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shRNAs Silencing DsRed Reporter in the Filamentous Fungus *Trichoderma reesei*

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ABSTRACT

RNA interference (RNAi) has been reported as an important process of down-regulation in post-regulation existed in plant, mammalian and fungi. In order to evaluate silencing efficiency of siRNAs on the DsRed targeted gene, a plasmid expressing siRNA was constructed and transformed into filamentous fungi DsRed-*T. reesei* strain. Worked as we developed RNAi silencing approach with DsRed as reporter in the *Trichoderma reesei* QM 9414. First, pANRed1 plasmids were transformed to *Trichoderma reesei* QM 9414 generating DsRed-*T. reesei* strain which are steadily expressing DsRed protein on PDA plates containing 100 μg/mL hygromycin B. Second, the pyruvate decarboxylase promoter (1534bp) and the cellobiohydrolase I terminator (702bp) were amplified from DNA of *Trichoderma reesei* 9414. The two fragments were respectively inserted between the PstI and SalI sites and between the BamH I and EcoR I sites of the plasmid pPHL generating a plasmid pPHL-Ppdc-Tcbh1. The fragments coding short hairpin RNAs targeting on DsRed specific sites were cloned into plasmid pPHL-Ppdc-Tcbh1 generating a plasmid pPHL-Ppdc-shRNAs-Tcbh1, and the vector expressing a random sequence was constructed as control. They were transformed to the DsRed-*T. reesei* strain generating the transformants those could be selected on PDA plates containing 100 μg/mL hygromycin B and 250 μg/mL phleomycin for 2–3 days. The results indicated that approximately 79% of transformants displayed a decrease in DsRed fluorescence. The DsRed expression in some transformants appeared to be fully suppressed. Characterization of ORF to genomic DNA was verified by PCR to randomly selected transformants. Real-time PCR quantification and Western blot analysis confirmed down-regulation of DsRed gene expression at different levels. The results demonstrate that RNAi exists in *T. reesei* and this technique is able to apply to investigation of gene expression regulation.

INTRODUCTION

Trichoderma reesei (Hypocrea jecorina) is a filamentous fungus that plays an important role in biodegradation and is widely used for the production of important hydrolytic enzymes. (Wilson, 2009). Most of those are cellulase and the major cellulase cellobiohydrolase I (CBHI), and forms about 64–80% of the extracellular proteins (Meyer et al. 2008; Schuster *et al.* 2010). In addition to its enormous secretion potential, *T. reesei* is able to glycosylate protein and has the function of post-translational modification. Therefore, *T. reesei* was considered to be an attractive expression host for recombinant protein production and expression. Currently, the yield of cellulase in *T. reesei* has been remarkably improved by random mutagenesis, and it is hard to further improve the yield of cellulose by this method. With publication of the genome sequence of *T. reesei*, it has become possible to introduce cutting edge DNA manipulation methods on the strain to improve cellulase production; (Martinez *et al.,* 2008). Therefore, developing an efficient molecular tool is a feasible way to further study the mechanisms of cellulase or hemicellulase gene regulation and improve protein production in *T. reesei*.

RNA interference (RNAi) is an efficient tool for knocking down the expression of a target gene in cells, yeast and fungi (Fire *et al.* 1998, Verdel *et al.* 2004; Simmer 2010, Cogoni 1999 ;). It has also been used to simultaneously silence homologous or even heterologous genes, by targeting a conserved sequence of a gene family or constructing a chimeric sequence derived from different genes (Salame *et al*. 2011,). The RNA interfering system with GFP or DeRed as reporter has been developed and reported in filamentous fungi (Wang *et al.* 2013, He *et al.* 2015). The first type of siRNAs found in fungi was siRNAs produced to silence exogenous sequences that are homologous to an endogenous gene (Cogoni et al. 1999). While it was reported to silence genes with synthetic exogenous dsRNAs or endogenously transcribed dsRNAs in various fungi effectively (Salame *et al.* 2011). In addition, dsRNAs were transcribed with an inducible promoter allows the study of gene expression at a specific stage of development (Barton and Prade 2008). Filamentous fungi commonly consist of multicellular or multinuclear hyphae and generally exhibit low gene targeting efficiency. Therefore, RNAi provides a flexible and versatile tool for gene knockdown in filamentous fungi, including *Trichoderma reesei* (Kiiskinen, 2004; Wang and Xia, 2011). To confirm the functions of fungal genes, or to improve the properties of industrial fungi, various genetic approaches have been used to disrupt target genes by

homologous recombination (Meyer 2008; Larrondo *et al.* 2009). However, in many types of fungi, the efficiency of homologous recombination is relatively low, varying from 0–40 % (Ruiz-Diez 2002; Michielse *et al.* 2005) because of the ectopic integration of the transformed DNA. The isolation of a gene-specific deletion can be an arduous undertaking. Therefore, development of alternative tools for genetic manipulation in filamentous fungi, such as RNAi, is necessary.

