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Gene silencing with RNA interference in the human pathogenic fungus *Aspergillus fumigatus*

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Abstract

Aspergillus fumigatus is an opportunistic pathogenic fungus which causes fatal invasive aspergillosis among immunocompromised patients. To obtain a better understanding of the key elements involved in *A. fumigatus* virulence and to identify possible drug targets, it is necessary to be able to generate gene-deletion strains. Unfortunately, the molecular techniques available do not include a rapid method to disrupt and identify essential genes. RNA interference, a process in which the presence of double-stranded RNA homologous to a gene of interest results in specific degradation of the corresponding message, has been successfully tested on *A. fumigatus*. We have shown that expression of double stranded RNA corresponding to portions of the *ALB1*/*PKSP* and *FKS1* genes results in reduced mRNA levels for those genes, with phenotypic consequences similar to that of gene disruption. The two genes could also be subjected to simultaneous interference through expression of chimeric double-stranded RNA. Use of RNA interference in *Aspergillus* will allow easier examination of the phenotypic consequences of reducing expression of a gene of interest, especially for essential genes.

Keywords: *Aspergillus fumigatus*; RNA interference; *ALB1*; *FKS1*; Essential genes

1. Introduction

Aspergillus fumigatus is a saprophytic fungus, normally associated with decaying organic matter. In immunocompromised patients, it usually causes a fatal invasive disease (Invasive Aspergillosis, IA), and in recent years *A. fumigatus* has become the most important air-borne fungal pathogen for such patients [1–3]. Understanding the pathobiology of this disease requires the identification of virulence factors by genetic manipulation of pathogenic strains and phenotypic analysis of the resulting mutants. In addition, identifying specific genes that are essential for growth in *A. fumigatus* is the first step in the discovery of new antifungal targets. Gene disruption is usually accomplished using homologous recombination. The most commonly used method is to replace a wild type gene with a version of that gene that has been interrupted or replaced by an antibiotic (hygromycin or phleomycin) [4,5] or an auxotrophic nutrients such as the uridine or lysine or arginine [6,7] (May, personal communication). However, the utility of this conventional approach for generating gene deletion strains in *A. fumigatus* is limited by the poor efficiency of homologous recombination in filamentous fungi [8–10] and the time required to construct the deletion vectors. In addition, disruption of essential genes is currently impossible in this species in the absence of a non-leaky inducible promoter. A diploid method has

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been published for assessing essential genes [11] but important deletions occur in the genome during this process, making this methodology unusable for targeted disruption (Chabane, Henry, unpublished observations). One recently developed method for specifically down-regulating gene expression is double-stranded RNA interference (RNAi). In this process, double-stranded RNA (dsRNA) induces the specific destruction of mRNA to which it is homologous [12,13]. The dsRNA "trigger" is thought to be cleaved into shorter fragments (21–25 nucleotides) [14,15], which then guide specific degradation of the corresponding mRNA, catalysed by a protein or protein complex with nuclease activity [16]. Specific inhibition of gene expression by RNAi has been demonstrated in a range of organisms, from the initial report on *Caenorhabditis elegans* [17], to trypanosomes [18,19], *Drosophila* [20], mammalian cells [21] and the pathogenic fungus *Cryptococcus neoformans* [22–24].

Three genes involved in the molecular mechanism of RNA silencing have been identified and characterized in the fungus *Neurospora crassa* [25]. The first of these is *QDE1*, which is similar to an RNA-dependent RNA polymerase found in the tomato [26,27]. The second is *QDE2*, a homolog of *RDE1*, which is essential for dsRNA interference in *C. elegans* [28,29]. The third is *QDE3*, a member of the RecQ DNA helicase family [30] that is similar to *AGO1* from *A. thaliana* [31]. Homologs of *QDE1, QDE2* and *QDE3* have been identified in the TIGR *A. fumigatus* database (http://tigrblast.tigr.org/ufmg/), with BLAST scores of 6×10^{-122}, 2×10^{-65} and 3×10^{-154}, respectively. The presence of these orthologs suggested that RNAi should be functional in *A. fumigatus*.

