RESEARCH LETTER

RNA silencing in the dermatophyte *Microsporum canis*

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Abstract

Dermatomycoses caused by *Microsporum canis* are frequent in domestic animals and easily transmissible to humans. Several proteases secreted by this fungus were identified as potential virulence factors, but the construction of deficient strains is required to investigate their role in the pathogenesis of the disease. Using target genes encoding two of these proteases, a first evaluation of the utility of RNA-mediated silencing as a reverse genetic tool in dermatophytes was carried out. *SUB3* and *DPPIV*, respectively coding for a subtilisin and a dipeptidyl peptidase, were both down-regulated, by means of two plasmid constructs designed to express an RNA hairpin that corresponds to part of their respective sequence. The degree of attenuation was evaluated by enzymatic assay of the transformants culture supernatants, and by real-time reverse transcriptase-polymerase chain reaction. Enzymatic activities and expression levels varied from less than 5% to 100% of that of control transformants obtained with plasmid without hairpin inserts. Inhibition was globally more efficient for *SUB3* than for *DPPIV*. These results show that RNA silencing can be used for functional genomics in *M. canis*, and particularly to circumvent the limits and technical difficulties of conventional disruption methods.

Introduction

Dermatophytic infections with the ascomycete *Microsporum canis* are common contagious skin mycoses in cats and dogs. In addition to being a frequent and worldwide concern in veterinary medicine, dermatophytosis caused by *M. canis* is also of great zoonotic importance. To date, little is known about the pathogenesis of the infection. Proteolytic enzymes are thought to be key factors in the invasion of keratinized structures like hair, nails, and *stratum corneum*. Among them, a family of metalloproteases (Brouta *et al*., 2002; Jousson *et al*., 2004a), a family of subtilisins (Descamps *et al*., 2002), and two dipeptidyl peptidases (GenBank accession nos. DQ286524 and DQ286525) have been characterized at the gene level. However, definitive evidence of their implication as virulence factors would require the construction of deficient strains.

Conventional gene disruption is widely used to knock out genes in filamentous fungi; however, this strategy is time-consuming and greatly hampered by the poor efficiency of homologous recombination in these organisms (Nakayashiki, 2005; Weld *et al*., 2006). In dermatophytes, the low transformation frequency (Gonzalez *et al*., 1989) constitutes an additional problem. In spite of these difficulties, functional gene investigation by means of gene disruption was performed in *Trichophyton rubrum* (Fachin *et al*., 2006; Ferreira-Nozawa *et al*., 2006) and *M. canis* (Yamada *et al*., 2006). In the latter study, the homologous recombination efficiency was 2%. An alternative method for gene silencing, RNA interference (RNAi), could be helpful for functional gene investigation. This technique is based on a natural phenomenon, by which a double-stranded RNA (dsRNA) induces enzymatic degradation of mRNAs in a sequence-specific manner. This post-transcriptional inhibition pathway was first described in the nematode *Caenorhabditis elegans* (Fire *et al*., 1998), and now it is known to operate in a wide variety of eukaryotic organisms, including filamentous fungi. In fungal systems, assessments of the silencing ability of different RNA species showed that double-stranded RNA in the form of a 500–1000 base pairs (bp) hairpin, with a relatively short intron-containing loop, was the more potent trigger for interference (Liu *et al*., 2002; Kadotani *et al*., 2003; Goldoni *et al*., 2004; Rappleye *et al*., 2004; Nakayashiki *et al*., 2005; Yamada *et al*., 2007).

Hairpin-induced RNA silencing for the functional investigation of fungal genes has never been utilized so far in dermatophytes. Its applicability was tested to silence two genes in *M. canis*: *SUB3* and *DPPIV*. *SUB3* encodes a major immunogenic and keratinolytic endopeptidase of the subtilisin family (Mignon *et al*., 1998a, 1999a, b; Descamps *et al*., 2002, 2003), and is inducible in a medium containing protein as the sole source of carbon and nitrogen. *DPPIV* encodes a dipeptidyl peptidase, which selectively removes an X-Pro dipeptide from the N-terminus of large peptides. The DPPIV protein is an analog of immunomodulatory and tissue-degrading factors (Beauvais *et al*., 1997; Yagishita *et al*., 2001; Boonacker & Van Noorden, 2003; Kumagai *et al*., 2005). In this study, *SUB3* and *DPPIV* were down-regulated by means of integrative plasmid constructs intended to express an RNA hairpin corresponding to a fragment of their specific sequence.

