e-ISSN:2320-3528
p-ISSN:2347-2286

Research & Reviews: Journal of Microbiology and Biotechnology

High Efficiency and Stable RNA Interference Vector Construction for *Penicillium* sp.

Haizhao Xue[1], Yibo Hu[1], Xiaoran Fang[1], Guangshan Yao[1], Xin Song[1,2]*

[1]State Key Laboratory of Microbial Technology, School of Life Science, Shandong University, Shan Da Nan Road 27, Jinan 250100, China

[2]National Glycoengineering Research Center, Shandong University, Shan Da Nan Road 27, Jinan 250100, Shandong, People's Republic of China

Research Article

Received date: 30/11/2016
Accepted date: 28/06/2016
Published date: 06/07/2016

***For Correspondence**

Xin Song, State Key Laboratory of Microbial Technology, School of Life Science, Shandong University, 27 Shandanan Road, Jinan, Shandong 250100, P. R. China, Tel: +86 531 88565610

E-mail: songx@sdu.edu.cn

Keywords: RNA interference, *Penicillium* oxalicum; filamentous fungi; Amylase, *Amy15A*, Plasmid, PHX-RNAi

ABSTRACT

RNA interference (RNAi), which can turn down gene expression effectively and specifically, has showed great potential in gene function research and gene modification. In this study, a RNA interference vector pHX-RNAi, which carries a hygromycin resistance cassette and a transcriptional unit for hairpin RNA expression was constructed. A gene *amy15A* which encodes the major amylase in a high cellulase producer strain *Penicillium oxalicum* A11 was targeted knocking-down. The results of SDS-PAGE and RT-qPCR showed that both the transcription and secretion levels of *Amy15A* were dramatically down-regulated, which implying that RNAi play a significant role in regulation of gene expression post-transcriptionally. All of the hygromycin-resistant transformants with the silencing constructs showed high degree of silencing in expression of *amy15A*. Considering the conservatism of RNAi machinery in fungi, the RNAi vector established in this study- pHX-RNAi could be applied to other filamentous fungi.

INTRODUCTION

RNA interference (RNAi) is a post-transcriptional gene silencing mechanism that widely exists in eukaryotes. RNAi is initiated by double-stranded RNA (dsRNA) or microRNA (miRNA) and degrades specific messenger RNAs (mRNAs) by RNA-induced silencing complex (RISC), and then it specifically impairs the expression of target genes [1,2]. Therefore, RNAi has been becoming an effective tool to explore gene function **(Figure 1).**

RNAi has been a remarkable technique in the study of animal and plant species [3,4]. However, this gene silencing tool has not been widely used in filamentous fungi. Generally, investigation of gene function is achieved by gene deletion in fungi. However, the traditional gene knock-out does not work well when polykaryocytosis and non-homologous recombination usually occur in filamentous fungi. Especially, some functional genes are essential for cell vitality, thus their deletion is lethal. Compared to gene knock-out which are relatively complicated and time consuming, RNAi is more suitable in studying those significant but lethal genes. DsRNA is necessary for targeting gene by RNAi. There are two strategies to generate dsRNA: one is to synthesize dsRNA *in vitro* and transfer it into the cell directly [5,6]; and the other is to introduce a sequence which can express a dsRNA or hairpin structure mRNA against the target genes into the genome [7,8]. The first one has been used in mammalian cell frequently, but it is not applied widely in fungi due to the instability of RNA interference [9]. Compared to the first one, the second strategy is more applicable in fungi because of the high transformation efficiency and sustained formation of dsRNA. An expression vector pSilent-1 has been reported to be applied in Aspergillus and some interference effects have been achieved for green fluorescent gene *egfp* and other genes [10]. Their study indicated RNAi could be a useful genetic modification tool in filamentous

fungi. However, pSilent-1 was unstable when it was applied in *Penicillium* sp. may due to its multi-copy functional elements (two copies of *trp*C promoter and terminator), which lead to self-recombination and functional elements elimination. Recently, Wang et al. enhanced the expression of total cellulase and xylanase through the RNA interference of *acel* gene in *Trichoderma koningii* [8]. And Qin et al. improved the recombinant lipase production via down-regulation of CBHI by RNAi in *T. reesei* [11]. It was strongly demonstrated that RNAi-mediated gene silencing could be wildly and efficiently applied in filamentous fungi.