We have previously reported the RNAi silencing system in the filamentous fungi *Trichoderma koningii* YC01. The system mainly consisted of an expression cassette for an intron-harboring hairpin RNA (ihpRNA) under the control of the *T. reesei* gpd gene promoter and cbh1 terminator (Wang *et al.* 2013). It contains two 430-bp DsRed fragments and a 209-bp spacer fragment including intron 2 of the *T. reesei egl*2 gene. These three fragments were ligated by overlap PCR, and the overlap-connected fragment was inserted into plasmid pPHL, generating plasmid pPHL-Redi. In this paper an efficient siRNAs derived from vector were applied in the industrially important filamentous fungi *Trichoderma reesei* QM9414 using the DsRed as a reporter. Our purpose is to confirm that 19 nt siRNAs derived from the vector are able to silence DsRed expression by targeting the specific sequence of DsRed in the DsRed-*T. reesei* strain.

HUMAN

MATERIALS AND METHODS

Strains and plasmids

Escherichia coli DH5a (supE44 ΔlacU169 (F80 lacZ ΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1) was used to propagate plasmids. *Escherichia coli* Top10F′ (Invitrogen) was used for plasmid construction and maintenance, and it was cultured in Luria–Bertani medium, with ampicillin (100 μg/mL) supplementation as necessary. The plasmid pANRed1 was generated from pAN7-1 (GenBank accession, Z32698), and it was reported by our group previously (Wang *et al.* 2013). The plasmid of pUC-19 (GenBank accession, M77789) was used for generating a recombinant plasmid pPHL. T. reesei QM9414 (ATCC 26921) was transformed with pAN-Red1 generating a transformant DsRed-*T. reesei* strain. The strain was grown for 5–6 days at 28^{0}C on potato dextrose agar plates (PDA) or PDA supplemented with 5 mM uridine when necessary. Minimal medium plus 100 lg/mL hygromycin B (Rothe, USA) was used as selective medium for screening of RNAi transformants.

Construction of RNAi vector

To construct the RNAi vectors, the expression cassette of phleomycin (PHL) resistance （2784bp） was amplified by PCR with primers of PHL-F and PHL-R in Tab.1 from template of the pPHL-Redi plasmid and the expression cassette was digested with *Hind*III and *Pst*I and then inserted into pUC-19 vector generating recombinant pPHL (W). The pyruvate decarboxylase promoter Ppdc (1534bp) was amplified by PCR from *T. reesei* DNA template with primers of Ppdc-F and Ppdc-R in Tab.1 and inserted between the *Pst*I and *Sal*I site in the plasmid pPHL generating a plasmid pPHL-Ppdc. The cellobiohydrolase I terminator Tcbh1 (702bp) was amplified by PCR from *T. reesei* DNA template with primers of Tcbh1-F and Tcbh1-R in Tab.1 and inserted between the *Bam*H I and *Eco*R I sites of pPHL-Ppdc generating a plasmid pPHL-Ppdc-Tcbh1. According to the *DsRed* sequence, the mRNA sequence of *DsRed* was input the Applied Biosystems Website to design siRNAs, we selected three siRNAs and they were designated as *red*-T3, *red*-T12 and a negative control *red*-neg. A 59-bp siRNA nucleotide region encompasses a 19-nt sense of the siRNA targeting different position of *DsRed*, a 9-nt loop and 19-nt siRNA anti-oligonucleotide sequences with *Sal*I and *Bam*HI sites were synthesized by (Life technologies, China) listed in Tab.2. These paired oligonucleotides were annealed in the thermocycler at condition of 95℃, 2 min, decreasing 0.1℃ per minute until 25℃, approximately 90 min to finish and then stored at 4℃. The three fragments were inserted into the *Sal*I and *Bam*HI on the pPHL-Ppdc´-Tcbh1 vector generating the pPpdc´-T3-Tcbh1, pPpdc´-T12-Tcbh1 and pPpdc´-Neg-Tcbh1 respectively.

Table 1. Primers amplified for phleomycin gene, Ppdc and Tcbh1

Primer	Primer sequence	RE site
PHL-F	5'-CCCAAGCTTGACGCAGAAGAAGGAAATCGCC-3'	*Hind*III
		*Pst*I
PHL-R	5'-CACTGCAGGAGGCTACTCTCGGATTGCCGGC-3'	
Ppdc-F	5’-CCAATGCATAGGACTTCCAGGGCTACTTG-3’	*Nsi*I
Ppdc-R	5’-ACGCGTCGACGATTGTGCTGTAGCTGCGCT-3’	*Sal*I
Tcbh1-F	5'-CGCGGATCCAGCTCCGTGGCGAAAGCCT-3'	*Bam*HI
Tcbh1-R	5'-CCGGAATTCGAGGCTACTCTCGGATTGC-3'	*Eco*RI

Underlined are restriction endonuclease site.