To evaluate RNAi methodology in *A. fumigatus*, we selected two genes that would produce clear phenotypes if the technique was successful. The first of these was *ALB1*/*PKSP*, which encodes polyketide synthase. This enzyme is involved in the melanin biosynthesis required for conidial pigmentation [32,33], so that the conidia of the ΔALB1 mutant obtained by gene replacement are white, instead of green like the parental strain. The second gene tested was *FKS1*, which encodes the catalytic subunit of β(1–3)glucan synthase [34,35]. This has been shown to be unique and essential in *A. fumigatus* [11].

2. Materials and methods

2.1. Strains and culture conditions

Aspergillus fumigatus strains used for this study were previously characterized: G10, a nitrate reductase mutant of strain CBS 144.89 [36], was used for transformation experiments in minimal medium [37]. *Escherichia coli* DH5α was used for plasmid propagation. Plasmid pUC19 was used in subcloning procedures. Ampicillin (100 μg/ml) or chloramphenicol (25 μg/ml) were added to the growth medium when required.

2.2. Cloning procedures, and DNA manipulations

Agarose gel electrophoresis, Southern blotting and subcloning of genomic DNA fragments into plasmids were performed according to standard protocols [38]. *A. fumigatus* DNA was isolated according to the procedure of Girardin et al. [39]. For PCR, samples in a 100 μl reaction volume containing 200 μM of dNTPs, 50 pmol of each primer, 10 ng of cDNA and 1 U of Taq polymerase (Pharmacia) were subjected to 30 cycles of amplification consisting of the following steps: 1 min at 95 °C, 1 min at 55 °C and 1 min at 72 °C. The PCR products were subcloned in PCR2.1 (TA Cloning Kit, InVitrogen) and sequenced.

2.3. Construction of an pRNAi vector containing the pGla promoter and the ALB1 terminator

pCB1004 [40], a kind gift of MH. Lebrun, was derived from plasmid pCB SK+ (Stratagene) and contains the *E. coli* hygromycin B phosphotransferase (hph) gene flanked by the trpC terminator and gpd promoter of *A. nidulans*. 1200 bp of the promoter of the glucoamylase gene of *Aspergillus niger* (pGla) [41] was amplified with primers 1-PGla1 and 2-PGla2 (Table 1) from plasmid pGUS 64 (a kind gift of CAMJJ Van den Hondel), and inserted into pCB1004 that had been digested with *Not*I and *Xba*I. 700 bp of the terminator of *A. fumigatus ALB1*/*PKSK* [32,33] was PCR amplified from genomic DNA with primer 3-TrpALB1ApaI and 4-TrpALB1KpnI and inserted in the resulting plasmid, after *Apa*I and *Kpn*I digestion. This final product was called pRNAi.

2.4. Construction of pALB1 (Fig. 1)

Constructs for RNA interference were designed with inverted repeats of 500 bp of coding sequence of the gene of interest separated by a spacer segment of green fluorescent protein (GFP) sequence [42]. A portion of the coding sequence of *ALB1* from nucleotides 704 to 1203 [32] was PCR amplified from CBS 144.89 genomic DNA, using primers 5-ALB1a and 6-ALB1b (Table 1) to add an *Hin*dIII restriction site at the 5′ end and a portion of GFP sequence at the 3′ end (Product A). A portion of the GFP coding sequence was PCR amplified from plasmid pMCB32 [42] using primer 7-ALB1c and 8-GFP (Table 1) to add a portion of *ALB1* sequence at the 5′ end and an *Cla*I restriction site at the 3′ end (Product B). PCR products A and B were then used as the template for a PCR with primers 5-ALB1a and 8-GFP to amplify a 750-bp fragment (Product C). This was then digested with *Hin*dIII and *Cla*I and ligated into

