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Transposon insertion mutation of Antarctic psychrotrophic fungus for red pigment production adaptive to normal temperature

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Abstract: Polar regions are rich in microbial and product resources. *Geomyces* sp. WNF-15A is an Antarctic psy chrotrophic filamentous fungus producing high quality red pigment with potential for industrial use. However, efficient biosynthesis of red pigment can only realize at low temperature, which brings difficult control and high cost for the large-scale fermentation. This study aims to develop transposon insertion mutation method to improve cell growth and red pigment production adaptive to normal temperature. Genetic manipulation system of this fungus was firstly developed by antibiotic marker screening, protoplast preparation and transformation optimization, by which transformation efficiency of ~50% was finally achieved. Then transposable insertion systems were established using *Helitron*, *Fot1*, and *Impala* transposons. The transposition efficiency reached 11.9%, 9.4%, and 4.6%, respectively. Mutant MP1 achieved the highest red pigment production (OD_{520} of 39) at 14°C, which was 40% higher than the wild-type strain. Mutant MP14 reached a maximum red pigment production (OD_{520} of 14.8) at 20°C, which was about twofold of the wild-type strain. Mutants MP2 and MP10 broke the repression mechanism of red pigment biosynthesis in the wild-type and allowed production at 25°C. For cell growth, eight mutants grew remarkably better (12%~30% biomass higher) than the wild-type at 25°C. This study established an efficient genetic manipulation and transposon insertion mutation platform for polar filamentous fungus. It provides reference for genetic breeding of psychrotrophic fungi from polar and other regions.

Keywords: Natural red pigment, Antarctic fungus, *Geomyces* sp., Protoplast transformation, Transposon mutagenesis

Introduction

Polar regions represent various extreme environments covering ultra-low temperature, acid–base stress, oligotrophic restriction, repeated freeze-thaw cycles, high salinity, seasonal dryness, multiple strong winds, high radiation, long time without sunshine, etc. (Hayward et al., 2021). Therefore, most terrestrial flora and fauna cannot live under these extreme conditions (Jesus et al., 2021). Polar species are dominated by lichens, mosses, algae, microorganisms, and some marine organisms that can adapt to the extreme conditions (Rosa et al., 2019). Different microorganisms in polar regions interact with other species and form a complex ecological network, including saprophytic, reciprocal, and parasitic ecological relationships (Godinho et al., 2015).

Studies have shown the diversity of microorganisms in polar regions, especially the psychrophiles and psychrotrophs (Harding et al., 2011; Ogaki et al., 2020). The unique physiological mechanism enables these microorganisms to produce novel compounds and enzymes and adapt to extreme environments (Gomes et al., 2018). Accordingly, polar microorganisms are reported to be promising producers of bioactive secondary metabolites with potential use for the development of new drugs and chemicals (Rosa et al., 2019). Polar fungi can produce various bioactive metabolites, including antibacterial, antifungal, trypanocidal, herbicidal, and antitumoral activities. (Furbino et al., 2014; Gomes et al., 2018; Gonçalves et al., 2015; Henríquez et al., 2014; Poveda et al., 2018; Purić et al., 2018; Vieira et al., 2018). Nevertheless, marketed products derived from polar microorganisms are limited. The special physiological characteristics of polar microorganisms make it difficult for artificial culture especially in large-scale culture. For example, *Geomyces* sp. WNF-15A is a typical cold-adapted filamentous fungus isolated from Antarctic soil, producing red pigment when cultured at low temperatures (Wang et al., 2013). The major content of this Antarctic *Geomyces* red pigment is geomycamine (Supplementary Fig. S1, Wang et al., 2015). It shows similar carbon skeleton structure to rubropunctamine and monascorubramine from *Monascus* spp. (Akihisa et al., 2005). The red pigment is easily soluble in water and its extinction coefficient is higher than that of cochineal red pigment (Jin et al., 2014). Also, it is more stable than cochineal red pigment in reducing agents, food additives, acid and alkali environments, and has good tolerance to ultraviolet rays, oxidants, and most metal ions (Jin et al., 2014). Besides, median lethal dose (LD_{50}) of the Antarctic *Geomyces* red pigment was over than 15 000 mg/kg, which meets level 1 of the acute toxicity dose classification of food toxicology (defined as nontoxic) in China (Wang et al., 2013). Also, citrinin content was not detected from *Geomyces* sp. WNF-15A culture (Supplementary Fig. S2). Of note, *Geomyces* sp.WNF-15A produced high-level red pigment below 14°C, but the production decreased by 60%–70% at above 20°C and almost blocked at 25°C (Huang et al., 2020).

Microbial breeding by genome modification is of great significance to biotechnology research and industry. In nature,

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spontaneous mutation and natural selective pressure lead to the continuous evolution and diversity of the life (Lee et al., 2012). Until today, many artificial mutagenesis strategies, including chemical and physical methods, have been extensively applied to improve mutation rates and screen beneficial varieties (Kodym & Afza, 2003). Nevertheless, these mutagenesis methods generally create mutant strains through random mutation, which is not easy to track the mutation sites that are useful for direction of rational modification. Transposon insertion mutation has become an effective tool for gene mutation and function analysis. Transposons can inactivate genes and cause changes in host phenotypes by random insertion. Then the mutant gene will be easily targeted by tracking transposon (Krishnan et al., 2018; Opijnen & Levin, 2020).

Till now, various fungal transposons have been reported. For example, *Impala* transposon was first identified in *Fusarium oxysporum* and is considered to be an ancient component of the *F. oxysporum* genome (Hua et al., 2001a). *Impala* is an active "cut-paste" transposon in the *Tc1/Mariner* family, which is about 1280 bp in length containing transposase coding gene flanked with 37 bp length of terminal inverted repeats (TIRs). Studies have shown that *Impala* can transpose in multiple heterologous hosts, including *Fusarium moniliforme* (Hua et al., 2001b), *Magnaporthe grisea* (Villalba et al., 2001), and *Aspergillus nidulans* (Nicosia et al., 2001). *Fot1* transposon is also a "cut-paste" transposon found in *F. oxysporum* with a length of 1928 bp, and its structure is similar with the *Impala* transposon (Nicosia et al., 2001). *Helitron* transposons belong to "copy-paste" DNA transposons and is ubiquitous in eukaryotes. *Helitron* can transpose by rolling-circle replication (Kapitonov & Jurka, 2007). The length of *Helitron* transposon in *F. oxysporum* was 6265 bp and the size of the recognition sequence at both sides was 322 bp and 94 bp, respectively (Thomas & Pritham, 2015). *Helitron* transposase does not contain RNase-H catalytic domain but with helicase domain and HUH endonuclease domain (Dyda et al., 1994; Kapitonov & Jurka, 2001; Grabundzija et al., 2016). It has been proved to be heterologously transposable in *Fusarium graminearum* (Wang et al., 2018).

