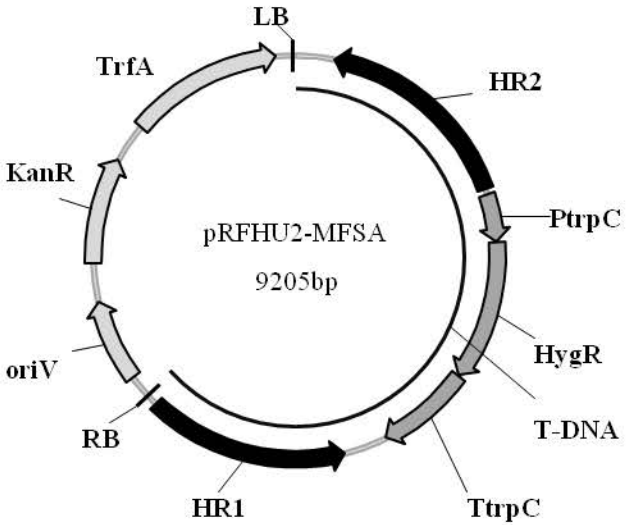
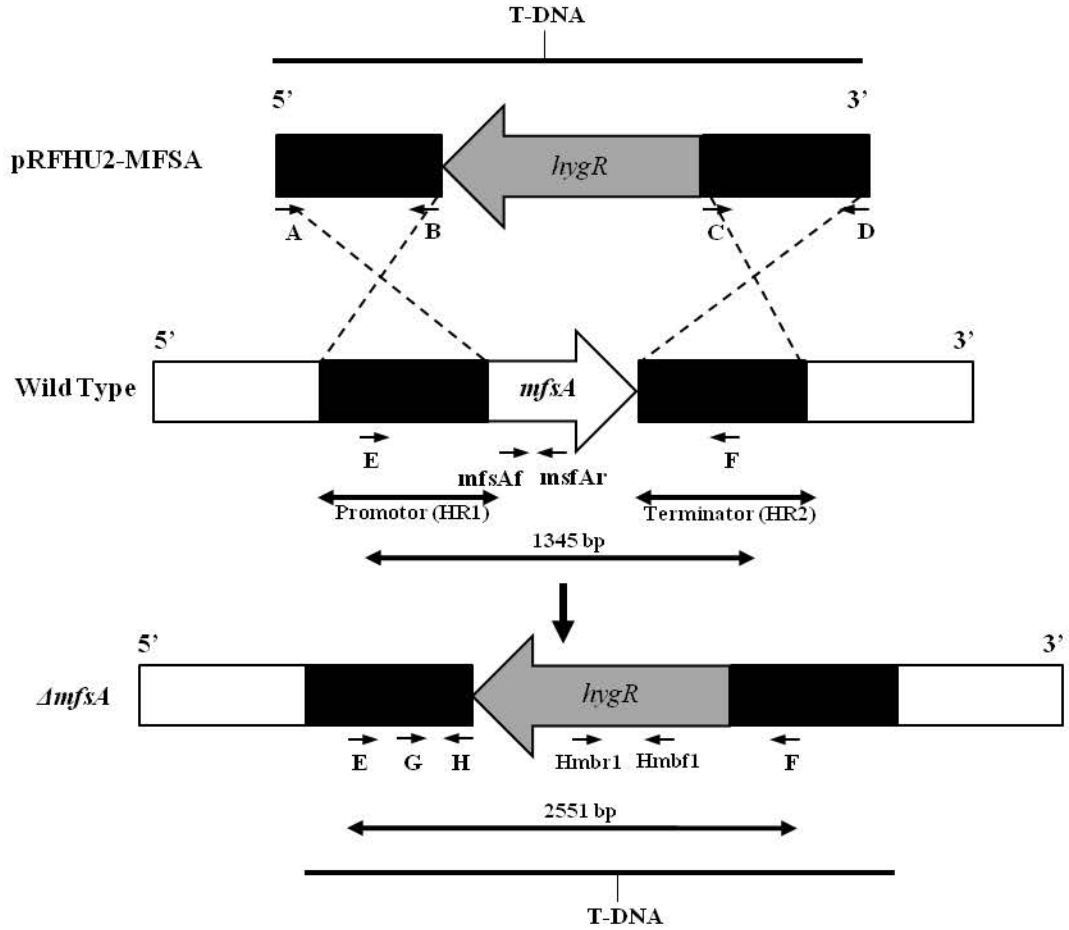
XBP1
>>>>>>>>>>>>>>>>>
LEU3
>>>>>>>>>>>>>>>>>>>>>>>>
ICGG_N10
>>>>>>>>>>>>>>>>>>>>>>>>>>>
GAL4
<<<<<<<<<<<<<<<<
RDS2
>>>>>>>>>>>>>>>>>>>>>>>>>>
GAL4
>>>>>>>>>>>>>>>>
HAP1
>>>>>>>>>>>>>>>>>
LEU3
201 CATAAGTCAGGCTGGCCGAGTACCAGGCTGTGTGGACACCTTTTAAGTGAAGAGCCCCTCAACTGATTCATGGCAAGCCTTTTTTGCTATCTTCCTATCA
<<<<<<<<<<<
MSN2
>>>>>>>>>>>
AOF1
>>>>>
PHD1
<<<<<<
DAL82
>>>>>>>>>>>>>>
GCN4
>>>>>>>>>>>
SKN7
301 CCGGCTGCCATCTTCGACCTCGGCC
>>>>>>>>>>>>>>>
AOF1


