



Original Article

Assessment of the function of *SUB6* in the pathogenic dermatophyte *Trichophyton mentagrophytes*

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Abstract

Trichophyton mentagrophytes is a keratinophilic pathogenic fungus that infects both humans and animals. Subtilisins are important for *T. mentagrophytes* virulence, particularly when invading the epidermal barrier of the host. Subtilisin gene *SUB6* belongs to a seven-member gene family (*SUB1–SUB7*) encoding the subtilisin serine proteases. Additionally, the *SUB6* gene product Sub6, which is thought to be the major allergen Tri r2 in *Trichophyton rubrum*, elicits both immediate- and delayed-type hypersensitivity (DTH) reactions in humans. To assess its gene function, *SUB6* was disrupted using the *Agrobacterium tumefaciens*-mediated transformation method. Polymerase chain reaction and Southern blot analyses were used to confirm the disruption. *In vitro* virulence analyses comparing the mutant with the wild-type strain showed that proteolytic activity was significantly increased in the *SUB6* gene disruption strain (*SUB6::hph*), which corresponded to the significantly increase in *MEP4* (metalloprotease gene) and *SUB3* expression of *SUB6::hph*. The *SUB6::hph*-infected animals showed attenuated clinical symptoms and pathological changes, and because of the persistently high level of immunosuppressive cytokine IL-10, the increase in DTH-related cytokines IFN- γ , TNF- α and IL-12 was delayed and lower than that in animals infected with the wild-type strain. These results suggested that *SUB6::hph* had attenuated virulence *in vivo*, and that a genetically-linked regulatory effect may account for the increase in proteolytic activity and the residual pathogenicity of the mutant strain.

Key words: *Trichophyton mentagrophytes*, *SUB6*, *Agrobacterium tumefaciens*-mediated transformation, pathogenicity.

Introduction

Dermatophytes are pathogenic fungi that are the most common agents of superficial mycoses worldwide [1]. They can invade keratinized tissue such as the skin, nails, and hair of humans and animals [2]. *Trichophyton mentagrophytes* is a zoonotic dermatophyte that is especially dominant in clinical cases of tinea pedis and onychomycosis, with a prevalence of 1.5–55.4% [3–9]. *T. mentagrophytes* is also a common cause of skin diseases in companion animals, livestock and wildlife [10], and is therefore considered a public health hazard because of the high possibility of zoonosis [11,12].

T. mentagrophytes secretes a number of proteases to invade the epidermal barrier and obtain nutrients to grow and multiply on the epidermal keratin tissue. The multiple endoproteases secreted by *T. mentagrophytes* are divided into the S8 (subtilisin, Sub) and M36 (fungalsin, Mep) families [13,14]. A seven-member subtilisin gene (*SUB*) family, encoding serine proteases, has been identified in *Microsporum canis*, as well as in *Trichophyton rubrum* and *T. mentagrophytes* [14,15]. Among these virulence genes, *SUB3* is involved in the adhesion to keratinized host structures by *M. canis* [16]; however, the functions of the other gene products remain unknown. The Sub and Mep families of secreted antigens induce both cell-mediated and humoral immune responses in the host [17,18]. Two unique antigens, Tri r2 and Tri t4, derived from *Trichophyton* species cause either immediate-type (IH) or delayed-type (DTH) hypersensitivity skin test reactions in different individuals [19]. Sub6, a *T. mentagrophytes* keratinase whose production is upregulated during infection, has been identified as the major allergen Tri r2 from *T. rubrum*. *SUB6* is the most likely candidate gene to encode this allergen [2,14]; however, the relationship between *SUB6* and the secretion of Sub6 both *in vitro* and *in vivo* remains unknown.

The reverse genetics approach is an effective way to investigate the function of a potential virulence gene by disrupting the target gene and analysing the mutant strain [20]. *Agrobacterium tumefaciens*-mediated transformation (ATMT) is a common method used for fungal gene transfer. It has been successfully applied to many kinds of filamentous fungi, including *Aspergillus nidulans*, *Neurospora crassa* and *T. mentagrophytes* [20–23]. Compared with other transformation methods, ATMT is simple and highly efficient [23].

The purpose of the current study was to evaluate the involvement of *SUB6* in the pathogenicity of *T. mentagrophytes*. A *SUB6* mutant strain was constructed and used for comparative studies with the wild type to learn more about the phenotype, keratinase gene expression, proteolytic activity and *in vivo* pathological changes caused by disruption of this gene.

Materials and methods

Strains and media

The wild-type *T. mentagrophytes* strain ATCC 28185 (teleomorph: *Arthroderma benhamiae* Ajello et Cheng, a gift from Ruoyu Li, Peking University First Hospital, Beijing, China) was cultured on solid Sabouraud glucose agar medium (SGA) supplemented with 50 mg ml^{−1} chloramphenicol (Sigma, St. Louis, MO, USA) at 28°C. Transformants were selected on solid SGA medium supplemented with 600 µg ml^{−1} hygromycin B (Sigma) and 500 µg ml^{−1} cephalixin (Sigma).

A. tumefaciens strain EHA105 (a gift from Dr K. J. Kwon-Chung, National Institutes of Health, Bethesda, MD, USA) was maintained at 28°C on solid yeast extract broth medium (YEB)[23] supplemented with 50 µg ml^{−1} rifampicin (Sigma) and 25 µg ml^{−1} chloramphenicol (Sigma). *A. tumefaciens* carrying binary vector pDHT/*SUB6::hph* was cultured on *Agrobacterium* induction medium (AIM) [24,25] supplemented with 50 µg ml^{−1} rifampicin (Sigma), 25 µg ml^{−1} chloramphenicol (Sigma), 50 µg ml^{−1} kanamycin (Sigma), and 200 µM acetosyringone (Sigma).

Construction of transformation vectors

The binary vector pDHT/*SUB6::hph*, used for site-directed mutagenesis, was constructed by inserting a 4.2-kb *XmaI/SpeI* fragment, digested from pAN7-1/*SUB6::hph* which contains a hygromycin B selectable marker (*hph*) flanked by the *SUB6* gene sequence, into *XmaI/SpeI*-digested pDHT/SK (a gift from Dr. K. J. Kwon-Chung) (Fig. 1) [20,26]. Two fragments (*SUB6*-I, nucleotide position: −408 to 187; *SUB6*-II, nucleotide position: 1058 to 1653) of the *SUB6* locus were amplified using primer pairs S6I-F/S6I-R and S6II-F/S6II-R, respectively, to construct the *T. mentagrophytes SUB6* homologous gene-targeting vector pDHT/*SUB6::hph*. Single restriction enzyme recognition sites were included on both ends of the primers. The amplified fragment *SUB6*-I, along with vector pAN7-1, was double-digested with *NheI/PspXI*, and then the digested products were ligated, forming the vector pAN7-1/*SUB6*-I. The amplified fragment *SUB6*-II, along with vector pAN7-1/*SUB6*-I, was double-digested with *XbaI/SbfI*. The digested products were ligated, forming the *SUB6* homologous vector pAN7-1/*SUB6::hph*.