This study aims to develop transposon insertion mutation method for red pigment production in *Geomyces* sp.WNF-15A adaptive to normal temperature. We first established genetic manipulation system of this wild type psychrotrophic fungus. Then transposon insertion mutation method was developed based on the three transposons of *Impala*, *Fot1*, and *Helitron*. Screened by colony growth and color, mutants that can grow and synthesize red pigment at room temperature were successfully obtained and further evaluated in submerged culture. This work provides a new strategy for strain improvement of polar *Geomyces* sp.WNF-15A. It also offers enlightenment for genetic breeding of other polar psychrophiles and psychrotrophs.

Materials and Methods

Strains and Culture Conditions

Geomyces sp. WNF-15A was donated by Dr. Nengfei Wang, First Institute of Oceanography, Ministry of Natural Resources, China. *Geomyces* sp. WNF-15A and its mutant strains were stored in 20% (wt/vol) glycerol at −80°C. *Escherichia coli* Top 10 was preserved in our laboratory. The composition of various media is listed in Table 1.

For culture of *Geomyces* sp. strains, 100 μL spore suspension was inoculated into 5 mL seed medium and cultured in dark at 20°C and 130 r/min for 36 h. Then 1 mL inoculum was inoculated in 50 mL seed medium with 20 glass beads (diameter of 4 mm), and

Table 1 Composition of Various Media Used for Protoplast Preparation and Cell Culture

Seed medium (g/L)	PDB medium[a] (g/L)	Medium R[a] (g/L)	YPD medium (g/L)	LB medium (g/L)	Substrate medium (g/L)	Soft agar medium (g/L)	Medium S (g/L)
Glucose10	Potato extract8	Potato extract8	Tryptone2	NaCl10	Glucose10	Glucose10	Soluble starch[b]28
Mannitol[b]20	Glucose20	Glucose20	Yeast extract[c]1	Tryptone[c]10	Mannitol[b]20	Mannitol[b]20	Typtone[c]1.85
Maltose[b]20	Agar (solid)10	Mannitol109.3	Agar (solid)10	Yeast extract[c]5	Maltose[b]20	Maltose[b]20	
Sodium glutamate[b]10		Agar (solid)10		Agar (solid)20	Sodium glutamate[b]10	Sodium glutamate[b]10	
Yeast extract paste[b]6					Yeast extract paste[b]6	Yeast extract paste[b]6	
$MgSO_4{\cdot}7H_2O$0.3					$MgSO_4{\cdot}7H_2O$0.3	$MgSO_4{\cdot}7H_2O$0.3	
$KH_2PO_4$0.5					$KH_2PO_4$0.5	$KH_2PO_4$0.5	
Agar (solid)30					Agar (solid)20	Agar (solid)7.5	

[a]Potato dextrose broth (PDB) medium was purchased from Hangzhou Microbial Reagent Co., Ltd., China.
[b]These reagents were purchased from Sinopharm Chemical Reagent Co., Ltd., China.
[c]These reagents were purchased from OXOID.

cultured for 3 days under the same conditions to prepare the first-order seed. It was transferred to 50 mL seed medium at 10% inoculation and cultured under the same conditions for 36 h to prepare the second-order seed. Afterwards, 200 μL second-order seed was evenly spread on agar seed medium, and cultured in an incubator with 80% humidity and at 20°C for 3 days.

For shake flask culture, the second-order seed was inoculated in 75 mL medium S by 6% (vol/vol), and 20 glass beads with a diameter of 4 mm were added. It was then cultured in dark at 130 r/min for 16 days (Huang et al., 2020). Three biological replicates were set for each experiment.

Construction of Plasmids

The backbone vector pFC000 was constructed by removing the open reading frame of Cas9 protein on the pFC332 (Nødvig et al., 2015), which was donated by Prof. Reinhard Fischer of the Karlsruhe Institute of Technology, Germany. Then transposable plasmids of pFC001 and PFC002 were constructed by inserting the complete transposable elements of *Impala* and *Fot1* between the promoter and terminator of PFC000. Based on pFC332, donor plasmid with hygromycin B phosphotransferase (*hph*) gene inserted between the *Helitron* recognition sequences (TIRs) as well as the helper plasmid containing *Helitron* transposase were constructed. The complete transposon of *Impala* (GenBank: AF282722.1) and *Fot1* (GenBank: AF443562.1) were synthesized by Suzhou Genewiz Biological Technology Co., Ltd. The *Helitron* transposon (Wang et al., 2018) was donated by Prof. Xiaoping Hu, State Key Laboratory of Crop Stress Biology in Arid Zone, College of Plant Protection, Northwest A&F University. Primers used for plasmids construction are listed in Supplementary Table S1 and synthesized by Shanghai Tsingke Biotechnology Co., Ltd. Construction of plasmids was mainly based on standard protocols of PCR (Cat. No. R011, TaKaRa) and seamless cloning (ClonExpressTM II one-step cloning kit, Vazyme Biotech Co., Ltd., China). The plasmid was purified from *E. coli* using routine procedures and dialyzed against distilled water prior to use. Transformation and other standard recombinant DNA operations for *E. coli* were performed as described previously (Green et al., 2012).

Preparation and Transformation of Protoplasts

Mycelia preparation

The mycelia on the plate were gently washed and scraped off with 5 mL YPD medium, and inoculated into 50 mL YPD medium for culture at 20°C and 130 r/min. The inoculum was pumped and filtered with a sand core funnel G1 covered with mirror paper. Mycelia of 1.5 g was weighed and put into a clean centrifugal tube for use.