Table 2. Sequences of siRNA targeting on *DsRed* gene

shRNA	Oligonucleotide sequences	Targeting position
DsRed-T3-F	5'-tcgacCGGCCACGAGTTCGAGATCTTCAAGAGAGATCTCGAACTCGTGGCCGtttttg-3'	67-86
DsRed-T3-R	5'-gatccaaaaaaCGGCCACGAGTTCGAGATCTCTCTTGAAGATCTCGAACTCGTGGCCGg-3'	
DsRed-T12-F	5'-tcgacGUGGGAGCGCGUGAUGAACTTCAAGAGAGUUCAUCACGCGCUCCCACtttttg-3'	274-193
DsRed-T12-R	5'-gatccaaaaaaGUGGGAGCGCGUGAUGAACTCTCTTGAAGUUCAUCACGCGCUCCCACg-3'	
DsRed-Neg-F	5'-tcgacGAACAAGACGGGCTTGGGCTTCAAGAGAGCCCAAGCCCGTCTTGTTCtttttg-3'	NA
DsRed-Neg-R	5'-gatccaaaaaaGAACAAGACGGGCTTGGGCTCTCTTGAAGCCCAAGCCCGTCTTGTTCg-3'	

Underlined are loops

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Fungal transformations and analysis of the transformants

Protoplast preparation and transformation of *T. reesei* were performed using the polyethylene glycol method as described previously (Penttilä *et al.* 1987) with the following modifications. Lysing enzymes (10 mg/mL) from *Trichoderma harzianum* (Sigma-Aldrich, Brondby, Denmark) in 1 mol/mL $MgSO_4$ was used for protoplast formation. For transformation, 20 µg of plasmid DNA (pANRed1 or pPpdc-shRNAs-Tcbh1) and 200 µL of PEG buffer (60 % PEG 4000, 50 mmol/mL $CaCl_2$, 10 mmol/mL Tris–HCl, pH 7.5) were used. Transformants were selected on PDA plates that contained 100 µg/mL hygromycin B (for pAN-Red1) or 100 µg/mL hygromycin B and 250 µg/mL phleomycin (for pPpdc-shRNAs-Tcbh1) for 2–3 days. The monoconidium of candidate transformants was picked up with toothpick and inoculated on fresh PDA selective plates for growth of another 2–3 days. The monoconidia of transformants were transferred to selective medium and their selection and growth was observed for six generations. DNAs of each strain for PCR identification were extracted to verify that the shRNA expression cassette was integrated into the genome of the transformants.

Nucleic acid isolation

Mycelia in liquid culture were harvested by centrifugation, frozen in liquid nitrogen, and stored at −80°C. Genomic DNA was extracted as described previously (Seiboth *et al.* 2004). Total RNA was isolated from the frozen mycelia with a Universal Plant Total RNA Extraction Kit (BioTeke, Beijing, China) using the manufacturer's protocol. To remove the genomic DNA, RNA preparations were treated with DNase I (Fermentas, Vilnius, Lithuania). The quantity and quality of the extracted RNA were assessed on a GeneQuant 1300 spectrophotometer (GE HealthCare, Uppsala, Sweden) and by agarose gel electrophoresis.

Reverse transcription and quantitative PCR

Approximately 500 ng of total RNA was subjected to reverse transcription using an RT-PCR kit (Takara Bio, Dalian, China), which contained a blend of oligo (dT) and random hexamer primers. Quantitative PCRs (qPCRs) were performed using an ABI Prism 7300 System (Applied Biosystems, Foster City, CA, USA). Each 20 µL reaction contained 2 µL of template (1:60 dilution of the reverse transcriptase (RT) reaction product), 10 µL of 2× SYBR Premix Ex Taq (Takara), 300 nmol/L forward and reverse primers (Table 1), and nuclease-free water. The PCR protocol consisted of 30 s of initial denaturation at 95°C, followed by 40 cycles of 5 s at 95°C and 31 s at 60°C. A melting curve was performed after each run to check the PCR product specificity. All PCRs were carried out in triplicate within a plate. The data obtained from the ABI Prism 7300 Sequence Detection System were analyzed according to the standard curve method for relative quantization protocol specified by the manufacturer. The expression level of DsRed in these strains was normalized with an endogenous control, actin1, and then divided by the quantity of the control strain. Thus, the control strain was designated as an expression level of 1, and all other quantities were expressed as a ratio relative to the control sample.

Imaging and measurement of DsRed fluorescence

The DsRed fluorescence of transformants was observed with the Olympus BX51TRF System (Tokyo, Japan). The DsRed fluorescence was excited at 554 nm and was detected using a U-25ND6 optical filter. The fluorescent images were recorded and the density of red color was analyzed with Image-Pro Plus 5.0 software (Media Cybernetics, Silver Springs, Maryland).