The binary vector pDHT/*SUB6::hph* was transformed into *A. tumefaciens* strain EHA105 using the heat-shock method.

All primer sequences are listed in Table 1. All fragments amplified or inserted into the plasmid were verified by DNA sequencing.

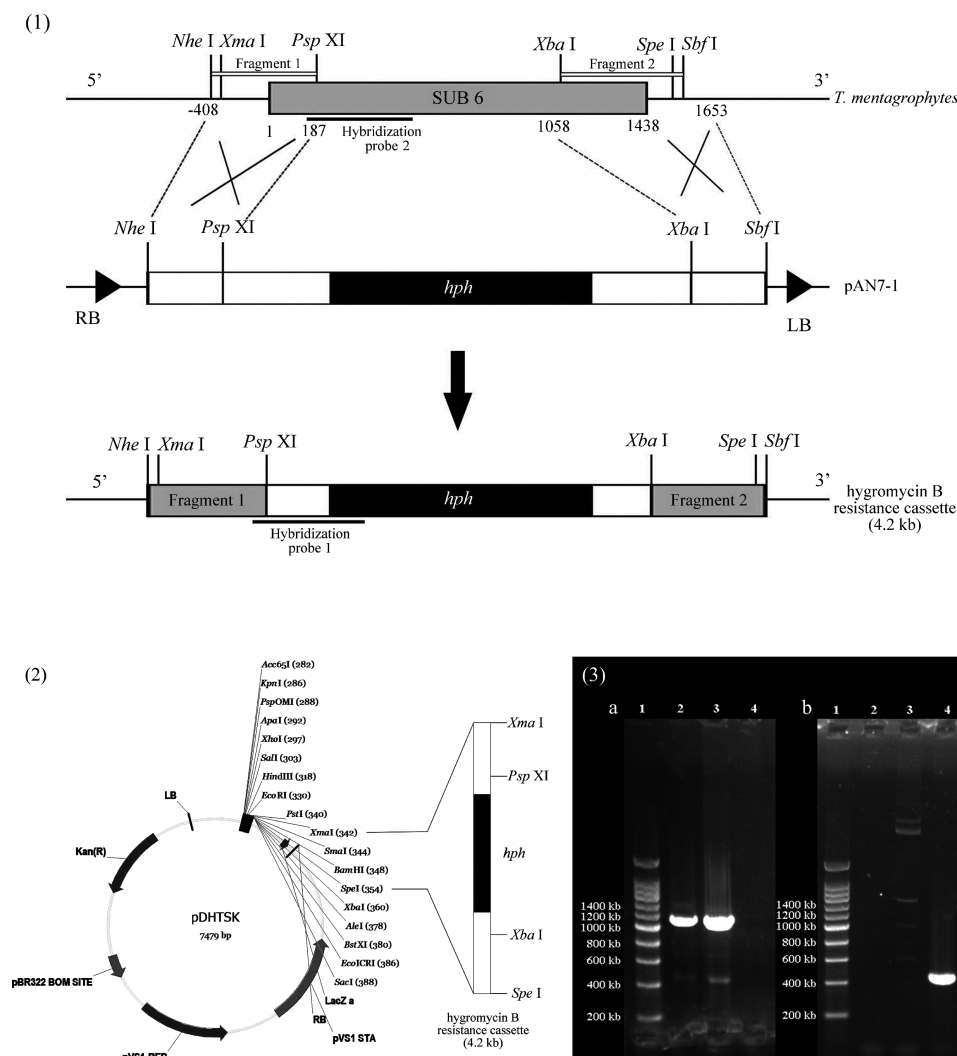


Figure 1. Construction of the transformation vectors. (1) Two fragments of the *SUB6* locus were amplified to construct the *Trichophyton mentagrophytes* *SUB6* homologous gene-targeting vector pDHT/*SUB6::hph*. Transformation vectors were constructed by double-digestion of the corresponding restriction sites. pAN7-1 is a plasmid that contains the hygromycin B selectable marker (*hph*). The hybridization probes consisted of: 1, a part of the *hph* gene with the flanking homologous *SUB6* fragment, and 2, a fragment of the *SUB6* locus, which should be replaced by *hph* if the transformation was successful. The fragments corresponding to probes 1 and 2 used for Southern analyses (black line), and the hygromycin resistance cassette (*hph*, black strip), are indicated. (2) Plasmids pAN7-1/*SUB6::hph* and pDHT/SK were digested with *Xma*I/*Spe*I, and the digested products were ligated, constructing the binary vector pDHT/*SUB6::hph*. (3) PCR analysis of transformants. (a) Amplification of *hph* (1084 bp) using the primers *hph*-F and *hph*-R. (b) Amplification of the gene fragment disrupted by *hph* (447 bp) using the primers S6q-F and S6q-R. Lane 1, DNA maker; lane 2, DNA sample from the *Trichophyton mentagrophytes* *SUB6* disruption strain (*SUB6::hph*); lane 3, the transformation vector pDHT/*SUB6::hph*; lane 4, DNA sample from the wild-type *T. mentagrophytes* strain 28185 (28185).

ATMT of *T. mentagrophytes*

A. tumefaciens strain EHA105 transformants carrying pDHT/*SUB6::hph* were cultured on solid YEB medium supplemented with 50 $\mu\text{g ml}^{-1}$ rifampicin, 25 $\mu\text{g ml}^{-1}$ chloramphenicol, and 50 $\mu\text{g ml}^{-1}$ kanamycin at 28°C for 48 h. The *A. tumefaciens* cells were resuspended in liquid AIM to an optical density at 660 nm of 0.7. Following the addition of 200 μM acetosyringone, the bacterial suspensions were incubated on a shaker (130 rpm, 28°C) for 6 h. The wild-type *T. mentagrophytes* strain 28185 (28185) was cultured on solid SGA medium at 28°C for 10 days, and the

plate was gently washed with sterile 0.9% sodium chloride solution and the concentration of conidia was adjusted to 1×10^7 colony forming units (cfu). A mixture of 100 μl *A. tumefaciens* suspension and 100 μl *T. mentagrophytes* conidial suspension was spread onto solid AIM medium (supplemented with 200 μM acetosyringone), which was then covered with sterilized nylon membrane (Sigma) and incubated at 24°C in the dark for 48 h. The nylon membrane was transferred onto a solid SGA medium plate supplemented with 600 $\mu\text{g ml}^{-1}$ hygromycin B and 500 $\mu\text{g ml}^{-1}$ cephalixin and incubated at 28°C for 3 days.

Table 1 Sequences of primers used in this study.