Protoplast preparation

Add 20 mL sterile deionized water to a centrifuge tube containing mycelia, and repeatedly blow and wash the mycelia (centrifuged at 4°C and 3000 *g* for 5 min). The mycelia were washed with 10 mL solution B, centrifuged at 4°C and 3000 *g* for 5 min, discarded supernatant. Repeated it again. Then add 10 mL solution A to the mycelia, blow and mix until there were no apparent large mycelial clusters in the enzymolysis solution, followed by placing on a rotary mixer for enzymolysis at a specific temperature. During the enzymolysis process, samples can be taken every 30 min to observe the release conditions of protoplasts. After enzymolysis, the enzymatic hydrolysate was filtered with glass wool (Hunan Yisi Technology Co., Ltd). The filtrate was centrifuged at 4°C and 700 *g* for 5 min (adjust both acceleration and deceleration to 1), then the supernatant was discarded. Solution B of 20 mL was added to wash the protoplasts and centrifuged at the same conditions and the supernatant was discarded and repeated. Then it was washed with solution C for one time. The protoplasts were suspended with 1 mL solution C to a final concentration of 10^{10} mL^{-1}. Then solution D was added by 20% volume and the final liquid transferred into 2 mL Eppendorf tubes (200 μL for each tube) for the following transformation experiments.

The solution used in the protoplast preparation and transformation was as follows. Solution A: dissolved 0.2 g/L cellulase (Shanghai Yuanye Bio-Technology Co., Ltd) and 0.15 g/L snailase (Beijing Solarbio Technology Co., Ltd) in solution C, mixed well and filter sterilized through 0.2 μm filters (Millex®GP, EMD Millipore, USA). Solution B: 52.596 g/L NaCl sterilized by autoclaving. Solution C: 52.596 g/L NaCl, 5.549 g/L $CaCl_2$, and 1.2114 g/L Tris (Shanghai Sangon Biotech Co., Ltd), pH was adjusted to 7.0 with HCl and sterilized by autoclaving. Solution D: 45% PEG3500 (Shanghai Macklin Biochemical Co., Ltd) was dissolved in solution C and filter sterilized through 0.2 μm filters.

Protoplast regeneration

The freshly prepared protoplasts were gradient diluted by 10 000 times with solution C, and the concentration of protoplasts in the final dilution solution was controlled at about 10^4 mL^{-1}. Then 10 μL protoplasts was added to 2 mL medium R, and then incubated at 20°C and 80% humidity for 2 days in dark. The medium was poured into a lukewarm agar medium R, mixed well and poured it into a clean plate, and cultured at 20°C and 80% humidity for 3 days. The control group was cultured on a standard PDB medium (without mannitol) to eliminate the influence of unbroken hyphae and spores. The number of colonies grown on medium R was denoted as R, the number of colonies grown on the standard PDB medium (without mannitol) was denoted as R_0, and the rate of protoplast regeneration *r* was calculated as *Equation 1*:

$$r(\%) = \frac{R - R_0}{protoplasts\ number} \times 100\% \quad (1)$$

Protoplast transformation

Add the plasmid to 200 μL protoplast solution and place the mixture on ice for 30 min. Add 700 μL solution D and leave for 20 min at room temperature. The obtained transformation solution was directly coated on the substrate medium and cultured at 20°C and 80% humidity without light. After a while, the second layer of soft agar medium containing antibiotics was added into the culture medium for 4–10 days. The number of colonies growing on the soft agar medium was recorded as T_1. After continuous culture for two generations, spore PCR was carried out with resistance primer and the number of colonies with the correct bands was recorded as T_2. The transformation efficiency of protoplasts *t* was calculated as *Equation 2*:

$$t\ (\%) = \frac{T_2}{T_1} \times 100\% \quad (2)$$

Analytical Methods

For spore PCR, the colonies were picked out and sucked in 50 μL TE buffer (Tiangen) repeatedly and mixed thoroughly. After heated in a microwave oven at high heat level for 2 min, the spore solution was placed on ice immediately for 5 min. Centrifuged the solution and then used supernatant as the template for PCR reaction.

Genomic DNA of the positive mutant identified by spore PCR was further extracted and used as the template for further PCR verification. The 2× Taq PCR MasterMix (GenStar) was used for the PCR reaction system. In each 15 μL reaction volume, 5 μL of supernatant was used as the template.

For liquid growth, 10 mL of inoculum was extracted and filtered to the weighing filter paper every 24 h, and the mycelia were repeatedly washed until the filtrate turned to colorless. Then, the filter paper with mycelia was dried (70°C) to constant weight. Record the initial weight of the filter paper as w_0 and the total weight after drying as w_1. The dry weight of the cell, w, was calculated according to *Equation* 3:

$$w\,(\mathrm{g/L}) = (w_1 - w_0) \times 100 \tag{3}$$

For colony growth, the spore solution (10^5 mL^{-1}) was vertically dropped into the middle of the agar medium, and cultured at 20°C and 80% humidity for 8 days without light. Measure the diameter of the colony in three different directions to calculate the average value every 24 h.

The production of red pigment was monitored by OD_{520} using a UV-2102 PC spectrophotometer (UNICO [Shanghai] Instruments Co., Ltd, China). The measurement method of red pigment production was performed as described previously (Huang et al., 2020). The number of protoplasts and spores was determined using a cytometer (Shanghai Titan Technology Co., Ltd). Denote the total number of the target object in the counting cell as M; the dilution factor is n, and the concentration m can be calculated according to *Equation* 4:

$$m(\mathrm{mL}^{-1}) = \mathrm{M} \times 2 \times 10^7 \times n \tag{4}$$

Optimization of Genetic Manipulations

Different conditions for genetic manipulations of *Geomyces* sp. WNF-15A were investigated. Different antibiotics were tested, including hygromycin B (300, 200, 100, 50, 30, 10, 5 μg/mL), oligomycin (200, 100, 10, 5 μg/mL), zeocin (100, 75, 50, 25 μg/mL), Geneticin (G418) (200, 100, 50, 30, 5 μg/mL), and oxazolidine (150, 100, 50, 25 μg/mL). Spore solution with a concentration of 1.07×10^6 mL^{-1} was coated on the agar seed medium supplemented with different concentrations of antibiotics and incubated at 20°C for 3 days.

The effects of culture time (12, 24, 30, 36, 48, 56, 60, 72, 84, 144 h), enzyme content (3% C, 1.5% S, 3% C + 1.5% S, 2% C + 1.5% S, 1.5% C + 1.5% S, 1% C + 1.5% S; C-Cellulase, S-Snailase), enzymolysis temperature (20, 22, 24, 26, 28, 30, 32°C), enzymolysis time (0.6, 1, 1.5, 2, 2.5, 3, 4 h), enzymolysis pH (5.5, 6, 6.5, 7, 7.5, 8, 8.5) on the number, and regeneration rate of protoplasts were investigated. Besides, the parameters of protoplast transformation, such as incubation time (0, 12, 24, 36, 48, 72, 96 h), exogenous vector concentration (0.5, 1, 2, 2.5, 3, 3.5, 4 μg DNA), and PEG concentration (30%, 45%, 50%, 60%, 65%, 70%) were also optimized.