Materials and methods

Strains and culture conditions

For plasmid multiplication, *Escherichia coli* TOP10 (Invitrogen) were grown in Luria–Bertani medium, with 100 μg mL^{-1} ampicillin when selection was required. *Microsporum canis* strain IHEM 21239 was used for fungal transformation. RNA used as a template to obtain gene fragments was extracted from the fungus grown on a feline hair-based medium (Mignon *et al*., 1998b). Fungal mass for the production of protoplasts was obtained by inoculation of 50 mL liquid Sabouraud's medium (Sab; 2% glucose, 1% peptone) with a plug of mycelium grown on S10 plates (0.2% glucose, 0.1% peptone, 0.1% $MgSO_4.7H_2O$, 0.1% KH_2PO_4), and subsequent incubation at 27 °C for 3–5 days. All newly obtained transformants were first grown in Sab containing 100 μg mL^{-1} hygromycin in order to confirm their resistant phenotype. They were then transferred in sterile-distilled water for short-term conservation at room temperature. For subsequent analysis, pS1-*SUB3* transformants were grown in 2.5 mL of a keratin medium (KM; 50 mM phosphate buffer, 0.5% glucose, 0.05 mg mL^{-1} inositol, 0.01 mg mL^{-1} pyridoxine, 0.01 mg mL^{-1} thiamine, 2 mM $MgSO_4$, 0.25% powdered bovine keratin (ICN)) containing 100 μg mL^{-1} hygromycin. pS1-*DPPIV* transformants were grown in the same volume of Sab broth containing 100 μg mL^{-1} hygromycin. All cultures were carried out at 27 °C for 4–5 days.

Construction of silencing vectors pS1-*SUB3* and pS1-*DPPIV*

Two constructs were obtained. The first one coded for an RNA hairpin with sequences identical to a part of *SUB3*. The second one coded for an RNA hairpin with sequences identical to a part of *DPPIV*. For this purpose, inverted