Table 1
Primers used in this study

	Sequence (5′–3′)	Description
1-PGla1	ATAAGAAT**GCGGCCGC**CTACCTGGCCTCATACTTC	*Not*I [a]pGla underlined
2-PGla2	CTAG**TCTAGA**CCATGGCTGAGGTGTAATG	*Xba*IpGla underlined
3-TrpALB1Apa1	CC**GGGCCC**TGGGGTGAAGTTCCTAGG	*Apa*I site
4-TrpALB1Kpn1	GG**GGTACC**CTTGCTTGGTAAGACATTG	*Kpn*I site
5-ALB1a	CCC**AAGCTT**GCGGTTGGATCGAAAGGT	*Hin*dIII
6-ALB1b	CAGCTCCTCGCCCTTGCTCACCATGTTGCATTGGGAGCGGCC	GFP underlined
7-ALB1c	GGCCGCTCCCAATGCAACATGGTGAGCAAGGGCGAGGAGCTG	GFP underlined
8-GFP	CC**ATCGAT**AAGTCGTGCTGCTTCATG	*Cla*I
9-ALB1d	CC**ATCGAT**GTTGCATTGGGAGCGGCC	*Cla*I
10-ALB1e	ACGC**GTCGAC**GCGGTTGGATCGAAAGGT	*Sal*I
11-FKSRNAia	GC**TCTAGA**GCCCGAGCCGCTTCCTGTG	*Xba*I
12-FKSRNAib	CAGCTCCTCGCCCTTGCTCACCATACTTGCGACGAGCCATCC	GFP underlined
13-FKSRNAic	GGATGGCTCGTCGCAAGTATGGTGAGCAAGGGCGAGGAGCTG	GFP underlined
14-GFPFKS	CCC**AAGCTT**AAGTCGTGCTGCTTCATG	*Hin*dIII
15-FKSRNAid	CCC**AAGCTT**ACTTGCGACGAGCCATCC	*Hin*dIII
16-FKSRNAie	CC**GGGCCC**GCCCGAGCCGCTTCCTGTG	*Apa*I
17-FKSRNAif	GC**TCTAGA**ACTTGCGACGAGCCATCC	*Xba*I
18-FKSRNAig	CCC**AAGCTT**GCCCGAGCCGCTTCCTGTG	*Hin*dIII
19-FKSRNAih	ACGC**GTCGAC**GCCCGAGCCGCTTCCTGTG	*Sal*I
20-FKSRNAii	CC**GGGCCC**ACTTGCGACGAGCCATCC	*Apa*I
ALB1RT1	CGCCTGGTCGTATCAACTAC	
ALB1RT2	CGGTATCGCAGTCATTCCTC	
ActinRT1	ATCGGCGGTGGTATCCTC	
ActinRT2	TCTTCGTGCCATTCGTCTG	
FKSRT1	CGCCAAGTTCAATAAGGAAG	
FKSRT2	TCGCAGTGTCCGTCAATAAG	

[a] Restriction sites are in bold.

the similarly restricted plasmid pRNAi. The antisense of *ALB1* was PCR amplified on genomic DNA CBS 144.89 with primers 9-ALB1d and 10-ALB1e to incorporate a *Cla*I site at the 5′end and a *Sal*I restriction site at the 3′ end and ligated and cloned into the appropriate sites of pRNAi containing the *ALB1* sense segment.

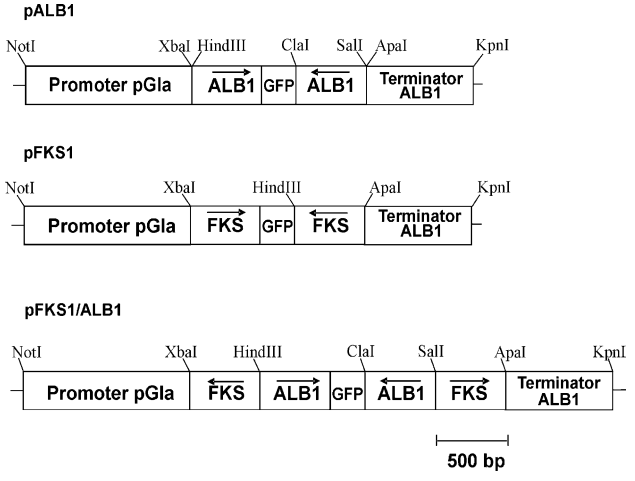

Fig. 1. Constructions used to test RNA interference. Constructs were designed with inverted repeats of 500 bp of coding sequence of the gene of interest separated by a spacer segment of green fluorescent protein (GFP) sequence. pALB1 was used to interfere with *ALB1* expression, pFKS1 to interfere with *FKS1* expression and pFKS1/ALB1 was used for tandem interference with both *ALB1* and *FKS1*.

