

# Development of transformation system for *Trichophyton rubrum* by electroporation of germinated conidia

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**Abstract** Dermatophytes are the fungi that can cause infections of skin, hair, and nails due to their ability to utilize keratin. The genetic transformation systems of dermatophytes were successfully applied to *Trichophyton mentagrophytes* and *Microsporum canis*. Here we describe the procedure for genetic transformation of *Trichophyton rubrum* by electroporation of their germinated conidia. A linearized transformation vector (pCHSH75-Pch/GFP/TtrpC) containing bacterial hygromycin B phosphotransferase gene (*hph*) and green fluorescent protein gene (*egfp*) was introduced into the germinated conidia of *T. rubrum* by electroporation. PCR reaction analysis showed that *egfp* gene was integrated randomly and Southern blotting analysis demonstrated a single integration of *hph* gene into the chromosomal DNA of randomly selected transformant. In this work we report the efficient transformation and selection of the stable *T. rubrum* transformants.

**Keywords** *Trichophyton rubrum* · Electroporation · Hygromycin B · Germinated conidia

## Introduction

Dermatophytes are a group of keratinophilic fungi which are responsible for dermatophytosis. According to WHO databases, mycoses concern about 40% of world human

population. Epidemiological studies conducted in Poland revealed that *Trichophyton rubrum* and *Trichophyton mentagrophytes* are responsible for most of the superficial infections (Macura et al. 2008; Wronski and Nowicki 2005). The knowledge about the mechanisms of dermatophyte pathogenicity and details of the mechanism of the host invasion is very limited. Studying the virulence factors, such as secreted aspartic proteases, keratinolytic proteases or fungalisins (Asahi et al. 1985; Brouta et al. 2001, 2002; Descamps et al. 2002; Mignon et al. 1998; Yamada et al. 2004) at the molecular level would be easier if the efficient systems of genetic transformation of dermatophytes were developed.

*Trichophyton rubrum* biology has entered the new area of functional genomics as more sequence data becomes available. In 2006 Wang et al. identified and described ESTs in *T. rubrum* libraries. Analysis of this data can give a lot of information about antigens, pathogenesis, metabolism, drug resistance and new drug targets (Yang et al. 2007) (<http://www.mgc.ac.cn/TrED>). Currently, the *T. rubrum* genome has been completely sequenced and the results will be available soon (<http://www.broad.mit.edu/science/projects/fungal-genome-initiative/status-fgi-projects>).

The actual methods are based on the transformation of protoplasts which cause several problems to researchers. Gonzales et al. (1989) was the first to apply polyethylene glycol (PEG)-mediated transformation procedure for *T. mentagrophytes*. They introduced successfully pH1S plasmid vector, containing bacterial hygromycin B phosphotransferase gene (*hph*) into *T. mentagrophytes*. However, there have been no additional reports of the genetic transformation of dermatophytes until 2004, when Kaufman et al. used restriction-enzyme-mediated integration (REMI) for stable expression of eGFP protein in a clinical strain of *T. mentagrophytes*. In the following year,

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Yamada et al. (2005) described a reproducible transformation system for *T. mentagrophytes* and *M. canis* also by PEG-mediated method using plasmid vectors containing both hygromycin B phosphotransferase gene (*hph*) and enhanced green fluorescent protein gene (*egfp*). The same transformation method was used by Ferreira-Nozawa et al. (2006) for *T. rubrum* using plasmid carrying modified *hph* gene and *pacC* gene which encodes a putative protein that is homologous to the family of pH signaling transcription regulators. In all cases protoplasts were used as the competent cells. Protoplasts preparation in standard transformation protocols is time consuming and technically difficult as it requires monitoring of the cell wall digestion. Alternative methods have been developed to eliminate this step. One of them is *Agrobacterium tumefaciens*-mediated transformation (ATMT) which was successfully used for *T. mentagrophytes* transformation (Yamada et al. 2008), another is electroporation of fungi conidia (Brown et al. 1998; Chakraborty et al. 1991; Dantas-Barbosa et al. 1998; Robinson and Sharon 1999; Sanchez and Aguirre 1996).