Name	Sequence (5'-3') ^a
S6I-F	<u>CTAGCTAGCCCCGGGCCTCAGCCGAAACCTTAAT</u>
S6I-R	<u>CAGTGGACTCGAGTCCCAGGAAAGGATATCACC</u>
S6II-F	<u>GCTCTAGATACCGATTCCAATATCAGGCA</u>
S6II-R	<u>TCACCTGCAGGACTAGTGCCTGACTACCCTTAAGATAC</u>
<i>hph</i> -F	CTTCATCGCAGCTTGACTAAC
<i>hph</i> -R	GCGGTCGGCATCTACTCTAT
S6q-F	GCTCAGAGGCAGGAGAAGA
S6q-R	TGCTAAGTGGGGCAAGAAC
probe1-F	TGCCGTTGTAGATGAGCTTG
probe1-R	AAAGCAGGAGAGGCACGATA
probe2-F	TGCCGTTGTAGATGAGCTTG
probe2-R	AACGTTCCCTCTTGGGGACTT
M1M-F	GCCACGATGAGATGAGAAG
M1M-R	GGCCGGTGTAGAAAGAGTT
M2M-F	AACCCTTACCGCTTTGCCT
M2M-R	TGCCATCACGTCCGATGT
M3M-F	ACGGCGTTCCACAGGAT
M3M-R	CGGTTGCGGGCATCATA
M4M-F	AGATGGGTCTGGCGTAAAC
M4M-R	AGTCAGTAGCACGGGTATCTC
M5M-F	TGGCTCTGGCACTAACAATG
M5M-R	CGGTGAGACGGTTGGAAA
S1M-F	AGAACGGCGGCACTAAGAA
S1M-R	CGGGAGCGAAGAGGTCAAT
S2M-F	GTGCCAAGTACCAGCCATT
S2M-R	GCATTGTTGCCACCCTTC
S3M-F	ACCCACAGCCTCAACAAG
S3M-R	GAGACTCTGCCCAAACCC
S4M-F	TGCTCAACAGCGTGGTATC
S4M-R	GGAGTAGTTACGGGCGTCT
S5M-F	TCACTCGGAGGCACATACAG
S5M-R	GTTGTGACCGGGAGCATAA
S6M-F	CGAGACCATCCCAAACAAGT
S6M-R	CAACAAAGTCGGGCTCAA
S7M-F	TGGTGTCTTCTTGGCTGTTG
S7M-R	CTTGGCGGAGGTGATAGAGT
actin#219F	CGAGCGTGGCTACAGCTTCT
actin#279R	CTCCTTGATGTCACGGACGAT

Note: a, Underlined text indicates inserted restriction enzyme recognition site.

Single fungal colonies were selected randomly and maintained on solid SGA medium supplemented with 600 $\mu\text{g ml}^{-1}$ hygromycin B at 28°C for 10 days, and referred to as the *T. mentagrophytes* transformants [20,22,23].

Total DNA isolation and hybridization analyses

All the *T. mentagrophytes* transformants were analyzed by polymerase chain reaction (PCR) and Southern blot analyses to confirm disruption of *SUB6*. Total DNA from 28185 and the *T. mentagrophytes* transformants was extracted from growing mycelia using sterile acid-washed glass beads (Sigma), as previously described [27]. The inserted *hph* gene

was detected with primers *hph*-F and *hph*-R and specific gene disruption was confirmed with primers S6q-F and S6q-R. Successful homologous recombination was determined by two Southern hybridization analyses. The first hybridization probe was designed to bind the *hph* fragment (1018 bp; see Table 1 for primer sequences), while the second probe was used to detect the *SUB6* deletion fragment, which should be replaced by *hph* if the transformation was successful (954 bp; see Table 1 for primer sequences).

The transformed strain that was confirmed by both PCR and Southern blot analyses was referred to as the *SUB6* disruption strain (*SUB6::hph*) for subsequent phenotypic characterization.

Total RNA extraction and quantitative real-time PCR analyses (qRT-PCR)

The effect of *SUB6*-disruption on the expression of the other *SUB* and *MEP* gene family members was explored by qRT-PCR. Conidial suspensions (200 μ l, containing 1×10^7 cfu) of 28185 and *SUB6::hph* were separately inoculated onto mineral medium supplemented with 0.05% (w/v) fox hair (from healthy adult foxes) and incubated for 18 days at 28°C [28]. Total RNA was isolated from the two strains using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). DNA contamination was removed using RNase-free DNase (Qiagen). cDNA was synthesized from 1 μ g of total RNA using M-MLV reverse transcriptase (Takara, Otsu, Japan). Primer pairs M1M-F/M1M-R to M5M-F/M5M-R, and S1M-F/S1M-R to S7M-F/S7M-R, were used respectively to amplify *MEP1–MEP5* (the resulting amplicon lengths were 264 bp, 268 bp, 211 bp, 287 bp, and 164 bp, respectively) and *SUB1–SUB7* (the resulting amplicon lengths were 255 bp, 250 bp, 224 bp, 160 bp, 228 bp, 263 bp, and 184 bp, respectively) in the qRT-PCR analyses. Actin was selected as the endogenous housekeeping gene, and was amplified using the primers actin#219F/actin#279R [28]. Data were analyzed using the $2^{-\Delta\Delta CT}$ method.

Proteolytic activity

One millilitre (1×10^7 cfu) of each of the conidial suspensions was added to separate 20-ml liquid basal medium [29] solutions containing 0.06 g of nail tissue from healthy adult foxes, and then incubated with shaking (130 rpm) at 28°C for 12 days. Centrifuged and filtered supernatant was reacted with 5 mg keratin azure (Sigma) as previous described [22]. Each 0.01 unit value change in absorbance was defined as one unit of proteolytic enzyme activity.

Animals and grouping

Adult white guinea pigs were purchased from Beijing Vital River Animal Laboratories (Beijing, China). Prior to the tests, the animals were housed for 7 days. All guinea pigs were reared, obtained, and housed in accordance with the laboratory animal requirements of our institute, and all of the experiments were performed with the approval of the China Agricultural University Animal Ethics Committee for Clinical Veterinary Science Experiments (protocol number CAU20130506). The guinea pigs were randomly divided into the 28185, *SUB6::hph* and negative control (CTL) groups. Animals within each group were then equally divided into the skin inoculation test and injection assays.

Skin inoculation test

An area of 2.5×2.5 cm² on the abdomen of each animal was shaved using an electric shaver 1 day prior to inoculation. Following routine sterilization procedures, 200- μ l (1×10^7 cfu) aliquots of the 28185 and *SUB6::hph* conidial suspensions were applied separately to the skin within the shaved area of the abdomen, with sterile 0.9% sodium chloride solution applied to the control animals. All guinea pigs were housed in separate cages to avoid contamination, and clinical symptoms were observed and recorded daily. On days 1, 2, 3, 7, 14, 21, and 28 post-inoculation (PI), three guinea pigs were randomly selected from each of the test groups, with one animal randomly selected from the control group, and skin biopsies were taken using surgical methods. Skin infections were evaluated using histological examination [22,30].