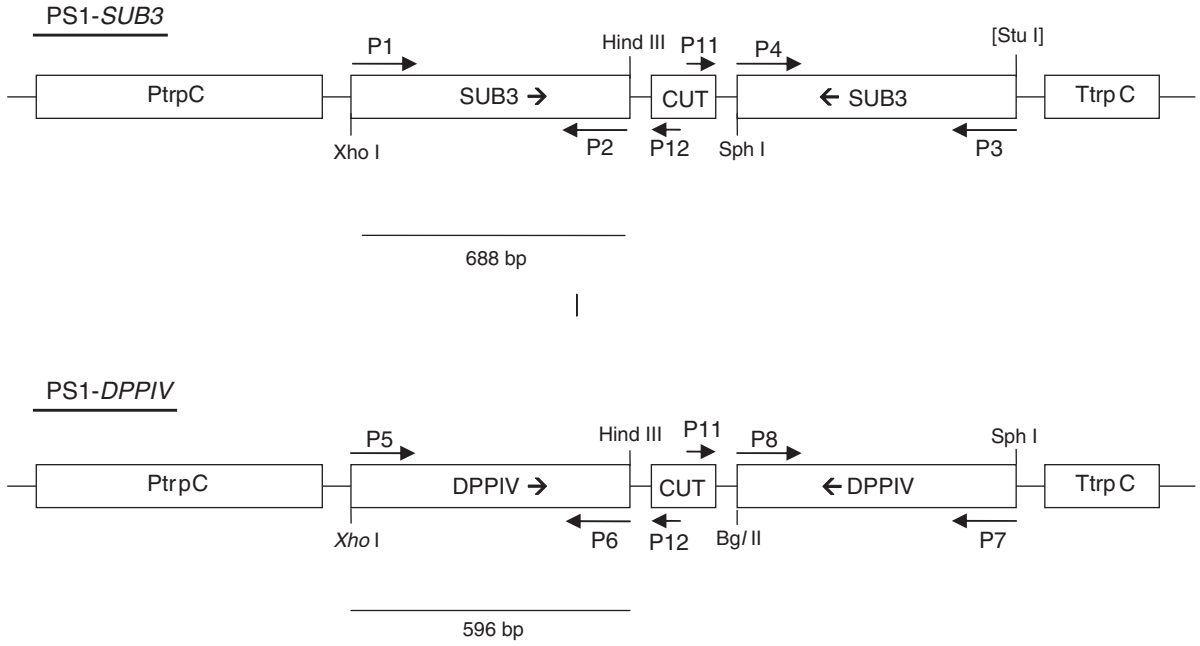

Fig. 1. Map of the constructions used for *SUB3* and *DPPIV* down-regulation. Inverted repeats of *SUB3* and *DPPIV* fragments are shown on both sides of the spacer segment constituted by an intron from the *Magnaporthe oryzae* cutinase gene (CUT). They are flanked by the trpC promoter (PtrpC) and terminator (Ttrpc) from *Aspergillus nidulans*. Oligonucleotide primers used for the obtention and detection of the constructions are represented as numbered arrows (see Table 1).

repeats were cloned into the plasmid pSilent-1 (Nakayashiki *et al.*, 2005), provided by the Fungal Genetics Stock Center (McCluskey, 2003). A map of each construct is shown in Fig. 1. The plasmid pSilent-1 carries the *E. coli* hygromycin B phosphotransferase gene (*HPH*) as a selection marker in fungus transformations, and a transcription unit driven by the *Aspergillus nidulans* constitutive trpC promoter, with two multiple cloning sites separated by an intron-containing spacer.

Inverted repeats of *SUB3* and *DPPIV* fragments consisted of stretches of 688 and 596 bp, respectively. They were amplified from *M. canis* RNA extracted with the RNeasy Plant Mini Kit (Qiagen) after grinding of mycelium under liquid nitrogen. RNA was treated with RQ1 DNase (Promega) and converted to cDNA with the iScript cDNA synthesis kit (Bio-Rad). Standard PCRs were performed (55 °C, 30 cycles) using *Taq* polymerase (Promega), or iProof DNA polymerase (Bio-Rad) when blunt-end ligation was planned. For each gene, two pairs of oligonucleotide primers were designed (primers P1–P2 and P3–P4 for *SUB3* and primers P5–P6 and P7–P8 for *DPPIV*, Table 1). Both pairs delimit the same DNA fragment, but include a tail with different restriction sites, to ensure cloning of the two fragments in opposite directions. PCR products were gel purified with Wizard SV Gel and PCR Clean-up System (Promega). The first fragment of each gene was simply ligated into pSilent-1 after creation of compatible ends and dephosphorylation of the vector with Shrimp Alkaline Phosphatase (SAP, Promega), using T4 DNA ligase (Invitrogen). The second fragment of *SUB3* and *DPPIV* had to be inserted into the recombinant plasmid by a two-step ligation, due to the inefficiency of conventional ligation. Vector was double-digested with SphI/StuI (for *SUB3*) and SphI/BglII (for *DPPIV*), dephosphorylated, and joined to the digested SphI end of each insert. Linear constructions that were obtained were then circularized by a second ligation, creating the silencing vectors pS1-*SUB3* and pS1-*DPPIV*. While pS1-*SUB3* circularization was blunt end, pS1-*DPPIV* was first digested with BglII. Intermediate and final vectors were propagated in *E. coli*, purified by plasmid mini-preparations (Nucleospin Plasmid Kit, Macherey-Nagel), and checked by a series of restriction digestions, followed by agarose gel electrophoresis (data not shown).