Inside
Outside
TM-helix (IN->OUT)
TM-helix (OUT->IN)
Reentrant region
TOPCONS
Reliability
1
0.9
0.8
0.7
50
100
150
200
250
300
350
400
450
Position

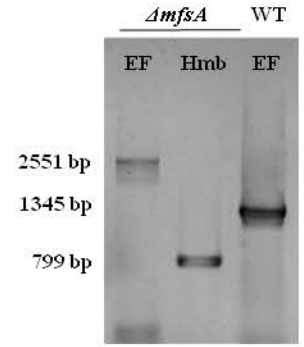
A)

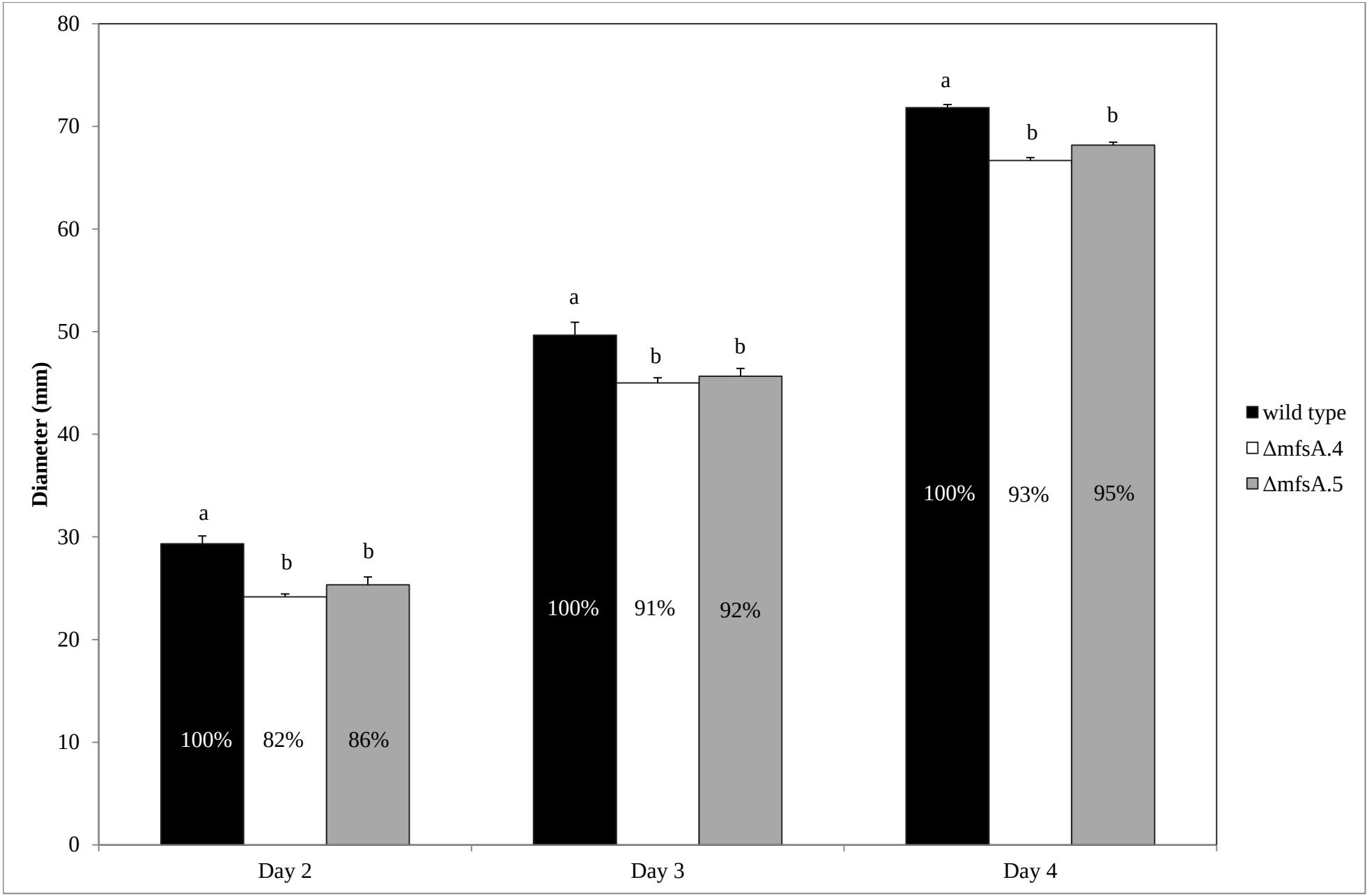

LB
TrfA
HR2
KanR
pRFHU2-MFSA
9205bp
PtrpC
HygR
oriV
T-DNA
RB
HR1
TtrpC

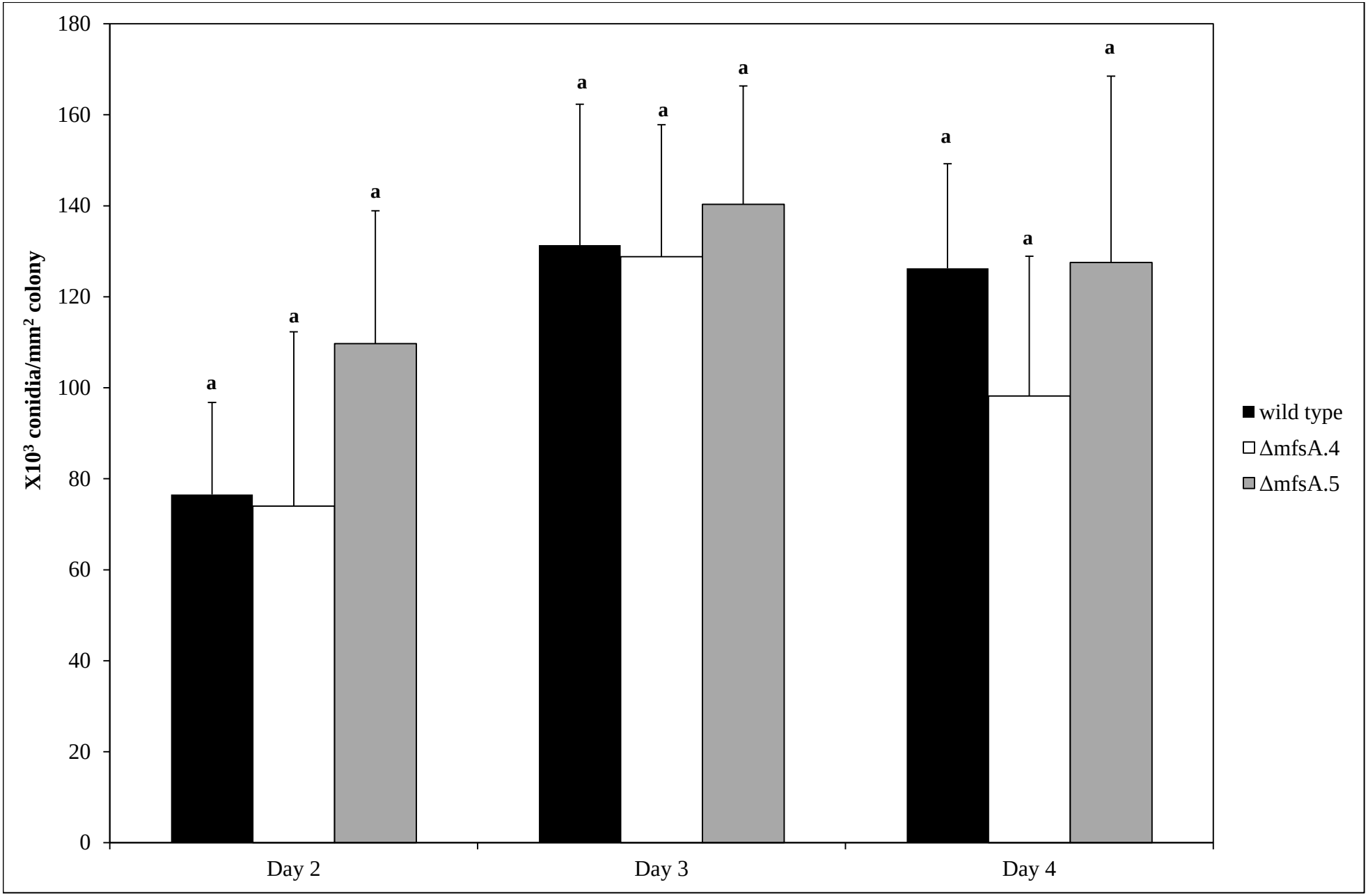
B)