Injection assays

Following routine sterilization procedures, the guinea pigs were injected with 50 μ l (1×10^7 cfu) of the 28185 or *SUB6::hph* conidial suspensions, or with sterile 0.9% sodium chloride solution, in the footpads of both hind limbs [31]. Three guinea pigs from each test group and one guinea pig from CTL group were randomly selected and sacrificed on days 1, 2, 3, 7, 14, 21, and 28 post-injection (PI). Sections of the footpads, popliteal lymph nodes and spleen were dissected and submitted for histological analyses. The spleen samples were used for enzyme-linked immunosorbent assay (ELISA) analysis.

Histological procedures

Tissue samples were embedded in paraffin, and then 3- μ m histological sections were stained with haematoxylin-eosin (HE) or Alcian blue/periodic acid-Schiff stain (AB-PAS).

Cytokine analyses

One hundred milligrams of spleen tissue were rinsed with $1 \times$ phosphate-buffered saline (PBS) and then homogenized in 1 ml of $1 \times$ PBS. The homogenates were centrifuged at 2–8°C for 5 min at 3500 rpm, and the supernatants were collected for testing. The levels of interferon (IFN)- γ , tumour necrosis factor (TNF)- α , interleukin (IL)-10 and IL-12 in the spleen homogenates were assayed by ELISA (R&D Systems, Minneapolis, MN, USA and CUSABIO, Wuhan, China) according to the manufacturer's instructions.

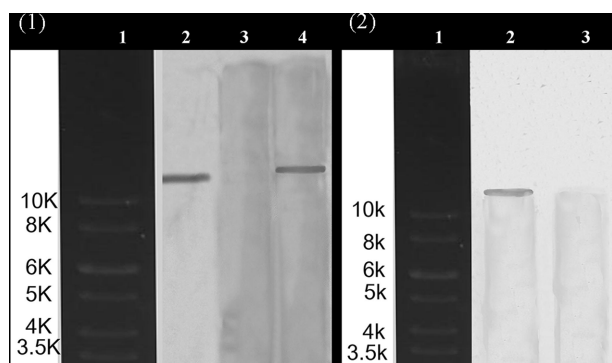
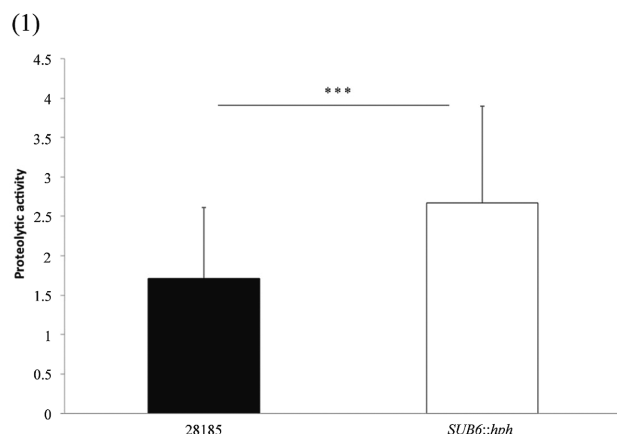


Figure 2. Southern blot analysis of genomic DNA from the *SUB6::hph*. Total genomic DNA of each strain was digested with *Hind*III overnight. The digested samples were separated by electrophoresis on 0.8% (w/v) agarose gels and transferred onto Hybond N+ membranes (Pharmacia, Kalamazoo, MI, USA). Probes were tagged with digoxigenin-dUTP using the DIG High Prime DNA Labelling and Detection Starter Kit II (Roche, Basel, Switzerland). Signals were hybridized and detected following the manufacturer's instructions. (1) A partial *hph* gene with the flanking homologous *SUB6* fragment (1018 bp) was used as a hybridization probe to verify the specific chromosomal position of *hph*. Lane 1, DNA maker; lane 2, the transformation vector pDht/*SUB6::hph*; lane 3, DNA samples from 28185; lane 4, DNA sample from *SUB6::hph*. (2) A partial *SUB6* gene fragment (954 bp) was used as a hybridization probe for detecting the deleted gene fragment. Lane 1, DNA maker; lane 2, DNA sample from 28185; lane 3, DNA sample from *SUB6::hph*.

Statistical analyses

All data were expressed as mean $\pm$ standard deviation, and one-way analysis of variance (ANOVA) was used for the statistical comparisons between different groups. The tests were performed using IBM SPSS v. 18.0 software (SPSS Inc., Chicago, IL, USA). $P < .05$ was considered to be statistically significant.



Results

Confirmation of the *T. mentagrophytes SUB6* mutant

Both PCR and Southern blot analyses confirmed that the transformed plasmid had undergone homologous recombination in the recipient *T. mentagrophytes* cells, resulting in *hph* inserted into the *SUB6* open reading frame, with the *SUB6::hph* strain containing only a single copy of the transfer DNA. Figures 1 (3) and (2) show the results of the molecular analyses used to confirm the disruption of *SUB6* in the *T. mentagrophytes* mutant strain. There were no morphological differences between *SUB6::hph* and 28185 when grown on solid SGA medium for five generations. The *hph* gene appeared to have stably integrated into the chromosome and was consistently expressed by each generation of strain *SUB6::hph*.

Changes in expression of keratinase genes in *SUB6::hph*

qRT-PCR analysis showed a significant increase in the expression of *MEP4* and *SUB3* in *SUB6::hph* compared with 28185 ($P < .01$), while the expression of *MEP1*, *MEP2*, and *MEP5* was significantly reduced. There were no significant changes in the expression of *MEP3*, *SUB4* or *SUB5* (Fig. 3), while no expression of *SUB1*, *SUB2*, *SUB6* or *SUB7* was detected.

Proteolytic activity

The proteolytic activity assay showed that the activity of *SUB6::hph* was significantly higher than that of 28185 ($P < .01$) (Fig. 3).

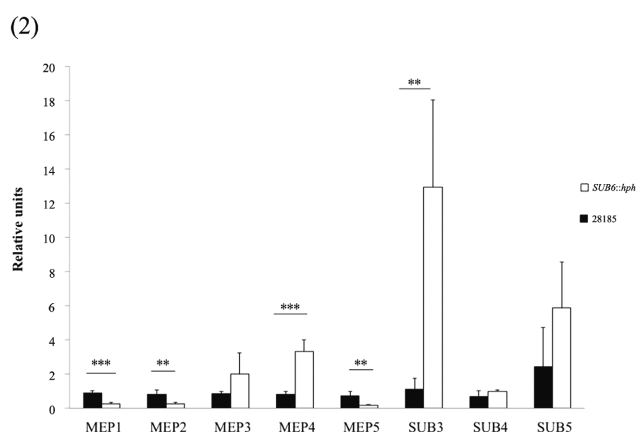


Figure 3. (1) Proteolytic activity of 28185 (28185) and *SUB6::hph* (*SUB6::hph*). (2) mRNA profiles of keratinase genes from *SUB6::hph* (*SUB6::hph*) and 28185 (28185) grown on mineral medium supplemented with fox hair. Data are representative of three independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