SDS-PAGE and Western blot analysis

About 1×10^5 spores of the transformant DsRed-*T. reesei* were inoculated into liquid medium and incubated with shaking 250 r/min at 28℃ for 72 hours. The sample was subjected to a grinding in a mortar with liquid nitrogen and then 1ml lysis buffer [40 mM Tris–HCl buffer pH 8.5 with protease inhibitor (Sigma)] was added with 3 min of vigorous vortexing and ice-bathed for 30 min and centrifuged with 12000 g at 4℃ for 2 min. The supernatant was transferred to fresh 1.5 ml Eppendorf tube. After protein extraction, protein concentration was quantified using the Bio-Rad Protein Assay method (Bio-Rad) and bovine serum albumin (BSA) as standard. Protein extract concentrations were determined using Bradford reagent (Sangon Biotech, Shanghai, China). Sixty micrograms of each protein extract was separated by SDS-PAGE and blotted onto a nitrocellulose membrane. Western blotting of the DsRed gene product was performed using a DsRed monoclonal antibody (Abbkine, CA, USA) using the protocol specified by the manufacturer. The second antibody Goat Anti-Mouse IgG-HRP (Abbkine, CA, USA) Anti-alpha Tubulin (Abcam, Shanghai,China)

Data analysis

Measurements from individual sample were carried out in triplicate. Results are expressed as the mean ± SD (standard deviation) of three independent experiments analyzed using the one-way ANOVA followed by the paired sample t-test. In all cases, p values <0.05 were considered statistically significant.

HUMAN

RESULTS AND DISCUSSION

Selection of a recombinant DsRed-*T.reesei* strain steadily expressing DsRed fluorescence

The plasmid pAN-Red1 was constructed and transferred into *T. reesei* QM 9414 generating the transformants growing on PDA plates that contained 100 μg/mL hygromycin B (for pAN-Red1) for 2-3 days. In order to obtain a single colony of transformant, a single-spore suspension by serial dilution of spore suspensions was further spread onto the selection plates to form single colonies. The strain was subcultured even after at least eight rounds on the selected medium and then it was named DsRed-*T.reesei* strain and used as a recipient for further study of siRNA silencing target DsRed reporter. This strain steadily exhibited strong red fluorescence under fluorescence microscope (Figure 1).

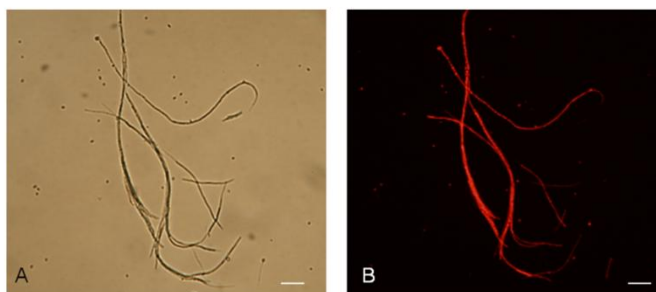

Figure 1 *T. reesei* QM9414 transformed by plasmid pAN-Red1 generating recombinant *DsRed-T.reesei.* strain

The images of the mycelia of recombinant strain *DsRed-T.reesei* under ordinary light (A) and fluorescent light (B) respectively. Bars indicate 10 μm (Magnification: 100×; exposed time under ordinary light: 2 msec; exposed time under fluorescent light: 50 msec).

Down-regulation of DsRed expression by RNAi in the *DsRed-T.reesei*

To observe the shRNA silencing DsRed expression in the *DsRed-T.reesei* strain, plasmid pPHL-Ppdc'-T3-Tcbh1, pPHL-Ppdc'-T12-Tcbh1 and pPHL-Ppdc'-Neg3-Tcbh1 were respectively co-transformed into the DsRed-*T.reesei* with pAN7-1, resulting in 38 transformants with both hygromycin B and phleomycin resistance including 23 DsRed-T3 transformants and 15 *DsRed*-T12 transformants. As a control, the plasmid pPHL-Ppdc'-Neg3-Tcbh1 carrying the phleomycin resistance cassette was transformed into the recombinant *DsRed-T.reesei* with pAN7-1 generating 17 DsRed-Neg transformants. The DsRed fluorescence of these transformants was measured following 3-day incubation in minimal medium by using fluorescence microscopy. Based on the intensity of DsRed fluorescence relative to that of the control strain, the transformants were categorized into three classes, and the proportion of each class to total transformants was measured (Tab.3). It was found that approximately 87% of the transformants silenced DsRed fluorescence in *DsRed*-T3 compared to the control strain, and thus were most severely silenced, while approximately 64% of the transformants showed silence of DsRed fluorescence in *DsRed*-T12 compared to the control strain. Whereas, 29.4% and 33.3% of the resulting drug-resistant transformants exhibited varying degrees of silencing (less than 80% DsRed fluorescence of the control strain) in DsRed-T3 and DsRed-T12.

Table 3. Silenced DsRed Transformants of DsRed-T.reesei after transformation with shRNAs

Transformants	Total	Strong silenced	Weak silenced	Not silenced	Silenced ratio (%)
*DsRed-T.reesei-*T3	23	13	7	3	86.96
*DsRed-T.reesei-*T12	15	4	5	6	64.0
*DsRed-T.reesei-*Neg	17	0	5	12	29.41

Fluorescence microscopy was carried out to observe red fluorescence of DsRed in the mycelia of transformants growing on PDA plates. The images showed that shRNAs derived from the pPHL-Ppdc'-shRNA-Tcbh1 plasmids mediated interfere and silence DsRed gene expression in DsRed-T3-11, DsRed-T3-19, DsRed-T3-12-4 and DsRed-T12-8 transformants efficiently (Fig.2).