2.5. Construction of pFKS1 (Fig. 1)

A portion of the coding sequence of *FKS1* from nucleotide 2649 to nucleotide 3148 [35] was PCR amplified from CBS 144.89 genomic DNA using primers 11-FKSRNAia and 12-FKSRNAib (Table 1) to add an *Xba*I restriction site at the 5′ end and a portion of GFP sequence at the 3′ end (Product A). A portion of the GFP coding sequence was PCR amplified from plasmid pMCB32 [42] using primer 13-FKSRNAic and 14-GFPFKS (Table 1) to add a portion of *FKS1* sequence at the 5′ end and an *Hin*dIII restriction site at the 3′ end (Product B). PCR products A and B were then used as the template for a PCR with primers 11-FKSRNAia and 14-GFPFKS to amplify a 750 bp fragment (Product C). This fragment was digested with *Xba*I and *Hin*dIII and ligated into the similarly restricted plasmid pRNAi. The antisense of *FKS1* was PCR amplified on genomic DNA CBS 144.89 with primers 15-FKSRNAid and 16-FKSRNAie to incorporate an *Hin*dIII site at the 5′

end and a *Apa*I restriction site at the 3′ end and ligated and cloned into the appropriate sites of pRNAi containing the *FKS1* sense segment.

2.6. Construction of the double construct ALB1/FKS1 (Fig. 1)

For the double construct, a portion of the coding sequence of the antisense *FKS1* gene was PCR amplified from CBS 144.89 genomic DNA using primers 17-FKSRNAif and 18-FKSRNAig (Table 1) to add a *Xba*I restriction site at the 5′ end and a *Hin*dIII restriction site at the 3′ end, and cloned into pALB1 digested with *Xba*I and *Hin*dIII. The sense *FKS1* fragment was PCR amplified from genomic DNA CBS 144.89 with primers 19-FKSRNAih and 20-FKSRNAii to incorporate an *Sal*I restriction site at the 5′ end and a *Apa*I restriction site at the 3′ end, and cloned into pALB1 containing the antisense *FKS1* fragment.

2.7. Transformation

Circular plasmids described above were used to transform *A. fumigatus* following procedures previously described [9]. After overnight expression of hph, transformants were selected on minimal medium containing 200 μg/ml of hygromycin B for 7 days at 25 °C.

2.8. RNA extraction and reverse transcription (RT)-PCR

Transformants and wild type strains were grown in liquid culture containing 1% yeast extract and either 2% xylose (to repress the pGla promoter) or 2% maltose (to induce the pGla promoter). After growth at 37 °C for 24 h RNA was isolated using the QIAGEN RNA/DNA Kit. Reverse transcription was carried out with the Invitrogen Reverse Transcription System Kit following the instructions of the manufacturer. A tube containing all the reaction components and heat-inactivated (10 min at 94 °C) AMV-RT (Avian myeloblastosis virus) was always included as a negative control to check for the presence of contaminating DNA.