Molecular Analysis of Mutants

Spore PCR was performed with primers Am-F/Am-R to verify whether plasmids were lost. Transformants not carrying the plasmids were used for further genotype verification. Genomic DNA was extracted using the Fungi Genomic DNA Extraction Kit (Solarbio). DNA samples were detected by PCR using primers listed in Supplementary Table S1. The theoretical PCR product size was 1253 bp (*Impala* transposon validated using primers of Im-F/Im-R), 1203 bp (*Fot1* transposon validated using primers of Ft-F/Ft-R), and 889 bp (*Helitron* transposon validated using primers of He-F/He-R), respectively. Sequencing results of PCR products were submitted as supplementary data.

Statistical Analysis

Each trial was performed in triplicate. The significances of the differences between treatments were determined by p-values using one-way analysis of variance (ANOVA). The result was recognized as significant at $P < 0.05$.

Results and Discussion

Development of *Geomyces* sp. WNF-15A Genetic Manipulation System

As a newly isolated polar fungus, *Geomyces* sp. WNF-15A is lack of reported genetic information and transformation methods. To implement transposon insertion mutation, we need to develop genetic manipulation system of this fungus first.

Antibiotic Sensitivity Analysis

Effective antibiotic resistance markers can increase the efficiency of genetic manipulation, so we first tested the sensitivity of *Geomyces* sp. WNF-15A toward commonly used antibiotics against filamentous fungi (Ruiz-Díez, 2002). The growth of *Geomyces* sp. WNF-15A was completely inhibited at 30 μg/mL hygromycin B and 200 μg/mL G418 (Supplementary Fig. S3), which can be used for the following screening of transformants. Accordingly, the *hph* and *neo* were selective as the marker gene against hygromycin B and G418, respectively. Commonly, oligomycin, phosphinothricin, and bleomycin may inhibit fungal growth at concentrations of 5–300 μg/mL, but we did not observe growth repression of *Geomyces* sp. WNF-15A with these three kinds of antibiotics.

Protoplast Preparation and Regeneration of *Geomyces* sp. WNF-15A

The genetic transformation of filamentous fungi is usually achieved by modification of protoplasts (Timberlake & Marshall, 1989). Enzymolysis has been proved to be a preferred method for protoplast preparation due to its high efficacy, safety, and mildness. However, many factors during the preparation process will affect the quantity and quality of protoplasts (Rehman et al., 2016). Therefore, we optimized protoplast preparation process in detail by mycelia harvest, enzyme composition, enzymolysis temperature, time and pH, etc.

In different growth stages, mycelia accumulation and fungal physiological activity are different, which will affect the enzymatic hydrolysis of cell wall (Wang & Zhao, 2007). We first tested the effects of mycelial age on protoplast preparation. As shown in Fig. 1a, the same amount of fungal biomass of various ages generated different amount of protoplasts. Generally, the number of protoplasts increased fast with cells cultured from 24–56 h, after which it decreased slowly until the end point. Cell wall of mycelia in the early stage is relatively weak, which made it sensitive to the enzymolysis reaction. To shorten the preparation period, we selected culture time of 36 h for mycelia collection and protoplast preparation in the following experiments. Cell wall composition is complex and strain dependent, so mixed enzymes are necessary for cell wall lysis (Wang & Zhao, 2007). We tested the effects of different combinations of cellulase and snailase on protoplast

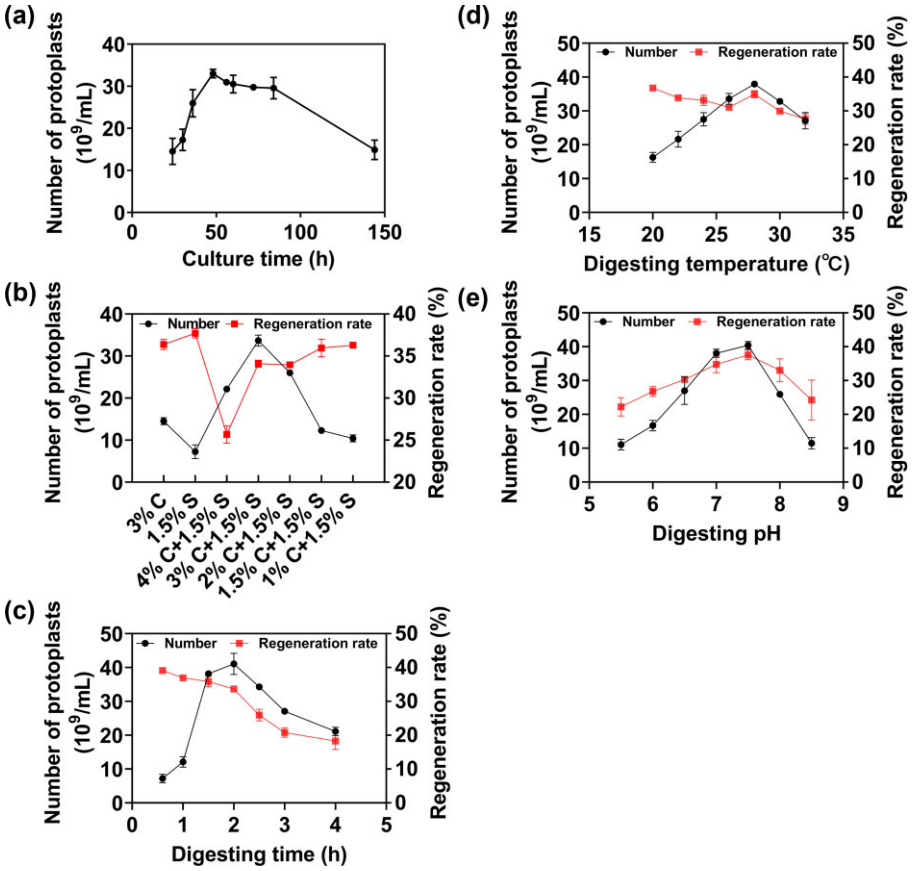

Fig. 1 The protoplast preparation of *Geomyces* sp. WNF-15A. (**a**) Effects of mycelial culture time on protoplast preparation. Wet mycelia (1.5 g) were digested for 1.5 h at 26°C by 2% cellulase and 1.5% snailase with pH adjusted to 7.0. (**b**) Effects of enzyme compositions on protoplast preparation. C, cellulase; S, snailase. Mycelia were digested for 1.5 h at 26°C with different enzymes with pH adjusted to 7.0. (**c**) Effects of digesting temperature on protoplast preparation. Mycelia were digested for 1.5 h by 3% cellulase and 1.5% snailase with pH adjusted to 7.0. (**d**) Effects of digesting time on protoplast preparation. The mycelia were digested at 28°C by 3% cellulase and 1.5% snailase pH adjusted to 7. (**e**) Effects of digesting pH on protoplast preparation. Mycelia were digested for 1.5 h at 28°C by 3% cellulase and 1.5% snailase.