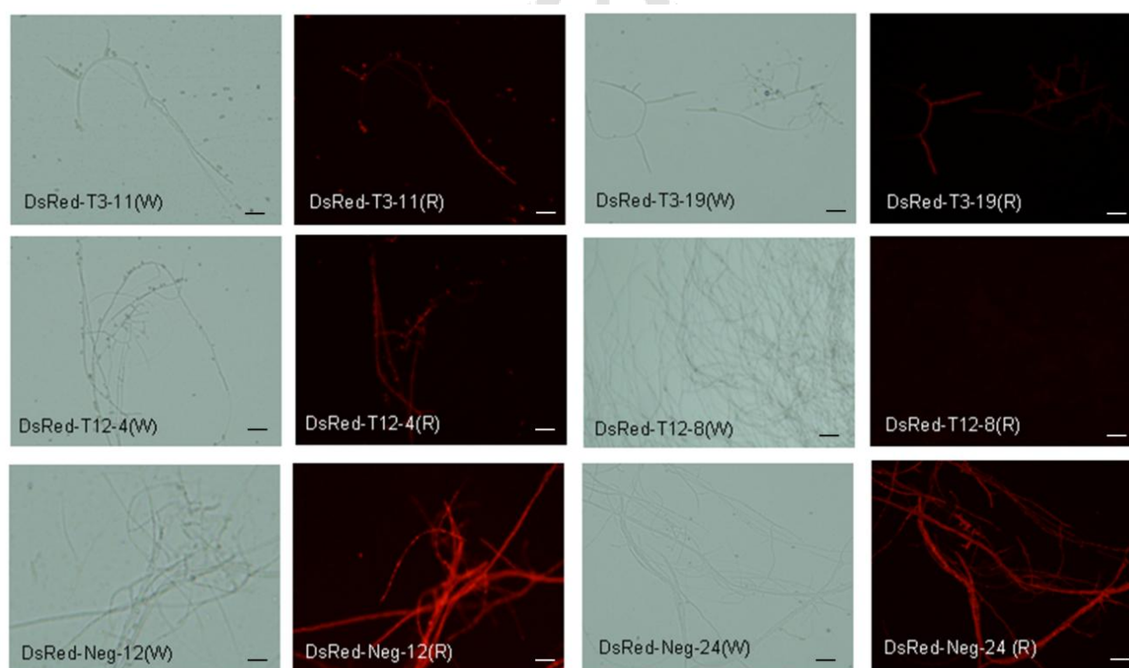

Figure 2 Silencing efficiency of DsRed by siRNAs in recombinant *DsRed-T.reesei*

W: First and third rows indicate image of the mycelia in bright field; R: Second and fourth rows indicate image of the mycelia in DsRed fluorescence. Bars indicate 10 μm. The first row

(top to bottom): *DsRed*-T3-11(W), *DsRed*-T12-4(W), *DsRed*-Neg-12(W); The second row (top to bottom): *DsRed*-T3-11(R), *DsRed*-T12-4(R), *DsRed*-Neg-12(R); The third row (top to bottom): *DsRed*-T3-19(W), *DsRed*-T12-8(W), *DsRed*-Neg-24(W); The fourth row (top to bottom): *DsRed*-T3-19(R), *DsRed*-T12-8(R), *DsRed*-Neg-24(R).

Analysis of the integrity of *DsRed* ORF and siRNA expression cassette

The results mentioned above indicated that siRNA was able to silence DsRed fluorescence in *T. reesei* effectively. To exclude the possibility that the recipient strain, or alternatively the transformation procedure itself, was responsible for the results observed, we conducted the experiments to confirm the integrity of DsRed gene and the integrity of the siRNA expression cassette after homologous recombination in DsRed-*T. reesei* with cotransformation with pAN-Red1 and pPHL-Ppdc'-shRNAs-Tcbh1. PCR was carried out to amplify the *DsRed* ORF with primers (Tab.4) from the DNAs extracted from *DsRed*-T3-11, *DsRed*-T3-19, *DsRed*-T12-4, *DsRed*-T12-8 and negative control *DsRed*-Neg-12 and *DsRed*-Neg-24 respectively. The results indicated that the 678 bp DNA fragments of *DsRed* ORF was amplified from the six DNA templates, suggesting the *DsRed* ORF is integrity after transformation (Fig.3A) and the siRNA expression cassette was also amplified with primers listed in Tab.4. The results indicated the siRNA expression cassettes are integrity（754 bp (Fig.3B). These results suggest that the intensity of the DsRed gene in transformants with siRNAs was due to the results of siRNA silencing.