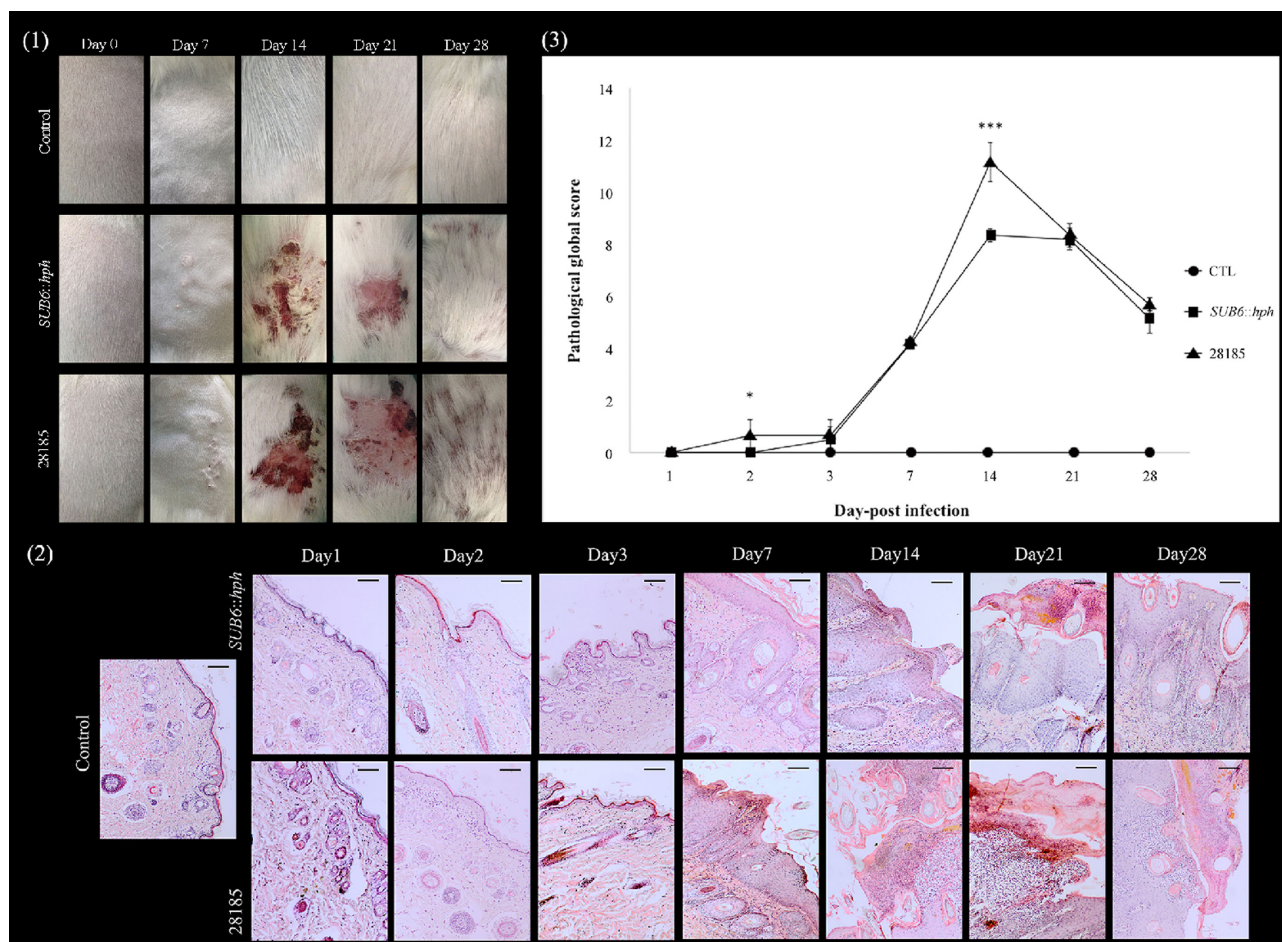


Figure 4. (1) Analysis of clinical symptoms of guinea pigs that were dermally inoculated with 28185 (28185), *SUB6::hph* (*SUB6::hph*) or sodium chloride solution (Control) respectively. Scurf and red papules were visible on day 7 post-inoculation (PI). Infiltrative erythema with crusts was seen on day 14 PI. Scabs were generated on approximately day 21 PI, and began to fall off on day 28 PI. No clinical symptoms were observed in the negative control (CTL) group. (2) Histopathological analysis of sections of skin infected with 28185 (28185), *SUB6::hph* (*SUB6::hph*) or sodium chloride solution (Control). Sections were stained with haematoxylin-eosin (HE). Scale bars = 100 μ m. (3) The histopathological changes were evaluated according to the degree of thickening and inflammation (0-absence, 1-mild, 2-moderate, 3-significant, 4-severe) of the epidermal layers (including stratum corneum, stratum spinosum and stratum granulosum), as well as the dermis. The global scores for the histopathological changes were the sum of the scores of each layer. Three guinea pigs were randomly selected from the 28185 and *SUB6::hph* groups, with one guinea pig randomly selected from the CTL group, and three skin biopsies were collected surgically from each animal. No pathological changes were observed in the CTL group. * $P < .05$; ** $P < .01$; *** $P < .001$.

In vivo skin inoculation test

Signs of dermatophytosis appeared in both the 28185 group and the *SUB6::hph* group, with the most severe symptoms observed on day 14 PI. Inoculation sites healed spontaneously in both groups by day 35 PI (Fig. 4). Samples of the skin lesions were inoculated onto SGA medium, and the resulting colonies were identified by PCR analyses as either 28185 or *SUB6::hph*.

In the histopathological analyses, inflammatory cell infiltration was observed on day 2 PI in the 28185 group, while pathological changes only became obvious in the *SUB6::hph* group on day 3 PI. Obvious hyperkeratosis, acanthosis and inflammatory cell infiltration appeared in both groups on day 7 PI. Oedema was observed at the

stratum granulosum, and inflammatory cells had spread to the dermis on day 14 PI. The pathology results were evaluated according to the degree of thickening and inflammation of the epidermal layers and dermis. The results suggested that the pathological changes in the 28185 group appeared earlier, and were significantly more severe than those of the *SUB6::hph* group on day 14 PI ($P < 0.001$) (Fig. 4).

In both the 28185 and *SUB6::hph* groups, the AB-PAS staining showed that conidia and hyphae were only present in the stratum corneum at the beginning of the experimental period. However, starting from day 7 PI, they could be seen in the epidermis and hair follicles. On day 28 PI, the conidia and hyphae were excluded as the scab began falling off (Fig. 5).