Penicillium oxalicum is a fast-growing cellulolytic fungus, and its mutant strains have been used in industrial cellulase production since 1996 [12]. The genome of *P. oxalicum* wild-type strain 114-2 has been sequenced [13]. The genes encoding transcription factors, chromatin remodeling proteins and G protein subunits have been annotated, and their functions in regulating gene expression of cellulases and amylases were elucidated [14,15]. Some genes annotated as transcription factors for cellulases expression have not been studied yet because their mutants can't be obtained by gene knock-out.

Amylase with an extremely high specific enzyme activity, accounts for over 40% of total extracellular proteins in *P. oxalicum* A11 when cultured on wheat bran-cellulose [16,17]. The aim of the present study is to construct a vector that can be used for down-regulation of genes expression through RNAi in filamentous fungus *P. oxalicum*. Here, we established the plasmid of pHX-RNAi from backbone plasmid pUC19, and *amy15A* was chosen as a reporter to verify the efficiency of pHX-RNAi because of the convenient detection. Moreover, no impact was observed on strain growth even though *amy15A* was deleted [17], which made it suitable to verify the effect of the vector constructed in this study.

METHOD AND MATERIALS

Strains and culture conditions

E. coli DH5α was used as the host strain for recombinant plasmid construction. The *E. coli* strain was cultivated in Luria-Bertani (LB) broth, in which ampicillin (100 μg/mL; Invitrogen) was supplemented when necessary. *P. oxalicum* 114-2(CGMCC 5302) [18], is a wild-type glycoside hydrolase-producing strain. *P. oxalicum* A11 was generated from *P. oxalicum* 114-2 by dominantly acitivating G protein PGA3 [19]. For conidia collection, the *P. oxalicum* was cultivated on wheat bran at 30°C for 3 d [20]. For genomic DNA extraction, the conidia were inoculated on Vogel's medium supplemented with 1% glucose as a carbon source and cultured for 48 h [21]. The genomic DNA of *P. oxalicum* 114-2 was used as the template for PCR. The transformants were selected by cultivating the protoplasts in the screening medium with 400 μg/mL hygromycin B. For amylase production, the strains were pre-cultivated in liquid glucose media (Vogel's medium with 1% glucose as the carbon source) for 18 h. And then, 0.5 g wet mycelia was transferred into 100 mL amylase fermentation media (Vogel's medium with 1% soluble starch as the sole carbon source) and cultured at 30°C, 200 rpm. For cellulase production, the pre-cultivated wet mycelia (0.5 g) were transferred into cellulase fermentation media (Vogel's medium with 1% cellulose as the carbon source) and cultured at 30°C, 200 rpm.

Plasmids construction

Genomic DNA was isolated from the mycelia of *P. oxalicum*114-2 using the method previously described [17], and all the plasmids applied in this study were newly constructed as follows.

Construction of *hph* marker containing vector k-*hph*: the 1.9 kb *Hind*III/*Sph*I fragment containing *hph* ORF region with several restriction enzyme cutting sites was synthetized using the *hph* from the plasmid pSilent-1 as reference. Then the *hph* fragment was amplified with primer pairs *Hph*s-F (*Hind*III) and *Hph*s-R (*Sph*I). The *hph* fragment digested by restriction enzymes was inserted into the *Hind*III/*Sph*I sites of the original plasmid pUC19.

Construction of expression vector K-*hph*-P*pgm*C: the 470 bp terminator of *trp*C fragment containing *Eco*RI/*Sac*I was amplified by PCR from the plasmid pSilent-1 using primer pairs T*trp*C-F (*Sac*I) and T*trp*C-R (EcoRI). Then, a 879 bp promoter of *pgm*C containing *Sph*I/*Pst*I was amplified from the *P. oxalicum* 114-2 genomic DNA by using primers P*pgm*-F (*Sph*I) and P*pgm*-R (*Pst*I). Two DNA fragments (T*trp*C and P*pgm*C) digested by restriction enzymes were inserted into the plasmid K-*hph* through SacI/*Eco*RI and *Sph*I/*Pst*I respectively. The resulting expression vector was designated as K-*hph*-P*pgm*C.

Construction of RNAi expression vector PHX-RNAi: a 134 bp synthetic intron fragment of the *P. oxalicum* PGA3 gene that contains *Pst*I, *Sal*I, *Bam*HI and *Xba*I, *Kpn*I, *Sac*I was inserted into the *Pst*I/*Sac*I sites of the K-*hph*-P*pgm*C to generate the RNAi expression vector PHX-RNAi.