Table 1. Sequence of oligonucleotide primers (5′ → 3′) and length of amplicons (bp)

Amplification of *SUB3* and *DPPIV* inverted repeats		
P1	GTTGTTGTTGCTCGAGCGCCGAGGACTTCGACTCC	
P2	GTTGTTGTTGAAGCTTCGAAGATACCAGCATTCTG	688
P3	GTTGTTGTTGGGGCCCCGCCGAGGACTTCGACTCC	
P4	GTTGTTGTTGGCATGCCGAAGATACCAGCATTCTG	688
P5	GTTGTTGTTGCTCGAGTACCGTCACTCCTATTTTG	
P6	GTTGTTGTTGAAGCTTGACAATCTTCTCTGTATCC	596
P7	GTTGTTGTTGGCATGCTACCAACTACACCAAGCAG	
P8	GTTGTTGTTGAGATCTTGAACACGGTTAAAGGCAC	596
HPH detection		
P9	GCTTTCAGCTTCGATGTAGG	
P10	GCATCAGCTCATCGAGAGC	441
Hairpin constructs detection		
P11	GGAGACTTGTTGGCCATG	
P3	GTTGTTGTTGGGGCCCCGCCGAGGACTTCGACTCC	783
P7	GTTGTTGTTGGCATGCTACCAACTACACCAAGCAG	683
P12	GTGGGGACACTATTCGGC	
P1	GTTGTTGTTGCTCGAGCGCCGAGGACTTCGACTCC	774
P5	GTTGTTGTTGCTCGAGTACCGTCACTCCTATTTTG	682
Confirmation of endogenous *SUB3* and *DPPIV* integrity		
P13	ATGGGCTGCATCAAGGTTATC	
P14	CTATCTTCCACTTCCGTTGTAGAG	1402
P15	CCGCTCCAGCCCGGCATGAAG	
P16	TCATTCCTCTGCCTCTTCGCC	2343
Real time PCR experiment		
P17	TCCCAGAGCTCCACCCT	
P18	CGACGATGGGGCGAGAGC	282
P19	GTACCATCGTTGACATCTACGC	
P20	GTTCCTGGGTTCTTGATAAC	205
P21	ATGAAGTTCCTCTCGCTTCTTC	
P22	GGACACACTCCTTGTAGGTC	118

Restriction sites are underlined.

Transformation

Polyethylene glycol (PEG)-mediated transformation of fungal protoplasts was performed, with midi-preparations of plasmids (Quantum Prep kit, Bio-Rad). Mycelium was harvested on two layers of Miracloth (Calbiochem), extensively washed with Digestion Buffer (DB; 100 mM potassium phosphate buffer, pH 5.8, 0.8 M NaCl), and finely chopped with a scalpel blade. Cell wall was digested in portions of 10 mL DB added with 20 mg mL^{-1} Lysing Enzymes from *Trichoderma harzianum* (Sigma), under mild agitation at 33 °C. Protoplast formation was monitored under a microscope and stopped when the protoplast concentration exceeded 10^7 mL^{-1}. Protoplasts were separated from mycelial debris by filtration through four layers of Miracloth and pelleted by centrifugation (5 min, 1500 ***g***). The pellet was washed in DB and allowed to stand for at least 10 min at room temperature. It was then washed in cold STC buffer (10 mM Tris-Cl pH 7.5, 1.2 M sorbitol, 10 mM $CaCl_2$) and harvested; protoplast concentration was adjusted to $1–2 \times 10^8$ mL^{-1} with STC. Twenty micrograms of circular pS1-*SUB3*, pS1-*DPPIV*, or parent pSilent-1 were added to 120 μL of protoplast suspension and incubated on ice for 20 min. The suspension was mixed with 40 μL of 60% PEG 3350 (Sigma) in 10 mM Tris-Cl, pH 7.5, and 10 mM $CaCl_2$, and incubated on ice for 20 min; 400 μL extra PEG solution was then added to the suspension, which was further incubated on ice for 20 min. Transformed protoplasts were regenerated at 27 °C on Sab plates containing 1.2 M sorbitol

(Sab Sorb), as follows: they were poured on the plates in Soft Medium (Sab Sorb containing 0.4% agarose) and overlaid 24 h later with Soft Medium supplemented with hygromycin, to a final concentration of 100 μg mL^{-1}.