2.9. Real time quantitative PCR

Real time quantitative PCR was performed using an iCycler iQ system (Biorad) using intercalation of SYBR green as a fluorescence reporter. Reactions were carried out using the SYBR green Kit from Biorad following the manufacturer's protocol. PCR were done in duplicate, and to check for specificity of the PCR melting curves were analyzed for each data point. The level of expression of each gene of interest (Ct) was then normalized against the measured level of the RNA coding for actin determined in each sample. Primers were as follows: ALB1RT1-ALB1RT2, FKS1RT1-FKS1RT2 and ActinRT1-ActinRT2 (Table 1). These three pairs of primers should amplify 109, 130 and 117 bp fragments, respectively. Reaction mixtures (25 μl) consisted of 20 μl of SYBR Green I PCR master mix (Biorad) containing SYBR Green I dye, Amplitaq Gold DNA polymerase dNTP and optimized buffer components. Cycling conditions consisted of denaturation at 95 °C for 15 s and annealing at 55 °C for 15 s. Standard curves were created for each run using several 30-fold dilutions.

3. Results and discussion

We have investigated gene silencing using RNAi in *A. fumigatus*. To do this we used RNAi initiated by a hairpin construct, where duplicate sequences of 500 bp of a gene of interest were cloned as inverted repeats separated by a 250-bp spacer of GFP sequence (as described for *C. neoformans* in [22] (Fig. 1). To control the expression of interfering RNA we used the glucoamylase promoter, which is induced in maltose medium and repressed in xylose medium [41,43].

Protoplasts of *A. fumigatus* were transformed with the hph-marker circular interference construct pABL1 (Fig. 1) in order to obtain ectopic integration in the genome. Transformants selected on hygromycin were restreaked to a master plate and replica plated on maltose for induction of RNA interference phenotype, and on xylose for repression of RNA interference phenotype. No white conidia were found among hygromycin resistant transformants after integration of the control plasmid, pRNAi. In contrast, on maltose medium, 29% of the pALB1 transformants demonstrated a clear mutant phenotype attributable to the absence or the reduction of *ALB1* expression, with either white colonies (5%) demonstrating a complete phenotype or light green colonies (24%) indicating an intermediate phenotype (Fig. 2). In contrast, no strong phenotypic changes were seen on xylose medium. Mycelia from one white (2M strain) and one light green colony (5M strain) transformed with pALB1, and from the control parental strain, were further analysed. Southern blotting and PCR experiments showed that the endogenous *ALB1* gene was intact in the transformants and similar to the gene in the wild type strain, suggesting that the phenotype was indeed due to RNAi interference (data not shown). RT-PCR was used to compare qualitatively *ALB1* transcription in these three strains under maltose or xylose supplementation (Fig. 3(a)). The results show that *ALB1* was not detectable when white strains (2M) (complete phenotype) were replicated on the maltose medium which induced RNAi, in contrast to the xylose medium which repressed RNAi. This confirmed the observed phenotype. The quantitative RT-PCR also showed that the *ALB1* gene is expressed 4× less in the

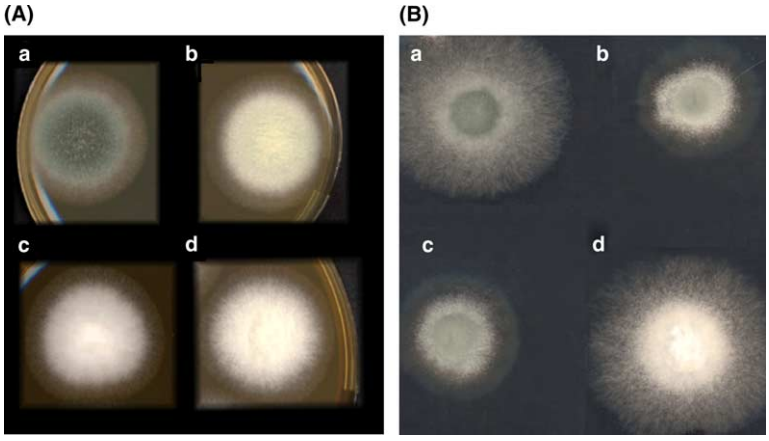

Fig. 2. Phenotype of transformants obtained after *ALB1* RNA interference. Transformants were streaked on medium containing maltose (A) or xylose (B) and incubated for 2 days at 37 °C. Three *ALB1* transformants and an *ALB1* disruption strain are shown: (a) wild type strain (green conidia); (b) 5M, intermediate phenotype (light green); (c) 2M, complete phenotype (white); (d) control ΔALB1 strain (kindly provided by A.A. Brakhage).