Construction of *amy15A* interference vector pHX-amyi: two DNA fragments (*amy15A*- and *amy15A*+) of *amy15A* (EPS34453) were amplified by PCR using two pairs of primers iamyF2 (*Sac*I)/iamyR2(*Xba*I) and iamyF1(*Pst*I)/iamyR1(*BamH*I), respectively. And then *amy15A*- and *amy15A*+ were inversely inserted into the RNAi expression vector PHX-RNAi by using the restriction enzymes *Sac*I/XbaI and *Pst*I/BamHI. This vector was designated as *amy15A* interference vector pHX-amyi.

All primers used in this study are listed in **Table 1.**

Fungal transformations

The protoplast transformation of *P. oxalicum* was performed as previously described [22,23]. 10 μL of plasmid DNA and 100 μL of protoplasts were spread onto the selection plates containing 400 μg/mL hygromycin B and the transformants were selected. The conidia of the candidate transformants were further spread onto hygromycin B selection plates to form single colonies.

e-ISSN:2320-3528
p-ISSN:2347-2286

Table 1. Primers used in this study; The restriction site sequences are underlined.

Primers	Sequence (5'-3')
Hphs-F(HindIII)	CCCAAGCTTCGACCTTAACTGATATTGAA
Hphs-R(SphI)	ACATGCATGCCAACCCAGGGCTGGTGACGG
T*trp*C-F(SacI)	CGGAGCTCACTTAACGTTACTGAAATCAT
T*trp*C-R(EcoRI)	CGGAATTCCTAGAGCGGCCGCAACCCAG
P*pgm*-F(SphI)	ACATGCATGCCCGACTCATTACATACCTCCA
P*pgm*-R(PstI)	AACTGCAGGAGGTGGTGAAGGTTGGATTTG
iamyF1(PstI)	AACTGCAGTGAAAAATGACCCTCTAATGAA
iamyR1(BamHI)	CGCGGATCCCCAAGCACCGGTGAAGGCG
iamyR2 (SacI)	CGGAGCTCCCAAGCACCGGTGAAGGCG
iamyF2(XbaI)	GCTCTAGATGAAAAATGACCCTCTAATGAA
*amy*15Ai-qF1	TACTCTCAAAGCCCTTGTGGAT
*amy*15Ai-qR1	TTGAACTTGGGCTCACCGAG
*amy*15Ai-qF2	GGTCGGTTCTATTTCTCAGCTCG
*amy*15Ai-qR2	ACTTGGCAGGGACGGTGTAGG
act-qF	CTCCATCCAGGCCGTTCTG
act-qR	CATGAGGTAGTCGGTCAAGTCAC

The analysis of *Amy15A* secretion by SDS-PAGE

SDS-PAGE was performed in polyacrylamide gel slabs and gels were stained with Coomassie Brilliant Blue R-250 (Sangon Biotech, China). 32 µL culture supernatant of each strain was loaded.

Phenotype analysis

Conidia of all strains were harvested after growing on PDA for 3 days at 30°C. Equal portions of conidia from all strains were inoculated on glucose, starch, or cellulose plates for 4 days at 30°C.

The analysis of enzymatic activity

Amylase activity was assayed according to the methods previously described [24]. The amounts of released reducing sugars were determined using the dinitrosalicylic acid method [25]. One enzyme unit (U) was defined as the amount of enzyme needed for releasing 1 µmol of glucose equivalent per minute.

The analysis of *amy15A* transcription levels by quantitative PCR

Total RNA extraction, cDNA synthesis and quantitative real-time PCR (RT-qPCR) were performed as previously described [26]. The RNAiso reagent (TaKaRa, Japan) was used for total RNA extraction, and PrimeScript RT reagent Kit With gDNA Eraser (Perfect Real Time) (TaKaRa, Japan) was used for cDNA synthesis. Quantitative RT-PCR was performed on Roche 480 LightCycler (Roche, Mannheim, Germany) using SYBR Premix Ex Taq™ (Perfect Real Time) (TaKaRa, Japan). Quantitative RT-PCR analysis of each gene was performed in triplicate by using the primers. Each amplification reaction conducted in a total reaction volume of 20 µL. The thermal cycling protocol was as follows: initial denaturation for 2 min at 95°C followed by 40 cycles of 10 s at 95°Cand 30 s at 61°C. The fluorescence signal was measured at the end of each extension step at 80°C. The transcript number of actin gene from the same sample was quantified as an internal standard. Each sample was performed with two biological replicates and three experiment replicates. The actin gene was used as the internal standard.