Here we report the development of the procedure for genetic transformation of *T. rubrum* by applying electroporation of germinated conidia using the hygromycin B resistance gene (*hph*) as a selective marker.

## Materials and methods

### Fungal strain and plasmid vector

The wild-type *T. rubrum* strain TR4 was a clinical isolate from 60-year-old woman with *onychomycosis* and identified based on traditional mycological method and restriction fragment length polymorphism of ITS1-5.8S-ITS2 region (Shin et al. 2003). It was maintained by cultivation on Sabouraud agar (1% w/v peptone, 2% w/v glucose and 1.5% w/v agar) with 50 µg/ml chloramphenicol and 500 µg/ml cyclohexamide. Transforming vector used in this study, pCHSH75-Pch-GFP/TtrpC (Yamada et al. 2005), has the *E. coli* hygromycin B phosphotransferase gene (*hph*) as a selection marker and enhanced green fluorescent protein gene (*eGFP*) as a reporter marker under the control of a promoter sequence from *Cochlibolus heterostrophus*.

### *T. rubrum* transformation

Transformation of *T. rubrum* was achieved by electroporation of germinated conidia according to the self-modified method of Sanchez and Aguirre which was used for transformation of *Aspergillus nidulans* (Sanchez and Aguirre 1996). Conidia of *T. rubrum* TR4 were harvested by centrifugation, washed twice in sterile distilled water and filtered

through BD Falcon Cell Strainer. Then they were adjusted to a density of  $1 \times 10^6$  cells/ml and purity of the obtained suspension was checked by microscopic observation. The obtained conidia was grown for 2 h at 37°C at 100 rpm in 20 ml YG medium (0.5% yeast extract, 2% glucose). Swollen conidia were collected by centrifugation, washed with 100 ml of sterile water and resuspended in 2 ml YED medium (1% yeast extract, 1% glucose, 20 mM HEPES adjusted to pH 8.0 with NaOH) and incubated for 45 min at 30°C at 100 rpm. Germinated conidia were collected by centrifugation and resuspended in 1 ml of electroporation buffer (50 mM mannitol, 1 mM HEPES). For electroporation, 1 µg of DNA (pCHSH75-Pch-GFP/TtrpC linearized by *Bgl*III) was added to 100 µl of the ice cold conidial suspension. The mixture was incubated on ice for 15 min and then transferred to a 0.2-cm cuvette. Electroporation was performed using the BioRad Gene Pulser. Voltage was adjusted to 1,000 V, capacitance to 25 µF and resistance was 400 Ω (pulse length varied between 8.1 and 8.5 ms). Under these conditions about 30–40% of the conidia were killed. Following electroporation, 1 ml of ice cold YED medium was immediately added to each transformation mixture which was kept on ice for 30 min then incubated at 30°C for 60 min at 100 rpm. Transformation mixtures were plated on Sabouraud medium containing 250 µg/ml hygromycin B and incubated 5–7 days at 28°C.

### Molecular analysis of transformants

Freshly harvested conidia from *T. rubrum* transformant colonies appearing on the Sabouraud medium, containing 250 µg/ml hygromycin B were inoculated into 5 ml YG medium without selective factor (germination step) and incubated at 28°C for 2–4 h. Then they were plated again on Sabouraud medium with 250 µg/ml hygromycin B and incubated at 28°C for 5–7 days. For sufficient selection of transformants this process was repeated to obtain fifth generation of *T. rubrum* transformants.

Genomic DNA was extracted from a small amount of mycelium by a rapid mini preparation method (Liu et al. 2000). PCR analysis of transformants was performed using GFP-F (5'-CGAACGGATCCATGGTGAGCAAGGGCG AGGA-3') and GFP-R (5'-AGCTAAAGCTTTTACTTGT ACAGCTCGTCCAT-3') primers (Yamada et al. 2005) that amplified a 712-bp fragment of *egfp* gene during 30 cycles of 94°C for 45 s, 60°C for 45 s and 72°C for 2 min.