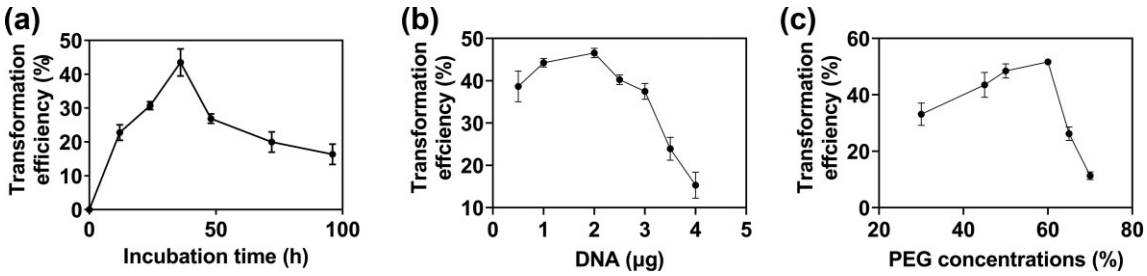

Fig. 2 Optimization of protoplast transformation in *Geomyces* sp. WNF-15A. (**a**) Effects of incubation time on protoplast preparation. Two micrograms of DNA was transformed with 45% PEG. (**b**) Effects of DNA amount on protoplast preparation. The protoplasts were transformed with 45% PEG and incubated for 36 h. (**c**) Effects of PEG concentration on protoplast preparation. Two micrograms of DNA was transformed and incubated for 36 h. The calculation of transformation efficiency is shown as *Equation* 2.

formation and regeneration. The results indicated that 3% cellulase + 1.5% snailase produced the maximum protoplasts and the regeneration rate also reached a high level (Fig. 1b). Enzymolysis temperature, pH, and duration may also affect the enzymatic reaction and the quality of protoplasts (Ma et al., 2014; Wu & Chou, 2019). Thus we investigated the changes in the number and regeneration rate of protoplasts under different enzymolysis conditions (Fig. 1c–e). The results finally suggested the optimal enzymolysis temperature of 28°C, optimal time of 1.5 h, and optimal pH of 7.5, respectively. Under the optimized conditions, protoplasts of 3.96×10^{10} mL^{-1} and regeneration rate of 36% were achieved finally. Microscopic photograph showed that the produced protoplasts were full in shape, evenly dispersed, and mycelia well digested (Supplementary Fig. S4).

PEG-Mediated Protoplast Transformation

Incubation culture is an essential process of protoplast self-repair and recovery of broken cell wall after transformation, which will directly affect the efficiency of transformation. With the extension of incubation time, the transformation efficiency increased gradually and reached the highest level of 46% at 36 h (Fig. 2a).

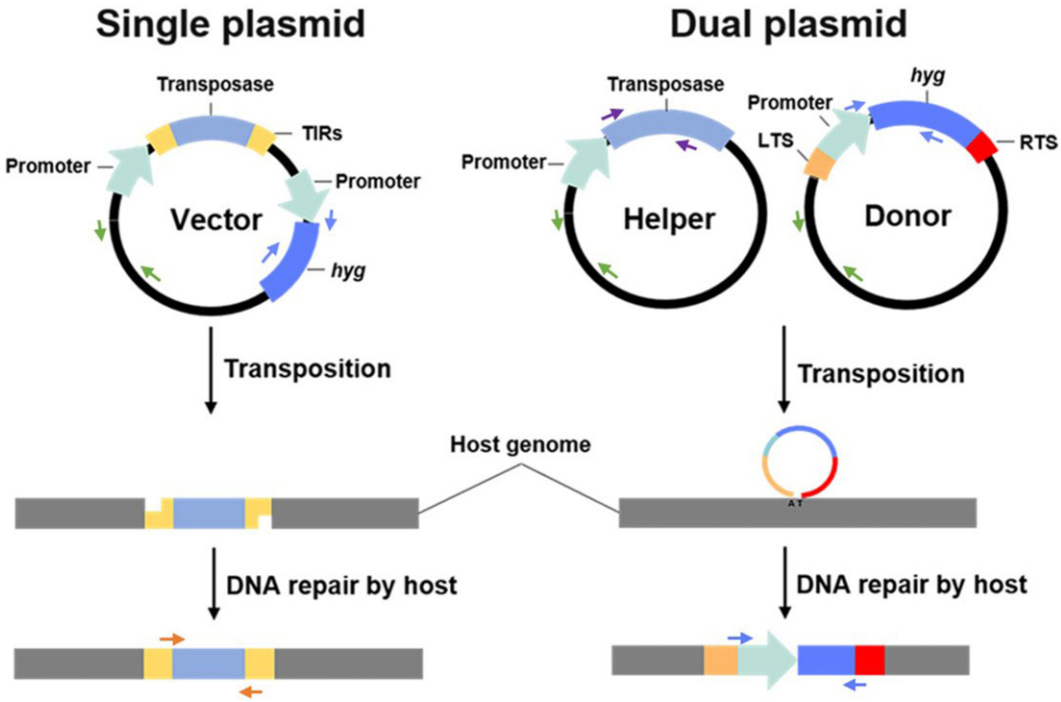

Fig. 3 Construction diagram of transposable systems. Promoter of P_{trpC} was used. TIRs, terminal inverted repeats of transposons. The transposase recognizes the TIRs to excise the sequence and insert it into a new genomic locus. LTS, left terminal sequence of transposons; RTS, right terminal sequence of transposons. The transposase recognizes the LTS and RTS to carry the rolling-circle transposition. *hyg*, hygromycin resistance gene. The short arrows represent the primer locations used for verification. Box lengths in the figure do not represent actual relative motif sizes.