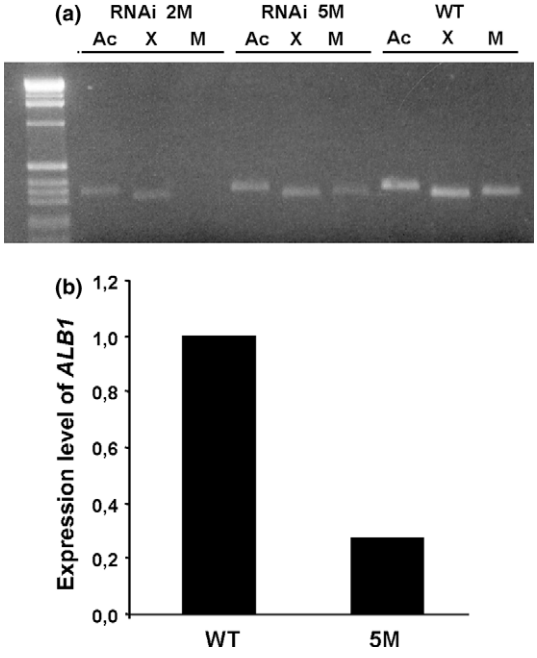

Fig. 3. (a) RT-PCR analysis of gene expression for an RNAi total phenotype transformant, an RNAi intermediate phenotype transformant, and a wild type phenotype transformant. Ac, actin; X, *ALB1* in xylose medium; M, *ALB1* in maltose medium. (b) Quantitative RT-PCR analysis of *ALB1* expression for the wild type (WT) and the intermediate transformants (5M). Results were first standardized against actin, with wild type expression set arbitrarily to 100.

light green strain (5M) (intermediate phenotype) than in the wild type strain on maltose medium (Fig. 3(b)).

Among the hygromycin resistant transformants, it not clear why a range of interference occurs, but the presence of intermediate and complete phenotypes among RNAi mutants has been observed in other microorganisms submitted to RNAi, including *C. neoformans* [22] and *T. brucei* [18]. One explanation in our experiments could be different sites of the integration event in the genome that induce variable expression of the plasmid; this could lead to variable efficacy in mediating mRNA degradation.

To examine the possibility of using dsRNAi to silence essential genes, we made an interference construct with inverted repeats of a portion of the *FKS1* gene (pFKS1, Fig. 1). Replication on maltose and on xylose medium (Fig. 4) showed that 1% of the transformants had a complete RNAi phenotype (58T), as demonstrated by growth on xylose medium and total lack of growth on maltose medium. Southern blotting and PCR experiments showed that the endogenous *FKS1* gene was intact in the transformants (data not shown). Growth on xylose medium of these transformants was reduced compared to a wild type strain. Quantitative RT-PCR

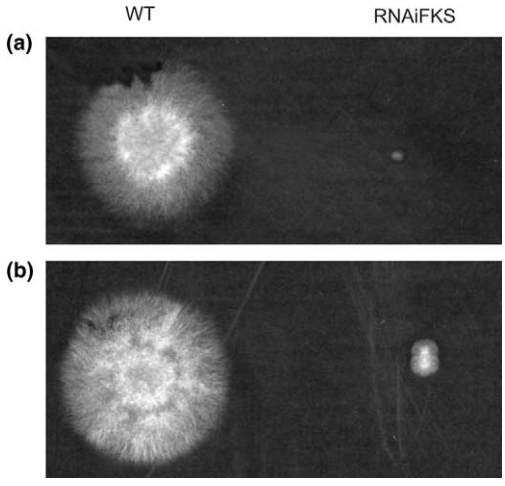

Fig. 4. Phenotype of transformants obtained after *FKS1* RNA interference. Wild type strain and 58T transformant were streaked on maltose 2% medium (a) and xylose 2% medium (b) and incubated for 2 days at 37 °C.