Southern blot analysis was performed on *Hind*III-digested genomic DNA, resolved in 0.8% agarose gels and transferred to positively charged nylon membranes (Sigma). Preparation of DIG-labeled probes, hybridization (50°C) and chemiluminescent detection were performed according to manufacturer's instructions (Roche). An internal sequence of the *hph* gene (990 bp) which was amplified

using hph-F (5'-AGTAAAAAGCCTGAACTCAC-3') and hph-R (5'-GTTTCCAATATCGGCGAGTA-3') (Yamada et al. 2005) was used as the hybridization probe.

## Results

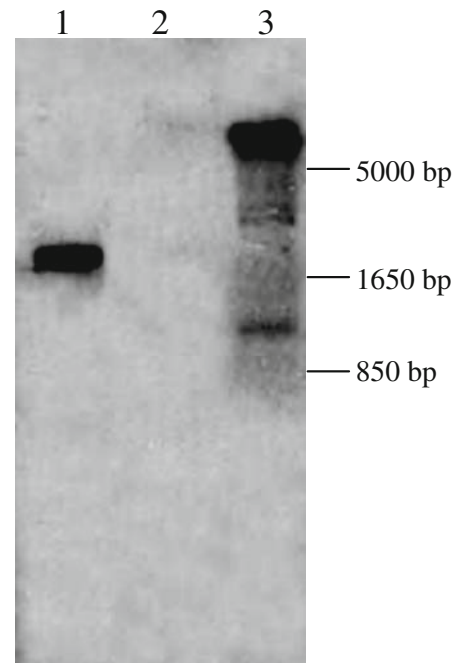
### Susceptibility of *T. rubrum* to hygromycin B

The application of an appropriate concentration of selective factor is one of the most important steps for the efficient selection of transformants. Mycelia and conidia of *T. rubrum* were inoculated into Sabouraud plates containing various concentrations of hygromycin B (10, 20, 50, 100, 200, 500 µg/ml). When the mycelia were plated on Sabouraud medium, after 7 days definite growth inhibition was observed at 500 µg/ml. On the other hand, plating of germinated conidia (previously incubated in YG medium) resulted in conidia mortality and lack of colony formation at the concentration of 200 µg/ml. In order to reduce the possibility of false positives, 250 µg/ml hygromycin B was used for selection in the transformation experiments.

### Mitotic stability of *T. rubrum* transformants

Germinated conidia ( $1 \times 10^6$ ) of *T. rubrum* which were transformed with 1 µg of the linearized pCHSH75-Pch-GFP/TrpC plasmid generated 33 colonies on the selective medium after 5–7 days. The efficient selection of the transformants was repeated, through the germination step, to obtain fifth generation of *T. rubrum* transformants. Twelve of the obtained colonies retained hygromycin B, resistant phenotype. Previously, the experiments on transformation of dermatophytes resulted in the lower efficiency of obtained transformants (Table 1).

The presence of *hph* gene in the randomly selected transformant of *T. rubrum* was confirmed by Southern blot analysis using the *hph* gene as a probe. To check the stability of the transformant, chromosomal DNA was isolated from mycelium cultivated in nonselective medium. The results indicated that the transformant from the fifth generation had retained the introduced DNA, which was integrated at



**Fig. 1** Southern blotting analysis of genomic DNA from *T. rubrum* transformant. Hybridization was performed using an internal fragment (990 bp) of *hph* gene as a probe. Lane 1 DNA sample from *T. rubrum* transformant, Lane 2 DNA sample from wild-type control of *T. rubrum* (negative control), Lane 3 DNA sample from pCHSH75-Pch-GFP/TrpC plasmid (positive control)

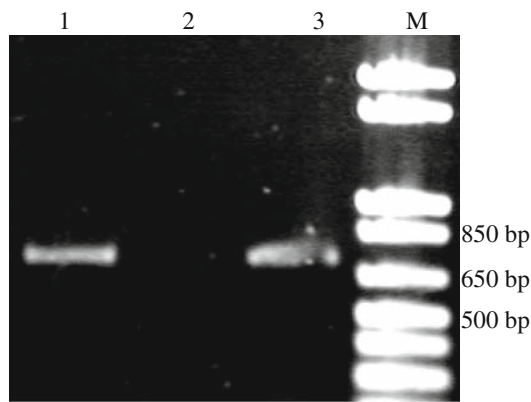
unique position, as only a single band within *Hind*III-digested chromosomal DNA was detected. A single band of ~2 kbp was detected in analyzed transformant (Fig. 1, lane 1). Additionally, presence of *egfp* gene in the genome of this transformant was confirmed by PCR with GFP-F and GFP-R specific primers which yielded a band of expected size of 712 bp (Fig. 2).