Table 4. Primers used in identifying for integration of shRNA expression cassette

Primer name	Primer sequence	Size (bp)
T3-YZ-F	5'-GCCACGAGTTCGAGATCTTCAAGAG-3'	754
T12-YZ-F	5'-GGGAGCGCGTGATGAACTTCAAGAG-3'	754
Neg-YZ-F	5'-ACAAGACGGGCTTGGGCTTCAAGAGAG-3'	754
Tcbh1-R	5'-CCGGAATTCGAGGCTACTCTCGGATTGC-3'	

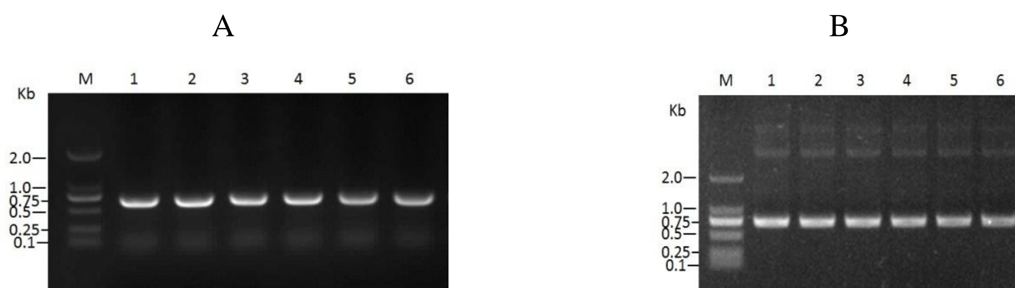

Fig. 3 Electrophoretic analysis of DsRed ORF and shRNA expression cassette for recombinant strains

Panel A: Electrophoresis of amplified fragments of DsRed ORF, Panel B: Panel A: Electrophoresis of amplified fragments of the shRNA expression cassette, M：DL2000bp DNA Marker；1~6: The recombinant strains DsRed-T3-11, DsRed-T3-19, DsRed-T12-4, DsRed-T12-8, DsRed-Neg-12, DsRed-Neg-24 from left to right respectively.

The integrity of the shRNA expression cassette was also examined in the genomic DNA of silenced transformants. PCR amplification and the DNA sequencing results showed that the shRNA expression cassette remained intact in the genomic DNA of silenced transformants (data not shown), eliminating the possibility that DsRed silencing in the silenced transformants was caused by genetic changes during the siRNA expression cassette transformation

Quantitative real-time PCR analysis of down-regulation of DsRed gene

For further to confirm and to characterize down-regulation of mRNA expression of the DsRed gene in transformants, quantitative real-time PCR was performed to detect mRNA expression level of the DsRed gene. The results showed a significant reduction of 67.83%，99.89%, 99.8% and 100% in the expression level of DsRed in DsRed-T3-11, DsRed-T3-19, DsRed-T12-4 and DsRed-T12-8 transformants compared to that of the original DsRed-*T. ressei* strain. The other two DsRed-Neg-12 and DsRed-Neg-24 transformants did not show mRNA level reduction in the expression of DsRed gene (Fig. 4).

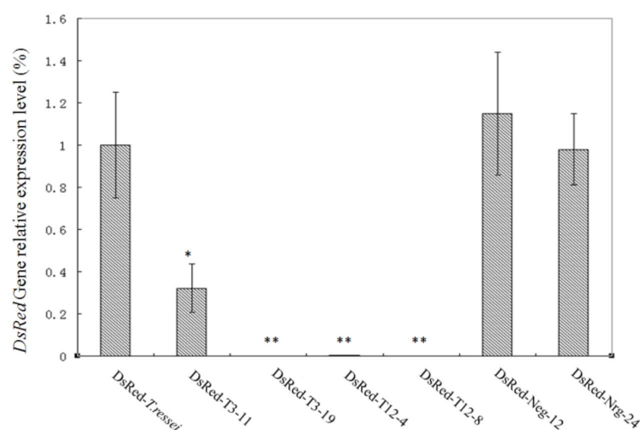

Fig. 4 Q-RT-PCR detection of mRNA expression level of DsRed in recombinant after incubation for 48h.

DsRed-*T. ressei*: relative expression level of DsRed mRNA of parent strain DsRed-*T. reesei*; DsRed-T3-11 and DsRed-T3-19: relative expression level of DsRed mRNA of recombinant strains containing disrupt sequence T3; DsRed-T12-4 and DsRed-T12-8: relative expression level of DsRed mRNA of recombinant strains containing disrupt sequence T12; DsRed-Neg-12 and DsRed-Neg-24: relative expression level of DsRed mRNA of recombinant strains containing negative control sequence. Error bars represent standard deviations (X±SD, n=3, *p< 0.05, ** p< 0.01).