Extension of the incubation time would reduce the selective pressure, produce more incorrect phenotypes and decrease transformation efficiency (Supplementary Table S2, Fig. 2a). Researchers usually used 2–12 μg DNA for PEG-mediated fungal transformation (Fincham, 1989; Wang et al., 2018). In this study, we tested 0.5–4 μg DNA for the transformation. Increase of DNA amount from 0.5–2 μg increased the number of transformants (Supplementary Table S3) and transformation efficiency slightly (Fig. 2b), but the number of transformants and transformation efficiency both decreased with DNA over 2 μg. Therefore, we confirmed the optimal DNA level of 2 μg. PEG is a polymer of ethylene glycol with high water solubility. During protoplast transformation, the ether bond carried by PEG has a negative charge, which can form an electrostatic attraction with Ca^{2+} in the buffer and the negative charge on the surface of the protoplast. It helps to reduce the polarity on the cellular surface and promote the aggregation of cells to form DNA-Ca^{2+} complexes to enter the cell efficiently (Wang et al., 2020). Fig. 2c and Supplementary Table S4 show the effect of PEG concentrations on the transformation efficiency of *Geomyces* sp. WNF-15A. PEG concentrations of 60% (wt/vol) brought an optimal transformation efficiency of 51%. With these efforts, we finally established genetic manipulation system of polar fungus *Geomyces* sp. WNF-15A, which built up a good basis for the following construction of transposon insertion mutation systems.

Screening and Construction of Transposable Systems

Stable and controllable engineering transposons are essential for transposon mutagenesis methods. Till now, scientists have characterized several transposons and identified the necessary elements for transposon DNA excision and integration (Opijnen & Levin, 2020). Here we employed three transposons, that is, *Impala*, *Fot1*, and *Helitron*, for development of transposon insertion mutation method in *Geomyces* sp. WNF-15A.

Impala and *Fot1* transposons are both "cut-paste" transposons composed of a coding gene and TIRs on the sides. The transposase recognizes the TIRs, excises the DNA, and inserts it into the new genome position (Munoz-Lopez & Garcia-Perez, 2010). We constructed single plasmid system for *Impala* (1280 bp) and *Fot1* (1928 bp) transposons (Fig. 2). Expression of transposase by P_{trpC} promoter will recognize TIRs, cut the entire transposable elements, and insert them into the host genome. *Helitron* transposon is "copy-paste" transposon that is different from *Impala* and *Fot1*. It proceeds through a circular intermediate, for which the sequence between left terminal sequence (LTS) and right terminal sequence (RTS) is duplicated by rolling-circle replication to produce a new copy and then inserted into the host genome (Kapitonov & Jurka, 2007). The coding gene of *Helitron* transposase is big (6265 bp), so we constructed a dual plasmid system for the *Helitron* transposon. The donor plasmid carries hygromycin B resistance cassette flanked by the recognition sequences, and the helper plasmid carries the expression cassette of *Helitron* transposase. Then, with the transposase expressed by the helper plasmid, the resistance marker expression cassette with LTS and RTS copied from the donor plasmid will be inserted into the host genome (Fig. 3).

With the recognition sequences in the transposable element, the inserted fragment may be transposed again under the action of the transposase. Therefore, after transposition, the self-replicating free plasmids need to be lost by passage cultivation to ensure the stability of the transposon insertion mutants. On the other hand, if there was endogenous homologous transposase in *Geomyces* sp. WNF-15A, it might also transpose inserted fragment continuously. So we compared the protein sequences of

Table 2 The Summary of the Mutants by Three Transposons

Mutants	Transposons
MP1, MP2, MP3, MP4, MP5, MP6, MP7, MP8, MP9	*Helitron*
MP10, MP11, MP12	*Fot1*
MP13, MP14	*Impala*
MP15, MG1, MG2, MG3, MG4, MG5, MG6, MG7, MG8, MG9, MG10, MG11, MG12	*Helitron*
MG13, MG14, MG15	*Impala*
MG16, MG17, MG18	*Fot1*

"cut-paste" or "copy-paste" transposases from different species, designed degenerate primers from the conserved sequences, and amplified DNA sequences using genome of *Geomyces* sp. WNF-15A as the template. BLAST research of 64 obtained DNA fragments indicated no conserved sequence related to coding genes of the three kinds of transposases (data not shown). Therefore, we speculated that the three exogenous transposons used in this study would not be affected by cross talk from endogenous transposase of the host.

Screening of Transposon Insertion Mutants

After transformation with transposable plasmids, the mutant colonies with bigger diameter or deeper red color were screened and further cultured in liquid seed medium without hygromycin B for three consecutive generations (3 days for each generation). Then most transformants can lose the transposable plasmids. Genotype verification showed that a total of 33 transposable mutant strains were obtained, including 21 *Helitron*, six *Impala*, and six *Fot1* mutants (Supplementary Fig. S5). We then tested colony growth and colony color (related with red pigment biosynthesis) of mutant strains in agar plate culture.

The mutants cultured at 20°C are shown in Supplementary Fig. S6a. A total of 15 mutants were screened as red pigment-related mutants, including 14 positive mutants (MP1–MP14) and one negative mutant (MP15). The positive mutants contain two *Impala*, three *Fot1*, and nine *Helitron* mutants. Compared with the wild-type, the positive mutants all showed prominent preponderance in the accumulation of red pigment. The negative mutant MP15 came from the *Helitron* transposon insertion mutation. At 25°C, we did not obtain the positive production mutant strains of red pigment production, but harvested several strains with growth advantage (Supplementary Fig. S6b). A total of 18 mutants with obvious growth advantage were obtained, including three *Impala*, three *Fot1*, and 12 *Helitron* mutants. The colony growth of these mutants in agar plate was further determined by maintaining the same number of spores and inoculation volume (Supplementary Fig. S7). After 10 days of culture, colony size of the mutants stopped extending but the continuous growth of aerial hyphae kept increasing the colony thickness. Nine mutants with significant growth improvement ($P < 0.01$ at all the three time points) was selected for further liquid culture analysis, including MG2, MG3, MG4, MG5, MG8, MG9, MG13, MG14, and MG15. Generally, the maximum diameter of mutant strains was 8–9 mm, compared with 7–8 mm of the wild-type colonies.