PCR testing of the transformants

Transformants were subjected to DNA extraction and standard PCR, in order to check for the presence of the hygromycin resistance gene (*HPH*), of the hairpin-encoding construct, and to confirm the integrity of endogenous target genes. Plugs of mycelium were digested with Lysing Enzymes, harvested by centrifugation, and resuspended in a 0.01 M phosphate-buffered saline (PBS). DNA was purified from lysed mycelium using the High Pure PCR Template Preparation Kit (Roche). PCR was performed with Go*Taq* polymerase (Promega). Primers P9–P10 were used for *HPH* detection; primers P13–P14 and P15–P16 were used for the amplification of endogenous *SUB3* and *DPPIV*, respectively (Table 1). Both arms of hairpin constructs were detected separately, using primers that hybridize in the spacer region (Table 1). As shown in Fig. 1, primer pairs P12–P1 and P11–P3 were used to detect arms of the *SUB3* construct, as were primer pairs P12–P5 and P11–P7 for arms of the *DPPIV* construct.

Enzymatic assays on culture supernatants

As mentioned above, pS1-*SUB3* and pS1-*DPPIV* transformants were cultured on KM and Sab broth, respectively. Culture supernatants were harvested by centrifugation. The total protein concentration was determined using the standard method of Bradford, with dilutions of Bovine Serum Albumine as standards. For the assessment of SUB3 activity, 20 μL of 5 mM *N*-succinyl–Ala–Ala–Pro–Phe–*p*-nitroanilide (AAPF–*p*Na, Sigma) was mixed with 20 μL of supernatant in a total volume of 200 μL. DPPIV activity was assayed by mixing 100 μL of 2 mM Gly–Pro–*p*-nitroanilide (GP–*p*Na, Sigma) and 100 μL of supernatant. A no-substrate blank was set up for each reaction. Tests were performed at 37 °C. Absorbances were measured at several time points during the linear phase of enzymatic kinetics, at 405 nm, with a Multiskan RC spectrophotometer (ThermoLabsystems). For each supernatant, enzymatic activity was calculated in arbitrary units, one unit corresponding to an increase of one absorbance unit per minute. Specific activity was then calculated by dividing the enzymatic activity by the total protein concentration. Residual specific activity levels were obtained, by expressing specific activities in percents with regard to the mean specific activity of four pSilent-1 transformants. A mean residual specific activity was then calculated for all tested pS1-*SUB3* and pS1-*DPPIV* transformants.

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Fungal RNA was extracted with TriPure reagent (Roche) according to the manufacturer's instructions, mycelium samples being homogenized by grinding under liquid nitrogen. Reverse transcription was performed using the iScript cDNA synthesis kit (Bio-Rad) after treatment of RNA solutions with RQ1 DNAse (Promega). Real-time PCR reactions were assembled with qPCR Mastermix Plus for the SYBR Green I – No ROX kit (Eurogentec) and subjected to the following protocol in an iCycler (Bio-Rad): 2 min at 50 °C, 10 min at 95 °C, and 45 cycles of 15 s at 95 °C, 20 s at 60 °C, and 40 s at 72 °C. *Microsporum canis* actin was used as the normalizer gene. Primer pairs P17–P18, P19–P20, and P20–P21 were used to detect actin, *SUB3*, and *DPPIV* mRNAs, respectively (Table 1); they were designed so that they did not hybridize within the gene regions corresponding to inhibitory hairpins. All assays were performed in duplicate. The specificity of each reaction was confirmed by agarose gel electrophoresis and by performing a melting curve. Results in terms of cycle thresholds were converted in folds actin expression using the $2^{-\Delta\Delta Ct}$ method, and then expressed in percents with regard to the mean value obtained for at least two control transformants per run. The method was validated by assaying serial dilutions of a series of samples.

Results

Gene silencing in *M. canis* was carried out by transforming the fungus with integrative plasmid constructs derived from the pSilent-1 silencing vector, which was previously developed for ascomycete fungi (Nakayashiki *et al.*, 2005). Constructions were designed to express part of the target genes as RNA hairpins of 688 and 596 bp, for *SUB3* and *DPPIV*, respectively, and bearing a loop constituted by a 147 bp intron from the *Magnaporthe oryzae* cutinase gene.