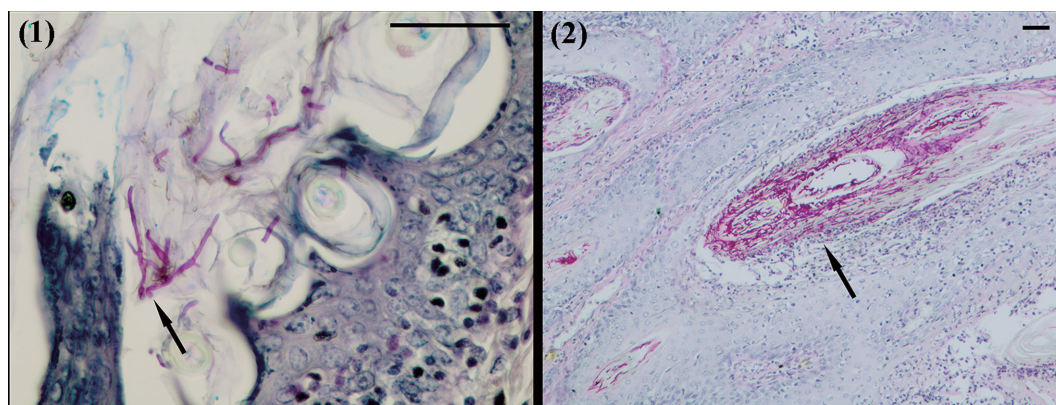


Figure 5. Histopathological analysis of sections of skin infected with *Trichophyton mentagrophytes* and stained with Alcian blue-periodic acid Schiff staining (AB-PAS). Conidia and hyphae (arrows) colonized the stratum corneum on day 1 post-inoculation (PI) (1), and hair follicles on day 14 PI (2). Neither conidia nor hyphae were found in the skin of CTL group animals. Scale bars = 50 μm .

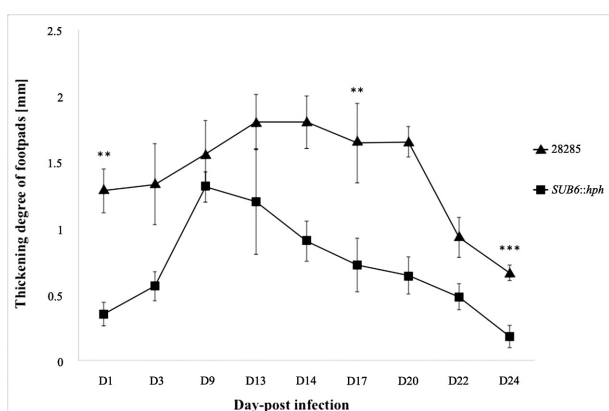


Figure 6. Thickening of footpads in 28185- (28185) and *SUB6::hph* (*SUB6::hph*) infected guinea pigs. Analysis was carried out using the calculation $T - T_{D0} = \text{mean}(T_C - T_{CD0})$, where T is the thickness of the footpads injected with 28185 or *SUB6::hph* on days 1, 3, 9, 13, 14, 17, 20, 22 or 24 post-injection (PI); T_C is the thickness of footpads injected with sodium chloride solution on days 1, 3, 9, 13, 14, 17, 20, 22 or 24 PI; T_{D0} is the thickness of footpads injected with 28185 or *SUB6::hph* on day 0; and T_{CD0} is the thickness of footpads injected with sodium chloride solution on day 0. * $P < .05$; ** $P < .01$; *** $P < .001$.

In vivo injection assays

The thickness of the footpads of the animals in each group was measured using Vernier callipers following injection of conidial suspensions or sterile 0.9% sodium chloride solution. Results suggested that footpads began to thicken starting from day 1 PI. The degree of thickening was most obvious on days 13–14 PI in the 28185 group, while thickening was greatest on day 9 PI in the *SUB6::hph* group. Overall, the degree of footpad thickening was greater in the 28185 group than in the *SUB6::hph* group. The most significant differences between the two groups were observed on days 1 and 17 PI (Fig. 6).

The histopathological sections showed abscess formation, inflammatory cell infiltration and polymorphonuclear

leukocytes at the interstitial tissue in both the 28185 and *SUB6::hph* groups. Granuloma formations were observed from day 14 PI onward, although pathological changes were relieved by day 28 PI (Fig. 7). AB-PAS staining showed that fungal elements could be observed from day 1 PI in both the 28185 and *SUB6::hph* groups (Fig. 7). Histopathological analysis of lymph nodes and spleens showed that neither fungal dissemination nor inflammatory reactions were present in any of the groups.

Cytokine analyses

Cytokine analyses indicated that on day 1 PI, levels of IFN- γ , TNF- α , IL-10, and IL-12 in the 28185 group were significantly higher than in the other groups, and that there were no significant differences between the *SUB6::hph* and CTL groups. The levels of IFN- γ , TNF- α and IL-12 began to rise on day 2 PI in the *SUB6::hph* group, and were significantly higher than in the CTL group. On day 3 PI, levels of IL-12 and TNF- α were higher in the wild-type *T. mentagrophytes* 28185 group than in the other two groups, while the level of IL-10 in the *SUB6::hph* group began to rise. The level of IL-10 in the *SUB6::hph* group was significantly higher than in the CTL and 28185 groups, and continued to rise until day 28 PI. On day 21 PI, there was a remarkable increase in the levels of all cytokines in the 28185 group, which were significantly different from the other two groups (Fig. 8).

Discussion

T. mentagrophytes is an important zoonotic dermatophyte that can cause pathogenic lesions in humans and animals. However, there are very few functional studies of the virulence genes of *T. mentagrophytes*. Disrupting the target