Analysis of the Mutant Strains in Liquid Culture

The 14 positive red pigment mutants were fully verified by shake flask fermentation (Supplementary Fig. S8, Table 2). Among them, red pigment production of MP1, MP2, MP3, MP4, MP5, MP7, MP8, and MP9 were obviously enhanced as compared with the wild strain when cultured at 14°C (Fig. 4a, b). For the mutants and wild-type, the red pigment production reached about a vertex after 10 days of liquid fermentation and then began to fall down. The MP1 achieved the highest level red pigment (OD_{520} of 39), which increased by 40% as compared to the wild-type strain. Some

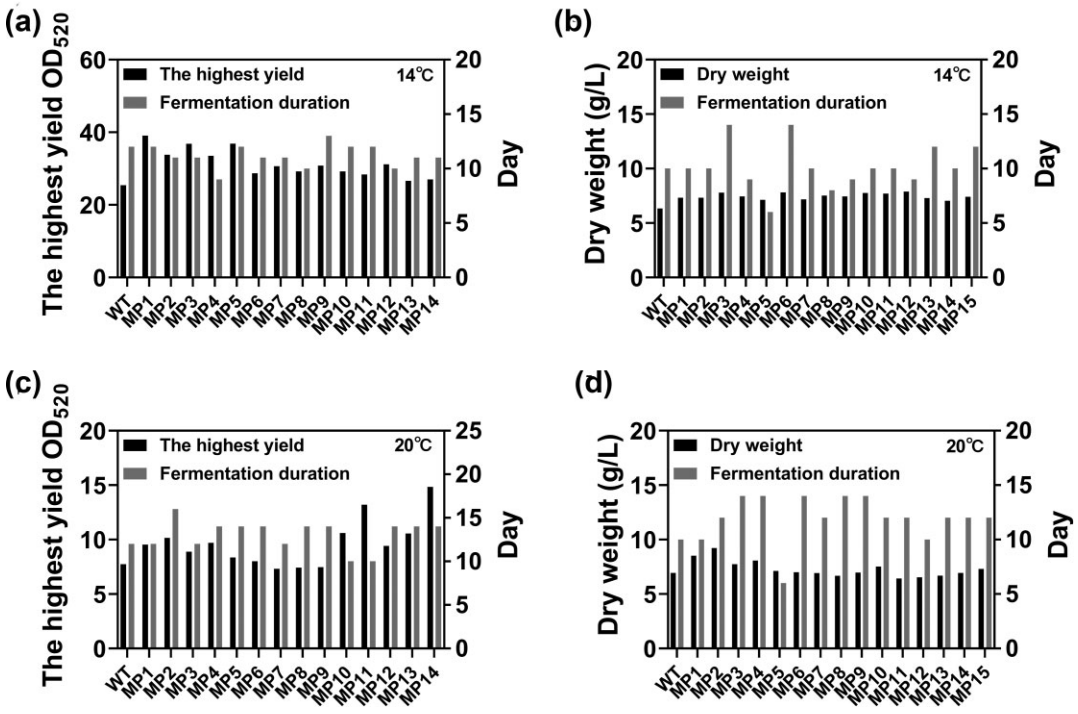

Fig. 4 Liquid culture of mutants for red pigment production in shake flask. (**a**) The highest red pigment yield and culture time of mutant strains at 14°C. (**b**) The maximum cell growth and culture time of mutant strains at 14°C. (**c**) The highest red pigment yield and culture time of mutant strains at 20°C. (**d**) The maximum cell growth and culture time of mutant strains at 20°C. Red pigment production was presented as absorbance at OD_{520}.

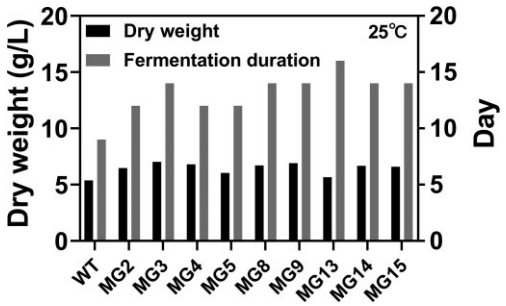

Fig. 5 Liquid culture of mutants for mycelial growth in shake flask. WT, wild type. Mutants of MG2, MG3, MG4, MG5, MG8, MG9, MG13, MG14, and MG15 were analyzed.

Table 3 The Efficiency of Three Transposons in *Geomyces* sp. WNF-15A

Transposon	Helitron	Impala	Fot1
Total number of transformants	1863	1629	1687
Total number of transposition mutants	223	154	86
Transposition efficiency (%)	11.9	9.4	4.6
Total number of positive mutants in red pigment[a]	8	2	3
Positive mutation rate in red pigment (%)	3.6	1.3	3.5
Total number of positive mutants in growth[b]	13	2	1
Positive mutation rate in growth (%)	5.8	1.3	1.2

[a]The mutants with variation percentage over 10% in red pigment production were considered as positive mutants, including MP1, MP2, MP3, MP4, MP5, MP7, MP8, MP9, MP10, MP11, MP12, MP13, and MP14.
[b]The mutants with variation percentage over 15% in cell growth were considered as positive mutants, including MP1, MP2, MP3, MP4, MP6, MP7, MP8, MP9, MP12, MG2, MG3, MG4, MG8, MG9, MG14, and MG15.

mutants such as MP1, MP2, MP3, MP6, MP7, MP8, MP9, and MP12 also showed better cell growth than the wild-type at 14°C.

We then investigated the shake flask fermentation of these mutants at normal temperature (Fig. 4c–d). The red pigment production of the 14 mutants decreased by 45–77% at 20°C compared to 14°C. The MP14 achieved the highest level of red pigment (OD_{520} of 14.8), which reached about twofold of the wild-type strain. The MP1, MP2, MP4, and wild-type strain grew better at 20°C than 14°C. When cultured at 25°C, most of the mutants showed no obvious synthesis of red pigment just as the wild-type, except that the culture medium color of MP2 and MP10 turned to red when cultured at room temperature. The red pigment production in liquid culture (OD_{520} of 2.2 for MP2 and OD_{520} of 3.2 for MP10) also proved the effectiveness of the mutation (Supplementary Fig. S9).

We also evaluated the nine strains with growth advantage at 25°C selected from agar plate in liquid culture. All the mutants grew better than the wild-type except the MG13 (Fig. 5). Generally, the wild-type strain reached a vortex cell weight after 10-day culture and then turned to decrease, but the mutants kept growing for two to four more days as compared to the wild-type. However, none of these mutants produced red pigment at 25°C. After culture for 14 days, the highest dry weight of MG3 reached 7.03 g/L, which was 30% higher than the wild-type strain at 25°C (Supplementary Fig. S10). It also reached almost the same dry weight as that of the wild-type at 14°C.