Forty-seven, 43, and 35 transformants were obtained with parent pSilent-1, pS1-*SUB3*, and pS1-*DPPIV*, respectively. All transformants showed a growth similar to that of the wild-type strain IHEM 21239 on Sab. Enzymatic activity towards AAPF–*p*Na was measured in culture supernatants of pS1-*SUB3* transformants grown in KM. Calculated residual specific activities were highly variable, ranging from 3.3% to more than 100% when compared with the mean value for pSilent1 transformants. The range of values obtained for residual specific activity, with a mean of 27.8%, is shown in Fig. 2. Only 1 colony out of 43 reached the mean level of the controls. Activity towards GP–*p*Na was assayed in the culture supernatants of pS1-*DPPIV* transformants grown in Sab broth. Residual specific activities varied from 45.6% to more than 100% of the pSilent-1 transformants, with a

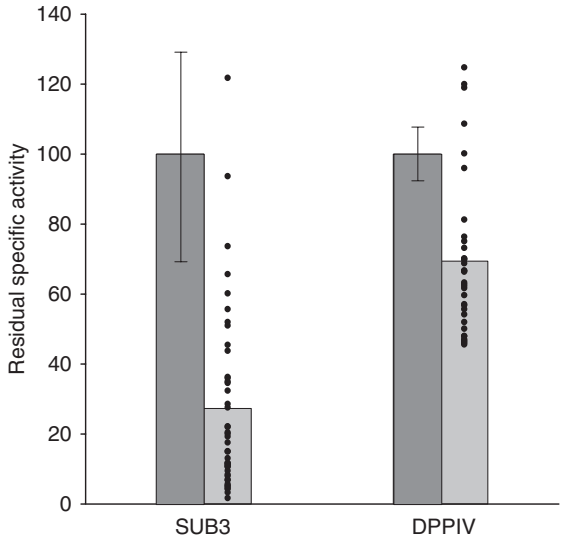

Fig. 2. Residual specific enzymatic activity (activity U μg^{-1} total protein) in culture supernatants of pS1-*SUB3* and pS1-*DPPIV* transformants. Dark bars represent the mean specific activity of control pSilent-1 transformants, arbitrarily set to 100 (+/− SD). The light bars correspond to the mean residual specific activity of silenced transformants, each particular value being represented by a point.

mean value of 69.4% (Fig. 2). Five colonies out of 35 reached or exceeded the level of controls.

Gene expression levels of *SUB3* and *DPPIV* were then quantified in 12 pS1–*SUB3* and seven pS1-*DPPIV* transformants, relative to expression levels of at least two pSilent-1 control strains. Tested transformants were called S-*SUB*-1 to S-*SUB*-12 and S-*DPP*-1 to S-*DPP*-7; they were selected among those that showed the lowest residual specific activities. S-*SUB*-1 to S-*SUB*-12 showed a decreased mRNA level compared with controls, while only S-*DPP*-5 among the S-*DPP*-tested strains exceeded the level of controls. The lowest observed values were 2% and 1.5% expression for S-*SUB*-1 and S-*DPP*-2, respectively. As for enzymatic activities, a whole range of inhibition levels was observed. Residual expression levels are shown in Fig. 3, in parallel with corresponding specific enzymatic activities.

PCR detection of the *HPH* gene and of the hairpin constructs was positive in S-*SUB*-1 to S-*SUB*-12 and 3/3 other pS1-*SUB* transformants, as well as S-*DPP*-1 to S-*DPP*-7 and 3/3 other pS1-*DPP* transformants. PCR amplification of endogenous *SUB3* and *DPPIV* complete coding sequences was performed for both genes on all these silenced transformants, and confirmed the integrity of these genes.