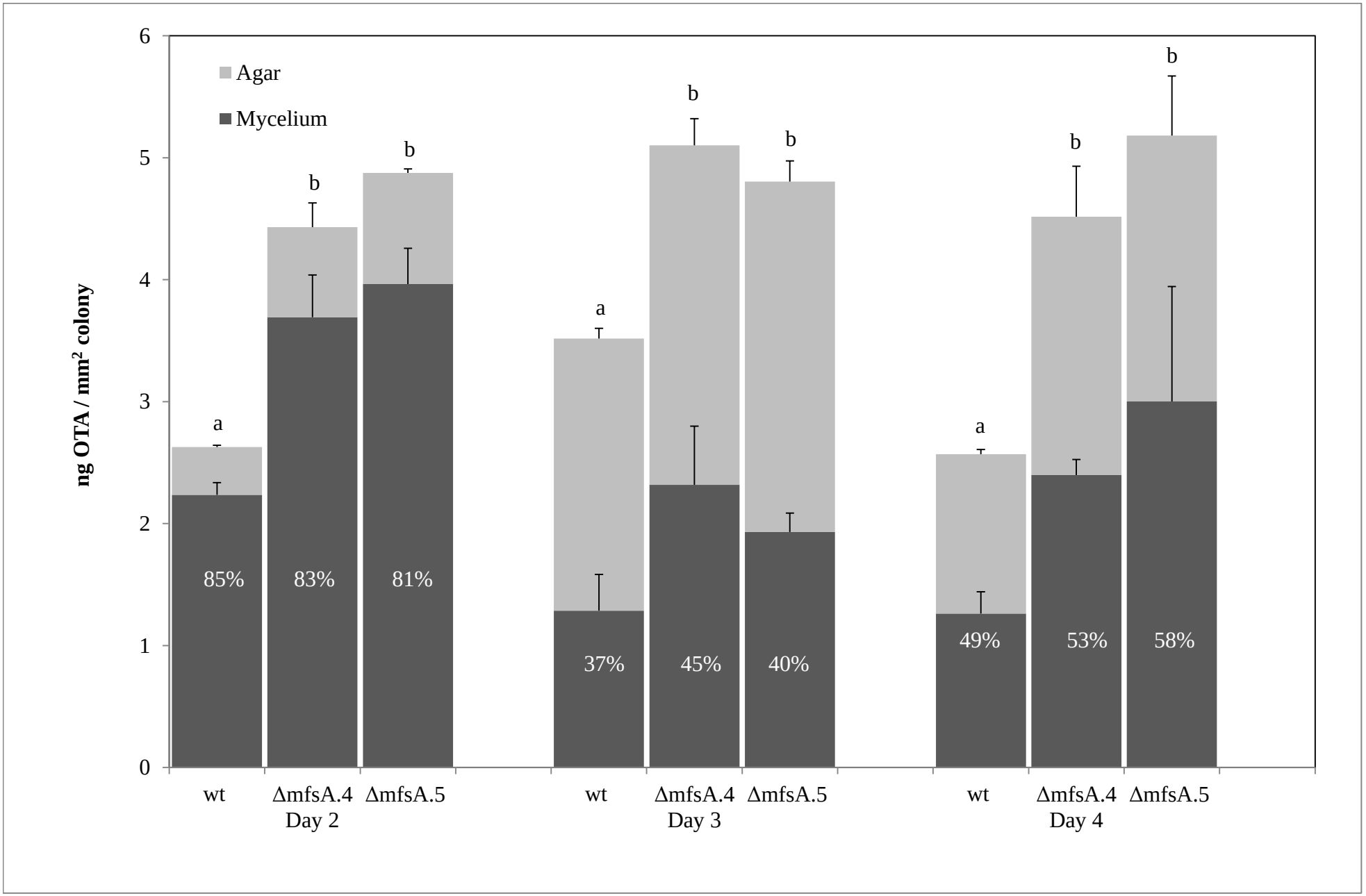

T-DNA
5'
3'
pRFHU2-MFSA
hygR
A
B
C
D
5'
3'
Wild Type
mfsA
E
mfsAf
msfAr
F
Promotor (HR1)
Terminator (HR2)
1345 bp
5'
3'
ΔmfsA
hygR
E
G
H
Hmbr1
Hmbf1
F
2551 bp
T-DNA