RESULTS

Construction of RNAi vector pHX-RNAi and pHX-Amyi

The codon optimized hygromycin B resistance gene *hph* was PCR-amplified and inserted into pUC19 and thus a vector k-*hph* containing selection marker gene *hph* was generated **(Figures 2A and 2B)**, and then T*trp*C and P*pgm*C were inserted into the K-*hph* to construct an expression vector K-*hph*-P*pgm*C **(Figures 2C and 2D).** The intron fragment from G protein subunit *PGA3* gene was inserted into the K-*hph*-P*pgm*C to construct a RNAi expression vector pHX-RNAi **(Figure 3A).** And then *amy15A*- and *amy15A*+ were inversely inserted into the RNAi expression vector pHX-RNAi in turn to construct an *amy15A*- silenced vector pHX-Amyi **(Figures 3B-3D).** The construction process of RNAi vector in this study is shown in **Figure 2.** PCR verification (data not shown) restriction enzyme digestion and sequencing analysis were performed to insure that the target fragments were inserted into the appropriate position of the vector and has no mutation **(Figure 4).**

Generation of *P. oxalicum amy15A*-silenced strains

To test the efficiency of PHX-RNAi, the gene *amy15A* was selected as the reporter. The recombinant plasmid PHX-Amyi was transformed into *P. oxalicum* A11 to knock down *amy15A* expression. A PCR analysis showed that the fragments of the expression cassette from upstream of the *hph* expression cassette to the promoter-*pgm*C were respectively amplified were applied to verify whether the PHX-Amyi expression cassette was integrated into the genome of the transformants completely. Finally, PCR analysis showed that all of seven randomly selected transformants contain complete PHX-Amyi expression cassette (data not shown). These seven positive transformants were assigned as *Amy15A*i-1, *Amy15A*i-2, *Amy15A*i-3, *Amy15A*i-4, *Amy15A*i-5, *Amy15A*i-6, and *Amy15A*i-7.

e-ISSN:2320-3528
p-ISSN:2347-2286

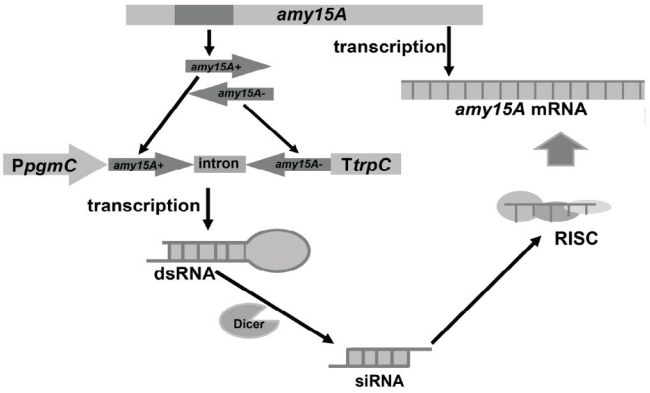

Figure 1. Schematic illustration of the RNAi approach by expressing a hairpin RNA.

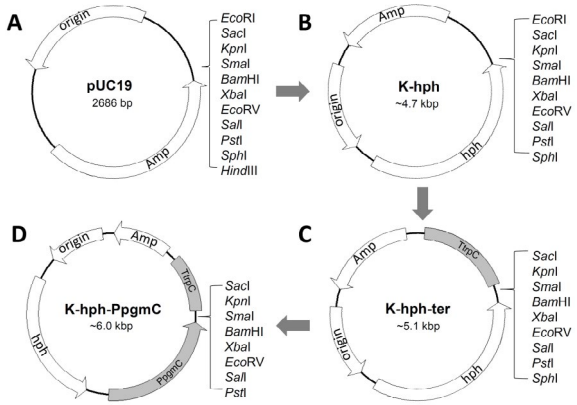

Figure 2. Schematic illustration of (A) pUC19; (B) K-hph; (C) K-hph-ter; and (D) K-hph-P*pgm*C.