Discussion

Here, RNA-mediated down-regulation is reported for two *M. canis* genes, *SUB3* and *DPPIV*, potentially involved in fungal virulence. This is believed to be the first evaluation of RNA silencing methodology in dermatophytes. The rate of inhibition was highly variable from one transformant to another using the same plasmid construction. However, selected transformants with reduced enzymatic activity showed a reduced transcription level of targeted genes. The observed variability of RNA silencing in *M. canis*, regarding phenotypic effects and mRNA depletion, is in agreement with the results of many other comparable trials involving fungi (Liu *et al.*, 2002; Kadotani *et al.*, 2003; Mouyna *et al.*, 2004; Rappleye *et al.*, 2004). The variability in the degree of interference is explained, at least partially, by the unpredictable fate of the transforming DNA in the fungal cell, and silencing efficiency is not correlated with the number of construct copies that are integrated in the genome (Rappleye *et al.*, 2004; Yamada *et al.*, 2007). The results of molecular analyses of silenced fungal strains suggest that interference strength depends on the region of integration of the construct, which can be non permissive to transcription, and by rearrangements that the construct undergoes (Liu *et al.*, 2002; Goldoni *et al.*, 2004).

Similar to the findings of Tanguay *et al.* (2006), who showed that transcript levels do not necessarily correlate with the inhibition of a particular phenotype, no strong correlation was observed between real-time RT-PCR and enzymatic assay results. This can be explained by slight differences in the growth stage of the transformants, because growth rate is difficult to normalize in the case of slow-growing fungi, such as *M. canis*. Furthermore, the ratios between RNA level and enzymatic activity can vary during fungal growth, e.g. RNA levels can remain constant while active enzyme is accumulating in the culture supernatant.

The overall inhibition was more efficient on *SUB3* than on *DPPIV*. The global efficiency of RNA hairpin-mediated silencing in filamentous fungi can vary from one silencing construct to the other (Goldoni *et al.*, 2004; Tanguay *et al.*, 2006). For instance, the length of the inverted repeats or the characteristics of the spacer region influence the yield of highly or fully silenced transformants. In addition, besides the fact that some fungi seem to lack the RNAi-like machinery (Nakayashiki *et al.*, 2006), not all fungal species respond equally to the same silencing strategy (Nakayashiki *et al.*, 2005). The relation between mRNA levels and phenotypic changes is particular to each target gene and may cause apparent variation in interference efficacy. Each target gene is also more or less amenable to repression, and some genes simply cannot be silenced in this way (Fraser *et al.*, 2000). In the case of *DPPIV*, it cannot be excluded that a still unidentified enzyme that is secreted under the conditions of this study cleaves the synthetic substrate used to assay enzymatic activity. It should, however, have little or no sequence homology to *DPPIV*, because the corresponding gene was shown to have no paralog in the *M. canis*