on xylose medium confirmed that FKS gene is 4× less expressed in the 58T strain than in the wild type strain (Fig. 5). This can be explained by the fact that the promoter pGla is leaky [43] and that even a slight reduction in the expression of *FKS1* was sufficient to inhibit normal growth. The total % of RNAi transformants obtained with *FKS1* was not as high as with *ALB1*. One explanation could be a loss of transformants during successive replica plating due to very slow growth caused by the leaky promoter.As in the case of *ALB1*, intermediate phenotypes (4%) were also seen among hygromycin resistant transformants. All of them exhibited a reduced growth on maltose medium. These results show that inducible RNA interference is a useful method to evaluate essential genes in *A. fumigatus*.

Investigation of novel genes by RNAi will require assessment of mRNA levels in a number of transformants before phenotypes can be examined with confidence that they result from interference. One way to mark transformants that exhibit interference is to interfere simultaneously with the gene of interest and with a marker gene which provides an easily tracked phenotype. Such an approach was successful in *C. neoformans* where Liu et al. [22] used tandem interference with two genes and found that 80% of the transformants obtained showed simultaneous interference of a gene involved in capsule synthesis and the *ADE2* gene which yields easily identified pink colonies. To exploit this approach, we tested RNA interference targeting both the *ALB1* and *FKS1* genes using an interference plasmid incorporating portions of each gene (Fig. 1). Four percent of the transformants displayed white conidia and reduced growth on xylose medium and were not able to grow on maltose medium, showing that both *FKS1* and *ALB1* were silenced by this construction. Twenty five percent of the transformants showed intermediate phenotypes for one or both genes. Our results show that RNA interference is a useful method for specific down-regulation of gene expression in *A. fumigatus* for several reasons. First, inducible RNAi can be used to identify essential genes. One of the weakness of the construct made is that the promoter, pGla is not totally repressed on xylose medium [41]. This could be however an advantage in a screen for essential genes in identifying small colonies. We are nevertheless analyse a new inducible promoter in our construct, the isocitrate lyase promoter that is totally repressed on glucose medium (kindly provided by M. Brock and A. brakhage, Hanover). It will be possible to use also RNAi in vivo if a system with a constitutive promoter is developped and the total absence of expression of the genes of interest verified before in vivo experiment. In addition, RNAi can produce cells with reduced expression of essential genes, yielding viable cells with intermediate phenotypes which could not be studied by a traditional disruption approach. Importantly, the effects of RNAi can be reversed using an inducible promotor, permitting in-depth study of gene function and avoiding the need to complement mutants with wild type genes, a process that is extremely difficult and time consuming in *A. fumigatus*. Tandem constructs incorporating *ALB1* and a gene of interest will simplify the process of identifying mutants of unknown phenotype in which RNAi is operating, and allow analysis of double mutants in *A. fumigatus*. In another application, because RNAi is a homology-based silencing system, it can also be used at the level of gene families. Simultaneous interference with homologous family members using a single dsRNA has been demonstrated in trypanosomes [19] for three genes encoding cAMP-specific phosphodiesterase. In *Drosophila*, this has also been achieved using a combination of dsRNA in the same experiment for a family of neuronal receptor tyrosine phosphatases [44]. Obviously, RNAi will be the approach of choice for genome-wide screens of gene function in *A. fumigatus*.

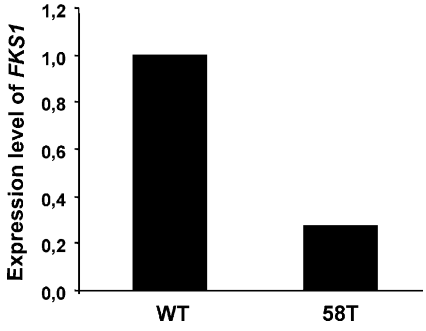

Fig. 5. Quantitative RT-PCR analysis of *FKS1* expression on xylose medium for the *FKS1* transformant (58T) compared to the wild type (WT). Results were first standardized against actin, with wild type expression set arbitrarily to 100.

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