The efficiency of three transposable systems was then summarized in Table 3. The insertion efficiency of *Helitron*, *Impala*, and *Fot1* reached 11.9%, 9.4%, and 4.6%, respectively in *Geomyces* sp. WNF-15A. To analyze the positive mutation efficiency, the mutants with variation percentage over 10% in red pigment production and over 15% in cell growth were recorded as positive mutants. Accordingly, the positive mutation rate in red pigment production was calculated as *Helitron* 3.6%, *Fot1* 1.3%, and *Impala* 3.5%; and the positive mutation rate in cell growth was calculated as *Helitron* 5.8%, *Fot1* 1.3%, and *Impala* 1.2%, respectively.

Our previous studies have shown that atmospheric and room temperature plasma (ARTP) is highly lethal to *Geomyces* sp. WNF-15A, with a fatality rate of up to 90% (Huang et al., 2020). We obtained a dominant mutant M210 using ARTP. Compared with the wild type, the red pigment production of M210 increased by 24.4% and the fermentation time was shortened by 33.3% when it was cultured at 14°C. Besides, red pigment production of M210 was increased by 292% when cultured at 20°C. Superficially, compared with the random physical mutagenesis of ARTP, the phenotypic advantage of the mutants obtained by transposon mutation is relatively weak. However, transposons can inactivate genes and cause changes in host phenotypes by random insertion, for which the mutant gene will be easily targeted by tracking transposon than random physical mutagenesis (Krishnan et al., 2018; Opijnen & Levin, 2020).

We summarized the reported transposition efficiency of the three transposons in the heterologous hosts (Table 4). *Impala* and *Fot1* transposons showed great transposable activity and efficiency in a variety of filamentous fungi. In these cases, the transposon element was inserted upstream of the nitrate reductase gene *niaD*. After the transposition, the nitrate reductase activity was restored so that the colony could grow on the medium containing nitrate. For *Geomyces* sp. WNF-15A, *Impala* and *Fot1* elements could be engineered into a dual plasmid system to improve transposition efficiency by hygromycin resistance. Although transposition efficiency was relatively low, it is more suitable for the wild-type strains from various habitats. Carr et al., (2010) demonstrated that the transposition of *Impala* could be induced by incubation at low temperatures, and the low temperatures clearly enhanced the transcript level of *Impala* transposase. High concentrations of copper sulfate (Ikeda et al., 2001), nutritional restriction (Morillon et al., 2000; Naas et al., 1995), trace antibiotics (Tomich et al., 1980), and cold or heat shock (Hey et al., 2008, Kretschmer & Cohen, 1979, Pfeifer & Blaseio, 1990) can also enhance the transposition. *Helitron* is a DNA transposon isolated from the *Arabidopsis* genome and widely exists in eukaryotes. There are several genes encoding *Helitron* transposase specific domains in *Fusarium oxysporum*, which can also transpose in *Fusarium graminearum*, but the transposition efficiency has not been clearly demonstrated (Wang et al., 2018). Generally, the transposon activity varies greatly among different hosts.

After over 10 years of development, transposon insertion mutation has been applied in many fields and successfully revealed much biological information, including gene function, pathogenic mechanism, drug targets, etc. Most of these studies focused on the bacteria field. Compared with fungi, the relatively small and dense genome of bacteria has more advantages in transposon identification and insertion site sequencing. Due to the few available transposons and the low activity of bacteria transposons in fungi, it is challenging to involve transposon insertion mutations in filamentous fungi (Opijnen & Levin, 2020). The unique physiological characteristics of polar microorganisms allow them as the potential source of novel and active natural products. However, the unknown genetic background and the lack of suitable genetic tools severely limited the research and application of these fungi (Díaz et al., 2019). In this study, we developed a stable genetic manipulation system for the polar filamentous fungus *Geomyces* sp.WNF-15A. Also, the activities of three exogenous transposons were tested and confirmed in this fungus, which provided a reference

Table 4 The Transposition Efficiency of Three Transposons in Other Filamentous Fungi

Transposon	Transposition efficiency[a] (%)	Host	Reference
Impala	25–54	*Fusarium oxysporum*	Hua-Van et al., 2001
	1.2	*Aspergillus fumigatus*	Firon et al., 2003
	90	*Aspergillus nidulans*	Nicosia et al., 2001
	77	*Magnaporthe grisea*	Villalba et al., 2001
	70	*Penicillium griseoroseum*	Queiroz & Daboussi, 2003
	80	*Fusarium moniliforme*	Hua-Van et al., 2001
	70	*Fusarium oxysporum f. sp. melonis.*	Migheli et al., 2000
	11.9	*Geomyces* sp. WNF-15A	This study
Fot1	70–75	*Fusarium oxysporum*	Migheli et al., 1999
	50	*Aspergillus nidulans*	Nicosia et al., 2001
	4.6	*Geomyces* sp. WNF-15A	This study
Helitron	N.A.	*Fusarium graminearum*	Wang et al., 2018
	11.9	*Geomyces* sp. WNF-15A	This study

[a]Transposition efficiency refers to the proportion of strains with correct transposable elements to all transformants. Selective marker gene was hygromycin B resistance gene *hyg* in this study, but nitrate reductase gene *niaD* in other reported studies.

for the mutation breeding of filamentous fungi based on transposon technology. The obtained mutants adapted to room temperature also laid a foundation for studying the low-temperature regulation mechanism of cold-adapted microorganisms.

Conclusions

Genetic manipulation system for polar psychrotrophic fungus *Geomyces* sp. WNF-15A was developed with ~50% transformation efficiency. Transposon insertion mutation methods were then established using *Helitron*, *Fot1*, and *Impala* transposons in this fungus. Mutants with cell growth and red pigment biosynthesis adapted to normal temperature were obtained and analyzed, proving the efficiency of the transposable systems. It provided a new strategy for the mutation breeding and industrial application of *Geomyces* sp.WNF-15A.

Supplementary Material

Supplementary material is available online at *JIMB* (*www.academic.oup.com/jimb*).

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Author Contributions

M. Cai conceived the project. L. Ding, H. Huang, and F. Lu conducted the experiments. J. Lu participated in the fermentation experiments. L. Ding and M. Cai analyzed the data and drafted the manuscript. Y. Zhang and X. Zhou reviewed the manuscript.

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Conflict of Interest

The authors declare that they do not have any commercial or associative interest to this work.

Data Availability

There is no data for submission in a public repository. Experimental materials and datasets for the current study are available from the corresponding author on reasonable request.

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