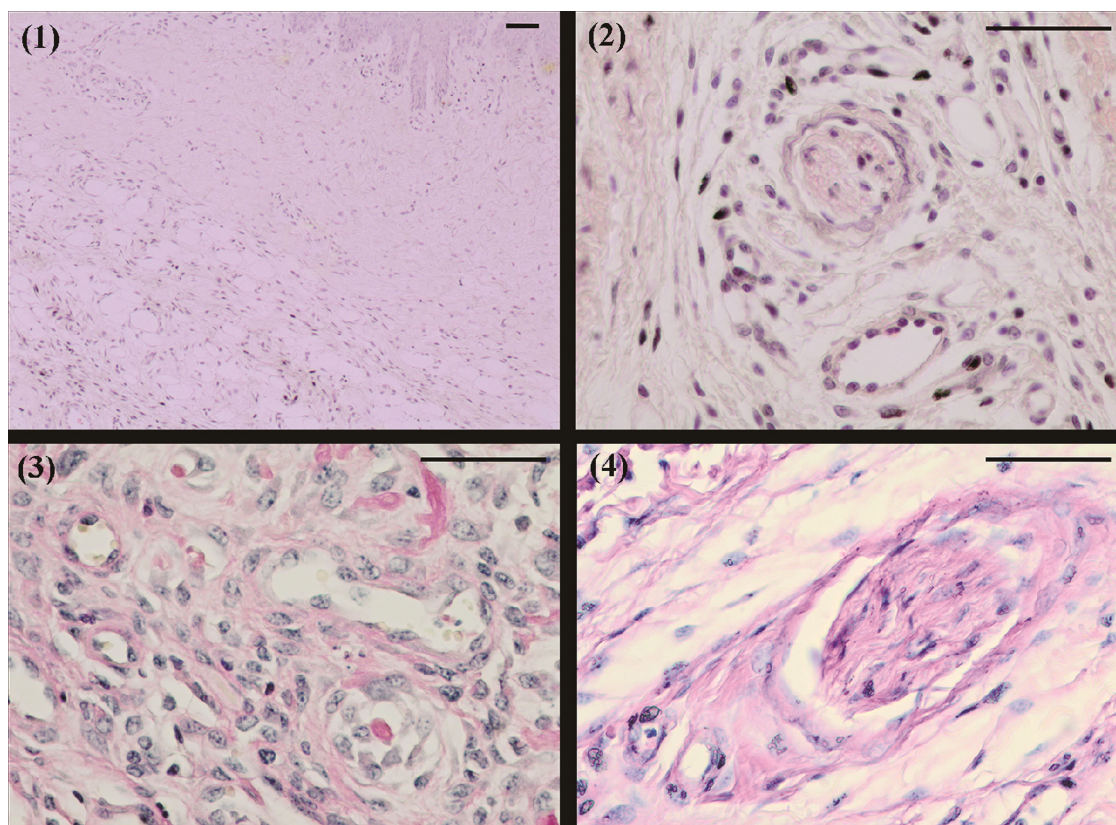


Figure 7. Histopathological analysis of sections of footpads injected with *Trichophyton mentagrophytes* strains. On day 14 post-injection (PI), sections of footpads from animals in the CTL (1) and 28185 (2) groups were stained with haematoxylin-eosin. Sections of footpads from animals in the *SUB6::hph* (3) and 28185 (4) groups were stained with Alcian blue-periodic acid Schiff. Scale bars = 50 μ m.

gene using ATMT, and observing the mutant phenotypes and pathogenic changes is the most commonly used method to study fungal gene function [20,21,23,26]. While there are many studies on specific gene disruption using ATMT in *T. mentagrophytes*, and the co-cultivation proportion, temperature and culture medium for fungi and bacteria have been optimized [22,23,32], the transformation frequency and likelihood of obtaining stable homologous recombination transformants remains low. This could be caused by the specific gene locus or by the size of homologous DNA fragments among various fungi [23,33]. For *T. mentagrophytes*, homologous flanking regions of 800 bp [22], 1500 bp [23] and 2000 bp [32] have been used in previous studies. Because of the small size of the *SUB6* gene (only 1438 bp), the external homologous segments of the *SUB6* locus were extended on both sides in the current study (see Fig. 1), which lengthened the homologous fragments and ensured the deleted sequences contained the exon regions at the same time. In this study, the transformation frequency was about 30 per 10^7 conidia, and 2% of transformants showed homologous recombination with disruption of the *SUB6* gene. The homologous segments used in the current study are the shortest sequences (~ 600 bp) that have successfully

been used for gene disruption in *T. mentagrophytes* via ATMT.

Keratinase, which is secreted by dermatophytes, is an important virulence factor. In *T. mentagrophytes*, the *MEP* and *SUB* gene families encode proteins that undertake the production and secretion of keratinase [13,14]. However, the relationship between these gene families remains unknown. Previous studies show that there is no difference in the expression of *SUB6* on media with or without keratin, and that the expression level *in vitro* is much lower than that *in vivo* [2]. The effect of *SUB6* gene disruption on proteolytic activity is unpredictable. In this study, we found that the proteolytic activity of the *SUB6::hph* was significantly higher than that of the 28185 on keratin-supplemented medium. According to the gene expression analyses, expression of *MEP4* and *SUB3* was significantly increased in the *SUB6::hph*; both of these genes are related to keratinase activity [2,14,34,35]. Previous studies indicated that the genetic defects in the *MEP4* mutant strain most severely affected the proteolytic activity of *T. mentagrophytes* [22,35]. *SUB3* is also involved in the adherence of *M. canis* to human and animal epidermis [16,36]. This increased gene expression explains the higher proteolytic

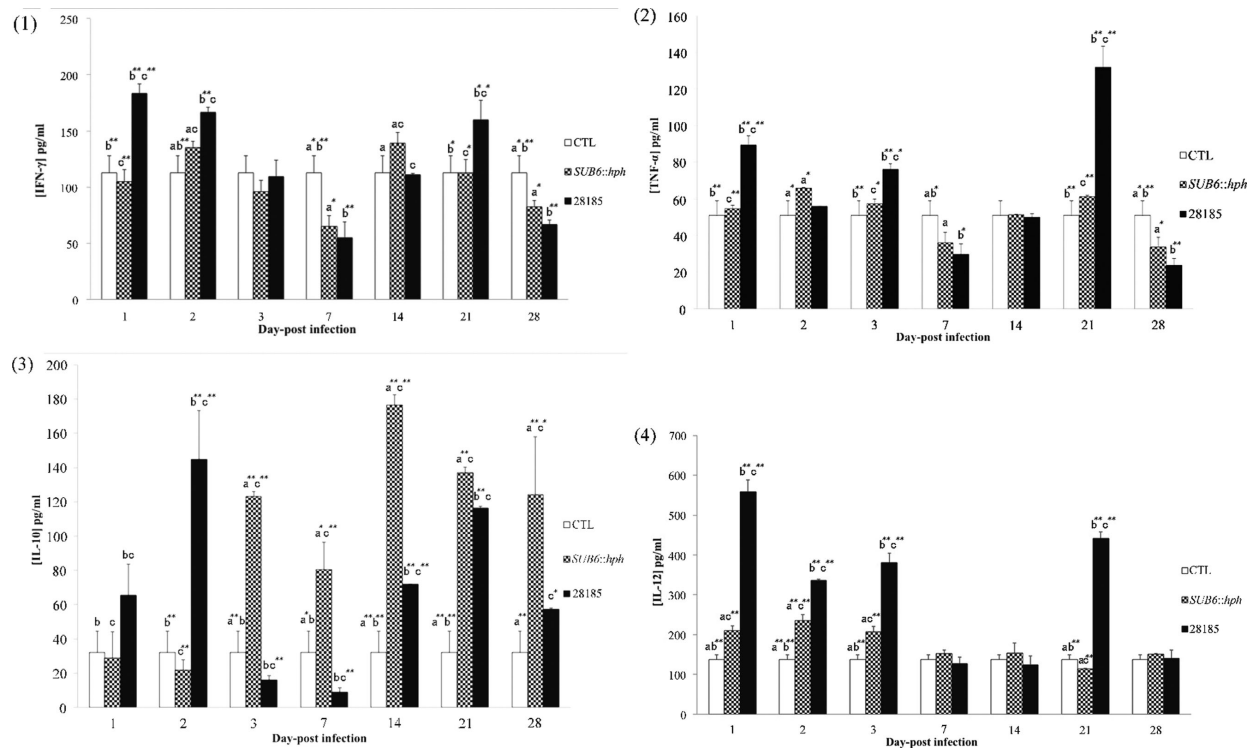


Figure 8. Levels of interferon (IFN)- γ (1), tumour necrosis factor (TNF)- α (2), interleukin (IL)-10 (3) and IL-12 (4) in the spleen homogenates of guinea pigs on days 1, 2, 3, 7, 14, 21 and 28 post-injection (PI). Each sample was analysed in duplicate. Statistical differences between two groups are indicated as follows: a, CTL- *SUB6::hph*, $P < .05$; a*, CTL- *SUB6::hph*, $P < .01$; a**, CTL- *SUB6::hph*, $P < .001$; b, CTL-28185, $P < .05$; b*, CTL-28185, $P < .01$; b**, CTL-28185, $P < .001$; c, *SUB6::hph*-28185, $P < .05$; c*, *SUB6::hph*-28185, $P < .01$; c**, *SUB6::hph*-28185, $P < .001$.