The mRNA expression level of the DsRed gene in transformants DsRed-T3-11, DsRed-T3-19 and parent strain DsRed-T.reesei were measured by Quantitative real-time PCR. As shown in Fig.5, the expression levels of DsRed mRNA in DsRed-T3-11 and DsRed-T3-19 transformants were respectively decreased to 33%, 30% and 32% and 0.2%, 0 and 0.01% compared to parental strain at 48, 72 and 96 h. The results suggest that silencing effects and mRNA expression level of the DsRed gene in DsRed-T3-11, DsRed-T3-19 transformant are very stable.

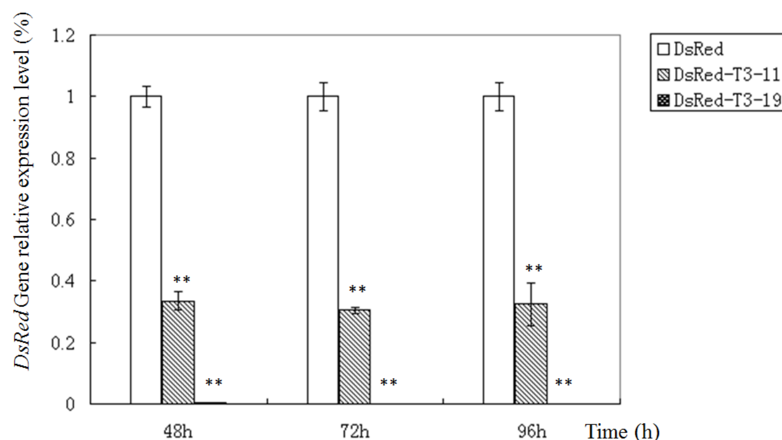

Fig. 5 Q-RT-PCR detection of mRNA expression level of DsRed gene in transformants after incubation for 48, 72 and 96 h respectively.

DsRed: DsRed mRNA level of parental strain DsRed-*T. reesei* cultured for 48 h, 72 h and 96 h; DsRed-T3-11: DsRed mRNA level of the recombinant strain DsRed-T3-11 cultured for 48 h, 72 h and 96 h; DsRed-T3-19: *DsRed* mRNA level of the recombinant strain DsRed-T3-19 cultured for 48 h, 72 h and 96 h. Error bars represent standard deviations (X±SD, n=3, *p< 0.05, ** p< 0.01).

Western blot analysis of DsRed-silenced transformants

To further correlate the levels of mRNA and protein synthesis in silenced transformants, Western blot analyses was performed. The protein samples were extracted from the transformant DsRed-*T. reesei* strain, the transformant DsRed-neg-12 and the transformant DsRed-T3-19 and as well as *T. reesei* QM 9414. As shown in Fig. 6, the intensity of the Western blot signals from the transformant DsRed-*T. reesei* strain, the transformant DsRed-neg-12 were significantly obtained and as expected, there was no the signals bands obtained from the *T. reesei* 9414 strain and the transformant DsRed-neg-12. The Western blot analysis for the DsRed-*T. reesei* parental strain, the transformant DsRed-neg-12 and the transformant DsRed-T3-19 was correlated perfectly with the results of Quantitative real-time PCR (Fig.4). The result indicate that RNAi exites in T. reesei and siRNAs are able to silence target gene efficiently.

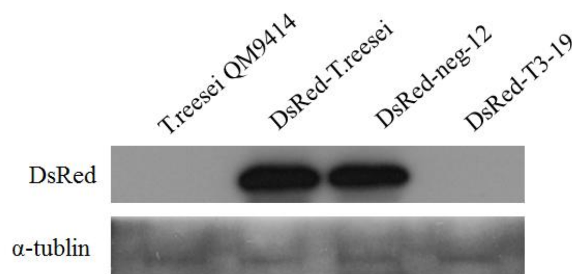

Fig. 6 Western blot assay of DsRed in recombinant DsRed-T3-19 silence and controls for 72h respectively.

T. reesei QM9414: Host cell; The transformant DsRed-*T. reesei* with DsRed is cultured for 72h; The transformant DsRed-neg-12 with negative shRNA is cultured for 72 h; The transformant DsRed-T3-19 with effective shRNA is cultured for 72 h.

DISCUSSION

RNA silencing systems have been developed previously in different species using the green fluorescent protein (GFP) as reporter, and screening of the silenced strains was performed by fluorescent microscopy or fluorescence plate reading (Nakayashiki et al. 2005; Kück *et al.* 2010). Although the GFP was used successfully in various microorganisms and fungi (Armesto *et al*.2012; Kalleda *et al*. 2013; Yang *et al*.2014), in all these cases, the host cells carried a gfp gene and showed a green fluorescence when observed by fluorescence microscopy. However, the parent strain of *T. koningii* frequently shows some degree of endogenous fluorescence at the wavelength of GFP, producing false-positive transformants (Wang *et al.* 2013). DsRed protein as an alternative reporter has been expressed in ascomycete fungi species *Penicillium paxilli*, *T. harzianum,* and *Trichoderma virens* and used to monitor the silencing process in *Acremonium chrysogenum* (Mikkelsen *et al*. 2003; Janus et al. 2007; Wang *et al.* 2013). But it has not been reported in *Trichoderma reesei*. To investigate function of siRNAs derived by vectors in *Trichoderma reesei*, an efficient RNA interference (RNAi) system has been established by using the DsRed protein as a reporter, and the results showed a stable silencing in the industrially important filamentous fungus *T. koningii* (Wang *et al,* 2013). Although it was able to silence target gene effectively, but the vector carry-over ihp-RNA is too long to construct vector harboring multiple different targeted shRNAs to silence target genes simultaneously. In this study, the pPHL-Ppdc'-shRNA-Tcbh1 plasmids were transformed into the DsRed-*T. reesei* strain, we reported a