### eGFP expression in *T. rubrum* transformants

Mycelia from five randomly selected *T. rubrum* transformants from third generation and the five from fifth generation were analyzed by fluorescence microscopy. GFP fluorescence was observed in all analyzed colonies that were obtained from the germinated conidia transformed with pCHSH75-Pch/GFP/TrpC. Microscopic images of the

**Table 1** The effect of different transformation method on the frequency of dermatophytes transformation

Number of cells	Number of Hyg <sup>R</sup> transformants per µg of transforming DNA	Transformation method	References
$1 \times 10^6$	0.004–6	PEG	Gonzales et al. (1989)
$1 \times 10^6$	0.06	PEG/REMI	Kaufman et al. (2004)
$1 \times 10^6$	7	PEG	Yamada et al. (2005)
$1 \times 10^6$	12	Electroporation of germinated conidia	This work



**Fig. 2** PCR analysis of *T. rubrum* transformant from fifth generation. Genomic DNA was subjected to PCR with the primers specific to the *egfp* gene. Lane 1 pCHSH75-Pch-GFP/TtrpC plasmid, Lane 2 wild-type of *T. rubrum*, Lane 3 transformant of *T. rubrum*, M the molecular marker (1 kb Plus)

two mycelia samples from third and fifth generation of transformants are shown in Fig. 3. In contrast, the wild-type control did not show GFP fluorescence.

## Discussion

The efficient transformation system is an essential tool for gene manipulation and studying the functional genomics of dermatophytes. The transformation via protoplasts was successfully used only for *T. mentagrophytes*, *M. canis* and *T. rubrum* (Gonzales et al. 1989; Kaufman et al. 2004; Yamada et al. 2005; Ferreira-Nozawa et al. 2006). The objective of present study was to develop an alternative transformation method which does not require the preparation of protoplasts which is time-consuming procedure. The

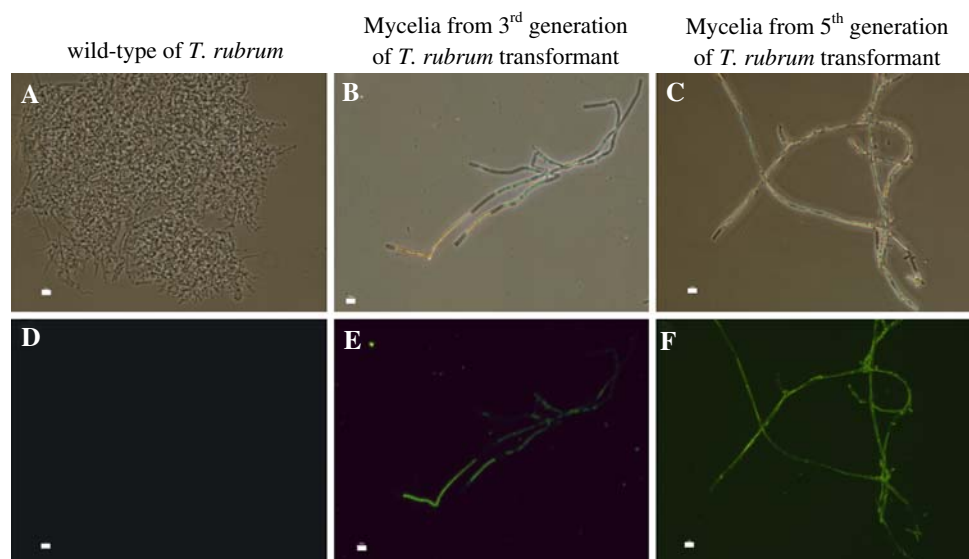
electroporation protocols have been described for the transformation of germinating conidia of many filamentous fungi. The results showed that the preparation of fungi conidia for transformation was more simple than protoplastization and allowed to generate large numbers of transformants (Meyer et al. 2003). For this reason we decided to use *T. rubrum* conidia as the competent cells.