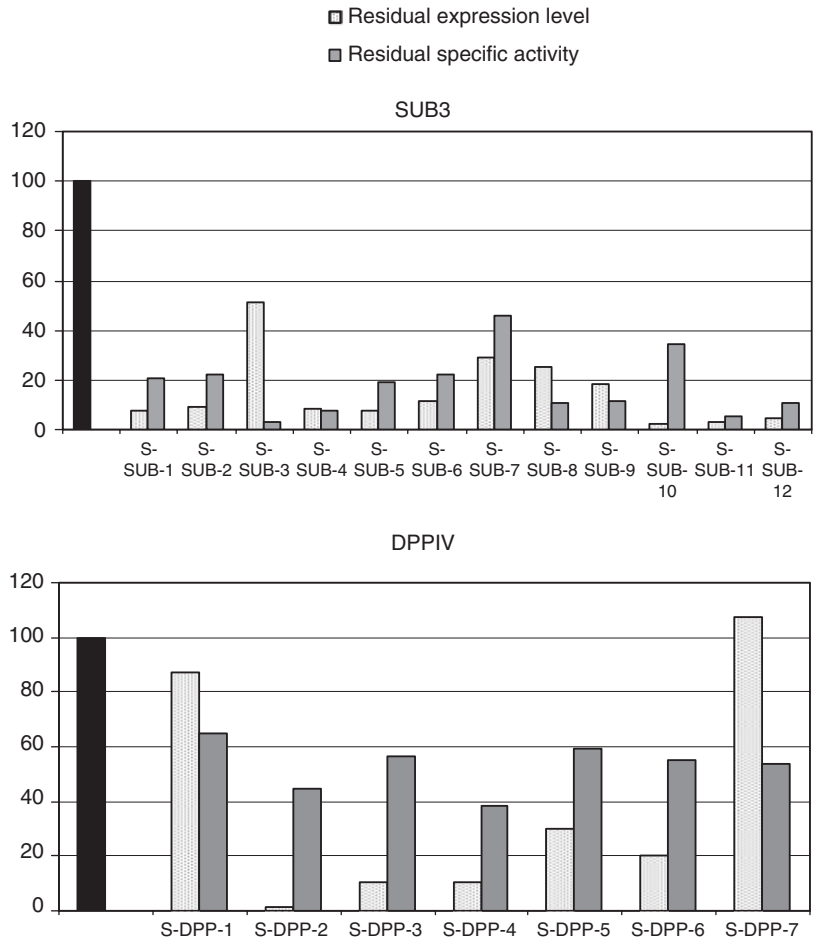

Fig. 3. Residual expression levels and residual specific enzymatic activities of pS1-*SUB3* and pS1-*DPPIV Microsporum canis* transformants, relative to control strains. The left dark bar represents the mean specific activity of control pSilent-1 transformants, arbitrarily set to 100.

genome (manuscript in preparation), as it was in *T. rubrum* (Monod *et al.*, 2005). Under that hypothesis, the efficiency of *DPPIV* silencing would be underestimated.

A single gene fragment corresponding to a conserved region is able to interfere with the expression of a whole gene family (Zoraghi & Seebeck, 2002; Miki *et al.*, 2005) or with multiple gene copies (Yamada *et al.*, 2007). However, studies demonstrate that small interfering RNA (siRNA)-mediated silencing can be efficient despite the presence of several mismatches (Jackson *et al.*, 2003; Saxena *et al.*, 2003). In the absence of clear knowledge of sequence-matching requirements for RNA-mediated inhibition in fungi, the *SUB3* construct was not designed with maximal homology to other family members, nor was possible attenuation of other *SUBs* checked for. Not less than seven *SUB* genes were found in the *T. rubrum* genome (Jousson *et al.*, 2004b) and the genome of other dermatophyte species so far encodes the same set of secreted proteases. Although the protein sequence of each ortholog is highly conserved across species, the level of secretion of each enzyme is species specific (Giddey *et al.*, 2007). Therefore, the residual activity of S-*SUB3* transformants could also be due to other subtilisins secreted by *M. canis* in minor amounts.

RNA silencing in *M. canis* and other dermatophytes can offer several advantages over conventional gene disruption or replacement. First, it circumvents the need for homologous recombination, which is arduous, and requires long flanking sequences. Disruption methods also require transforming uninuclear cells, which are not easily available in some dermatophyte species, including *M. canis*. By contrast, RNA-based silencing is able to induce the degradation of all similar mRNAs in a multinuclear cell, even if only one nucleus expresses the silencing construct (de Jong *et al.*, 2006). Second, this strategy allows the investigation of essential genes, whose disruption would be lethal. An inducible promoter may be used, to obtain conditional knockdown. A third advantage of the method is the possibility of achieving simultaneous silencing of several genes with a single conserved fragment, or with a single chimeric construct (Fitzgerald *et al.*, 2004). Therefore, RNA inhibition could be particularly helpful in dermatophytes, which possess large gene families coding for proteases.

A recent study (Henry *et al.*, 2007) showed that in *Aspergillus fumigatus*, the inhibitory construct could be lost or modified over successive transfers. Consequently, the evolution of silencing in *M. canis* transformants has to be verified further in the context of each intended downstream application. However, all the data presented here, including PCR detection of the integrated constructs, were collected with transformants that underwent two transfers. Indeed, all transformants were grown a first time in liquid medium in order to confirm hygromycin resistance. Then, they were transferred in distilled water for short-term conservation and picked from this stock for each subsequent analysis. These data give a first indication about the stability of the used constructs. In conclusion, RNA-mediated gene silencing in dermatophytes like in other fungi can be used as an alternative to disruption methods, for the functional investigation of available and forthcoming genome sequence information.

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