activity observed in *SUB6::hph*, and suggests that the absence of *SUB6* may affect the regulation of other related virulence genes. Many gene disruption studies have found that keratinase production is regulated by multiple genes in a gene family. When a single gene is disrupted from the gene family, pathogenicity is not completely abolished [22,36,37]. This suggests that to weaken or eliminate keratinase activity, the set of related genes must be disrupted at the same time [38]. Our results indicated that these closely related gene families regulate their expression and work together to ensure the basic pathogenic function of the mutant, which was also hypothesized for the *SAP* gene family of *Candida albicans* [37].

Dermatophytes can induce several immune responses following infection [39,40]. In general, IH reactions are associated with chronic, low-grade inflammatory lesions, while DTH reactions are associated with short-term, severe inflammations that usually resolve spontaneously [19,41]. Though the expression of *SUB6* is low *in vitro*, *in vivo* experiments have shown much higher levels of *SUB6* gene expression [2]. The *SUB6*-encoded protein Sub6 is the novel antigen Tri r2 in *T. rubrum*, which can induce both IH and DTH immune responses [2,14,19,39]. In previous animal inoculation studies, the skin of the test animals was

prepared using sandpaper, needle or tape prior to inoculation to ensure a high infection rate [30,42]. In the current experiment, a non-abraded skin technique was used for inoculation to avoid artificial damage that could skew the results [43]. Both the clinical and pathological changes indicated that all the inoculated animals had mycological infections, and the reproducibility using this technique was high. When compared with 28185 infection, *SUB6::hph*-infected guinea pigs showed delayed onset of clinical symptoms and pathological changes, with lower-graded lesions and inflammation. These findings indicated that the immune reaction induced by *SUB6::hph* was weaker than that of the wild-type. In contrast to previous studies, the histopathological results showed that subcutaneous injection of *T. mentagrophytes* did not cause deep fungal infection, which suggested that the deep tissue culture was necessary for diagnosis of deep fungal infection [31].

The DTH response relies on a T-helper (Th) 1 cell-mediated immune response, and a large number of cytokines are involved [41,44]. IFN- γ transforms monocytes into activated macrophages, and triggers the cell-mediated immune response [31,41,45]. IL-12 stimulates the production of IFN- γ in active T lymphocytes and natural killer cells [19,41], while TNF- α is a proinflammatory

factor and influences dendritic cell maturation [31,41]. IL-10 is an immunosuppressive cytokine that can induce the Th2-type response [31,44]. It suppresses the DTH response and prevents excessive production of TNF- α , IFN- γ and cytotoxic metabolites [31,41,45]. In the early-stages of the *in vivo* injection assays, levels of IFN- γ , IL-12 and TNF- α in spleen homogenates of the 28185 group increased, which suggested that the *T. mentagrophytes* antigen stimulated the Th1-type immune response. On day 2 PI, the level of IL-10 rapidly increased. Subsequently, the levels of IFN- γ , IL-12, and TNF- α began to decline, which was consistent with previous research findings [31]. Compared with the 28185 group, the increase in the levels of the DTH-related cytokines IFN- γ , IL-12, and TNF- α occurred later, and levels were lower in the *SUB6::hph* group, which correlated with the clinical and pathological results and indicated that the animals infected with *SUB6::hph* produced a milder hypersensitivity reaction. Additionally, on day 21 PI, levels of IFN- γ , IL-12, and TNF- α in the 28185 group began to increase again, which was not observed in the *SUB6::hph* group. This indicated that the DTH-related inflammatory response may have subsided earlier in the *SUB6::hph* group. These differences in the DTH-related cytokines all confirmed that the disruption of *SUB6* strain showed attenuated virulence *in vivo*. The increase in levels of the immunosuppressive cytokine IL-10 was delayed in the *SUB6::hph* group, but remained consistently higher, which could be the reason for the earlier subsidence of the DTH reaction [31,42].

Although strongly upregulated expression of *SUB3* and *MEP4* in *SUB6::hph* was observed on keratin-supplemented medium, and the proteolytic activity was higher than the wild-type *in vitro*, the pathogenicity *in vivo* depends on a variety of factors, among which the proteolytic activity is involved but less important than that *in vitro*. Because many genes related to proteolytic activity show lower expression *in vivo* than *in vitro* [2], the high proteolytic activity *in vitro* was not as pronounced pathogenicity *in vivo*. Additionally, the expression of antigen-related genes (such as *SUB6*) *in vivo* is also critical for pathogenicity, given that the antigen-induced histopathological changes are the chief factor to influence the evaluation of pathogenicity. The expression of *SUB6* and closely related gene *SUB7*, as well as *SUB1* (Sub1 has a C-terminal extremity that is longer than the other Subs and is rich in proline like lots of parasite surface antigens [46] and is a candidate for the immunomodulation target like Sub6 during dermatophytosis [14]) and *SUB2*, which were specifically upregulated in previous *in vivo* studies [2], was not detected in *SUB6::hph* *in vitro*. *SUB1*, *SUB2* and *SUB7* are most likely related antigens like *SUB6* [2,14,19,41], and the unexpression of these genes *in vitro* suggested that it was

inappropriate to deduce the direct effect of *SUB6* *in vivo*. Further research need to be done to investigate the changes in expression of these keratinase genes in *SUB6::hph* *in vivo*. The direct effect of *SUB6* *in vivo* was complicated; while the direct effect of disruption of *SUB6* on the pathogenicity and inflammatory reactions in the host cells was verified to be weak according to the clinical symptoms, pathological changes and DTH-related cytokines in the *SUB6* mutant-infected animals in this study.

Examining the immune response and developing immunotherapy techniques for dermatophytosis have been the focus of a large number of studies. In addition to the Th1 and Th2 immune pathways, the Th17 pathway is also involved [42,44]. As keratinase Sub6 can induce both IgE antibody and DTH responses simultaneously [2,19], it is important to study the effects of disruption of the keratinase gene *SUB6*. The pathogenic changes of *SUB6::hph* suggest that *SUB6* is involved in the induction of the immune response, while the mutant strain showed attenuated virulence *in vivo*, suggesting that this region may be a good target for vaccine research. Further research is needed to explore the genetically-linked regulatory effect in the keratinase gene family, which may account for the increase of proteolytic activity and the residual pathogenicity of *SUB6::hph*.

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Declaration of interest

The authors have no conflicts of interest to declare. The authors alone are responsible for the content and the writing of the paper.

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