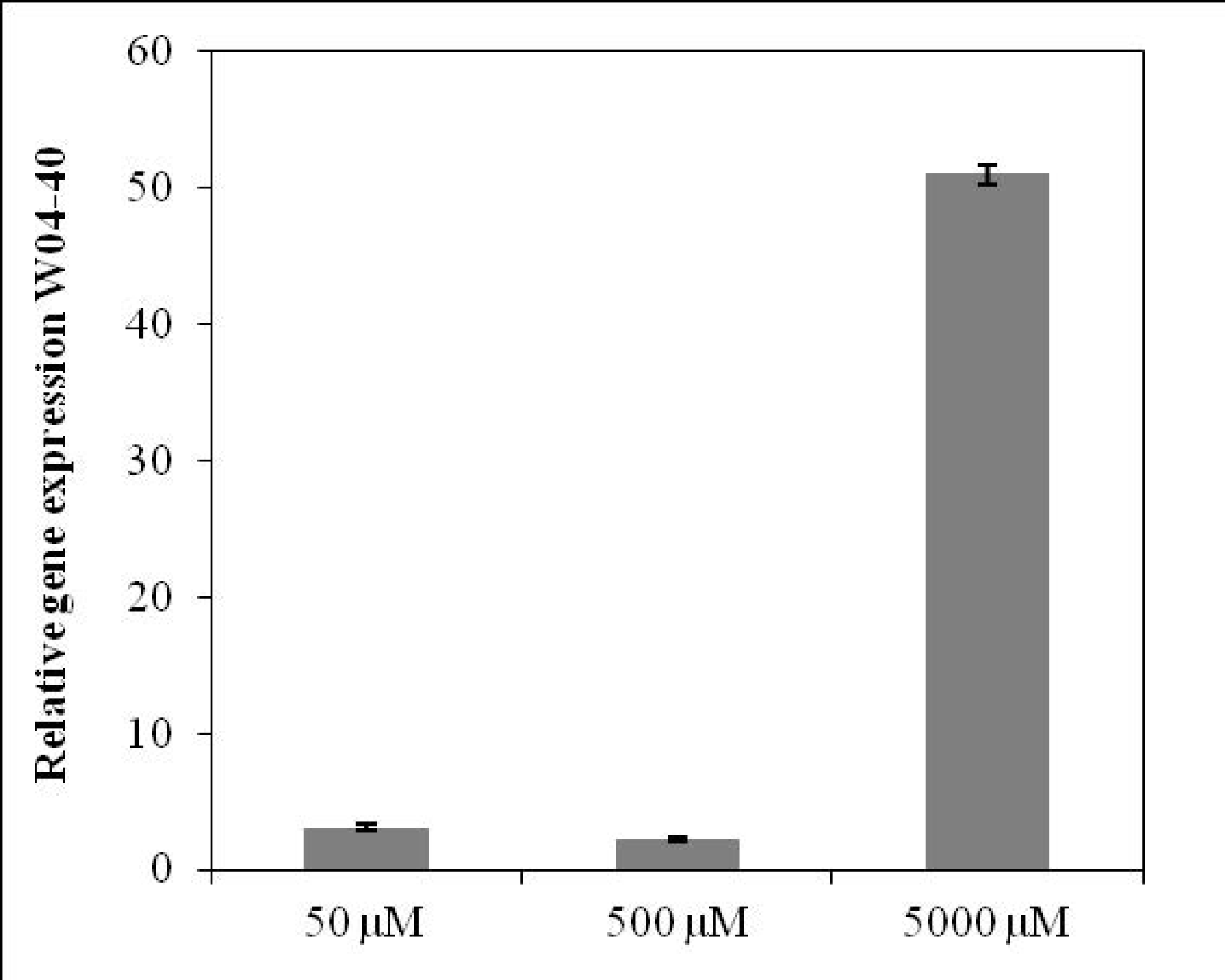
C)


ΔmfsA
WT
EF
Hmb
EF
2551 bp
1345 bp
799 bp


80
70
60
50
40
30
20
10
0
Diameter (mm)
a
b
b
100%
82%
86%
a
b
b
100%
91%
92%
a
b
b
100%
93%
95%
Day 2
Day 3
Day 4
wild type
ΔmfsA.4
ΔmfsA.5


180
160
140
120
100
80
60
40
20
0
X10³ conidia/mm² colony
a
a
a
a
a
a
a
a
a
Day 2
Day 3
Day 4
wild type
ΔmfsA.4
ΔmfsA.5


Agar
Mycelium
ng OTA / mm² colony
0
1
2
3
4
5
6
a
b
b
a
b
b
a
b
b
85%
83%
81%
37%
45%
40%
49%
53%
58%
wt
ΔmfsA.4
ΔmfsA.5
Day 2
wt
ΔmfsA.4
ΔmfsA.5
Day 3
wt
ΔmfsA.4
ΔmfsA.5
Day 4


Relative gene expression W04-40
60
50
40
30
20
10
0
50 µM
500 µM
5000 µM