plasmid pAN-Red1 containing DsRed gene under the control of the pki1 (pyruvate kinase-encoding) gene promoter and cbh2 terminator of *T. reesei* QM 9414, whereas the siRNA expression cassette in the pPHL-Ppdc'-shRNA-Tcbh1 plasmid is under control of the pyruvate decarboxylase promoter Ppdc and cbh1 terminator. Our results indicated both ORF of DsRed and the siRNA expression cassette are amplified by PCR. These results suggest that the integrity of DsRed gene and the siRNA expression cassette is holonomic (complete) after homologous recombination. In this work, we constructed the plasmid with Ppdc' promoter, cbh1 terminator and baring the shRNA. This plasmid is more efficient than previous results obtained with hairpin RNA-expressing plasmid in filamentous fungi (Kadotani *et al*. 2003; Nakayashiki *et al.* 2005; Janus *et al.* 2007).

Hypha with red fluorescence was clearly observed under fluorescence microscopy and DsRed provided a powerful marker for identifying silenced fungal transformants compared to that of control strain (Fig.2). The results suggest that shRNA derived from the plasmid pPHL-Ppdc'-shRNA-Tcbh1 can efficiently silence target gene expression and DsRed reporter provides a powerful marker for identifying silenced fungal transformants. Our results show that the silencing ratios are 72.67% in the transformants of *DsRed*-T3 and 53.13% in the transformants of *DsRed*-T12 respectively. The different silencing effects should be reason as following: First, target position will affect the silencing effects; Second, nonhomologous recombination take place in the transformants. Hoff *et al* reported that a high frequency of nonhomologous recombination took place in *A. chrysogenum*, and therefore, generate different knockdown strains (Hoff *et al.* 2010, Schmitt *et al.* 2004; Walz *et al.* 1993); Third, a different copy of homologous recombination may take place in recombinants. Young *et al* reported that pAN7-1 was inserted at a single site and was present as 4-6 copies arranged in a head-to-tail tandem array (Young *et al.* 1998; Nizam *et al.* 2010). Fourth, in filamentous fungi, RdRP (RNA-dependent RNA polymerase) is essential for the synthesis and amplification of sequence-specific siRNAs, providing the organism with a continuous source of siRNA. Such amplification could serve as one explanation for the potency and longevity of the gene silencing observed (Nicolas *et al*. 2010; Sijen *et al.* 2001; Makeyev *et al*. 2002). A BLAST search of the *T. reesei* genome using the Neurospora crassa qde-1 gene product as the query sequence revealed a putative RdRP with an amino acid identity of approximately 55%.

The sequence specificity and efficiency of the RNAi process and the convenience of the DsRed reporter, along with advances in proteomic and microarray analyses, have spurred the development of novel high-throughput approaches in fungal as well as numerous other biological systems (Neumann *et al*. 2006; Walter *et al.* 2010; Wheeler *et al*. 2005).

The efficient silencing of the DsRed reporter gene in *T. reesei*, coupled with high-throughput expression screening, promises to accelerate our understanding of fungal processes, permitting increasingly rapid targeted genetic manipulation and the continued optimization of current and future industrially utilized fungi. Our data clearly show that the RNA silencing approach described here for *T. ressei* is effective, and the DsRed reporter gene provides a convenient tool for identification of silenced fungal transformants by their DsRed fluorescence compared to the control strain. This system offers the possibility of generating multiple-knockdown strains in *T. ressei*, allowing studies of the functions of genes involved in a common biosynthetic pathway, and was used for simultaneous silencing of multi-targeted genes in *T. ressei*. It was recently suggested that in order to generate a fungal RNAi hairpin vector library, the Gateway (Invitrogen) in vitro recombination technology could be used for high-throughput cloning (Weld *et al*. 2006). The method developed in this study may make it possible to conduct siRNAs derived from vector silencing multi-target genes in metabolism pathway, so it is able to be used to silence multiple target genes simultaneously. In order to silence multiple target genes simultaneously, short hairpin RNAs (shRNA) should be selected and to be inserted into the vector.

CONCLUSION

In this work, the results demonstrate the siRNA derived from vector using to knock down DsRed gene expression in DsRed-*T. reesei* strain is effective, and the DsRed reporter provides a convenient tool for identifying silenced fungal transformants. The method here is able to apply to investigation of homologous or heterologous expression through down-regulation of some control protein expression of the host strain.

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