Specific selection of the stable transformants is one of the most important steps of transformation methods. Hygromycin B is an aminoglycoside antibiotic produced by *Streptomyces hygroscopicus* and it is effective against bacteria, fungi and higher eukaryotes. It is commonly used as a selective marker for analysis of fungi transformants. To test the sensitivity of *T. mentagrophytes* to hygromycin B, Gonzales et al. (1989) and Kaufman et al. (2004) used the protoplasts instead of mycelia which suggested that mycelia were probably resistant to this antibiotic. Such observation was also confirmed by Yamada et al. (2005).

In this work we report that the mycelia of *T. rubrum* and their conidia were resistant to very high concentrations of hygromycin B (500  $\mu\text{g/ml}$ ). For this reason we decided to relax cell wall structures of conidia through their germination step to make them more permeable to hygromycin B. Pretreatment of *T. rubrum* conidia by the introduction of a cell wall destabilization step during incubation in YG medium was found to be essential for successful transformation. Complete growth inhibition of the regenerating germinated conidia by hygromycin B was observed at the concentration of 200  $\mu\text{g/ml}$ .

Moreover, *T. rubrum* transformants whose mycelia were selected on Sabouraud plates containing 500  $\mu\text{g/ml}$  hygromycin B were unstable. We observed that transformants lost the ability to GFP fluorescence and hygromycin B-resistant phenotype in the next generations (data not

**Fig. 3** Growth of *Trichophyton rubrum* transformants expressing GFP. Mycelia of *T. rubrum* were observed by fluorescence microscopy. Differential interference contrast images (a–c), GFP fluorescence images (d–f) (Bar 10  $\mu\text{m}$ )



shown). We speculate that this may be related to the *hph* gene and *egfp* gene deletion or mutation within the promoter which controls both genes during recultivation of transformants in the consecutive cell generations. Therefore, growth of mycelia, which are resistant to very high concentrations of hygromycin B, can lead to deletion of the selection marker, thus making impossible to obtain stable homozygotic *egfplegfp* transformants in the next generations. However, loss of the hygromycin B-resistant phenotype in the next generation of transformants has not been analyzed at molecular level.

Based on these results and preliminary hygromycin B sensitivity tests of *T. rubrum*, we decided to change the selection of transformants through the step of conidia germination during successive recultivation. This method would thus be applicable for efficient selection of *T. rubrum* transformants. Southern blotting analysis confirmed that a single copy of the *hph* gene was integrated into the chromosomal DNA of *T. rubrum*. GFP fluorescence was confirmed in all colonies that were obtained from the germinated conidia transformed with pCHSH75-Pch/GFP/TtrpC. However, not all cells of *T. rubrum* transformants from third and fifth generation were fluorescing, but all of them had hygromycin B-resistant phenotype. Additionally, as shown in Fig. 3, we have also observed that not all sectors of a hyphae were fluorescing. This is not unusual, because it is known that not every cell in the culture demonstrates the same level of *egfp* expression in the presence of subsaturating inducer. The same phenomenon was observed in bacterial cells in which GFP expression from plasmid carrying *araBAD* promoter was regulated by the concentration of arabinose in the growth medium (Siegele and Hu 1997). Fluorescence measurements of cell suspensions showed intermediate GFP expression levels in cultures which resulted from the induction of small subpopulation among uninduced cells. This mechanism is analogous to the induction pattern of the *lac* operon described by Novick and Weiner (1957) over 50 years ago.

In order to explain the contribution of unstable transformants, the localization and number of copies of the integrated *hph* gene into the chromosomal DNA, and to understand the role of hygromycin B selective pressure in the next generations as well as the type of mutations which lead to the instability of *T. rubrum* transformants, it is necessary to obtain greater number of *T. rubrum* transformants. Experiments are currently underway in our laboratory.

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