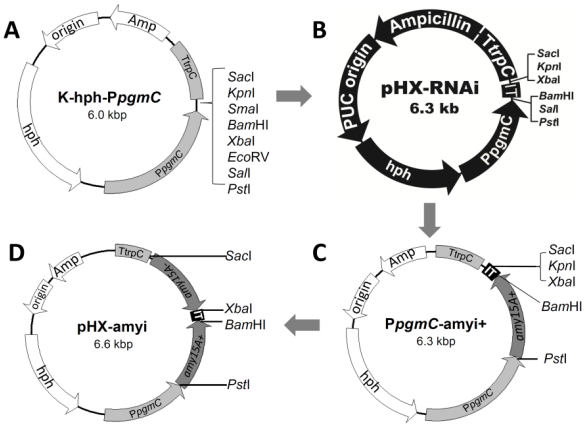

Figure 3. Construction process of RNAi vector. (A) K-hph-P*pgm*C; (B) pHX-RNAi; (C) P*pgm*C-amy+; and (D) pHX-amyi.

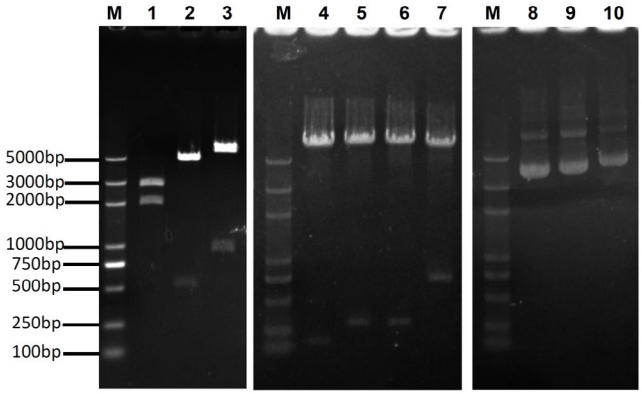

Figure 4. Recombinant plasmids analysis by using restriction enzyme digestion. Lane 1: K-hph digested by HindIII/*Sph*I; Lane 2: K-hph-ter digested by *Eco*RI/SacI; Lane 3 was K-hph-P*pgm*C digested by *Pst*I/*Sph*I; Lane 4: PHX-RNAi digested by *Pst*I/SacI; Lane 5: PHX-Amyi(-) digested by *Xba*I/SacI; Lane 6 and 7: PHX-Amyi digested by *Pst*I/BamHI and BamHI/*Xba*I, respectively; Lane 8-10: agarose gel electrophoresis analysis of plasmids PHX-RNAi, PHX-Amyi(-), and PHX-Amyi, respectively. Lane M: the DNA marker.

e-ISSN:2320-3528
p-ISSN:2347-2286

Down-regulation expression of *Amy15A* by RNAi mediated gene silencing

These positive transformants were cultivated in shake flasks with starch as sole carbon source. The extracellular proteins secreted by these transformants on culture medium supernatant were analyzed by SDS-PAGE. To ensure the equal loading of samples, 32 μL culture supernatant of each strain was loaded after cultivating in 1% (w/v) starch medium.

As shown in **Figure 5**, compared to the strain A11, the amylase ***Amy15A*** silencing of transformants showed a weakened band, the bands were around 70 kDa, which is consistent with the Amy15 protein composed of 636 amino acids [22]. The results demonstrated that hairpin RNA-induced silencing yields silenced strains displayed obvious effect in protein secretion.

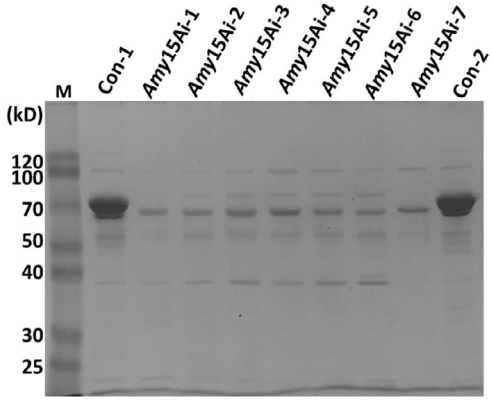

Figure 5. Extracellular proteins of A11 and mutants on starch analyzed by SDS-PAGE, 32 μL culture supernatant of each strain was loaded after cultivating in 1 % (w/v) starch medium. con-1/con-2 was extracellular proteins of A11. Lane M: the molecular weight marker

The original strain A11 *amy15A* silencing strains Amy15Ai were cultured on agar plates to study the growth characteristics and carbon source utilization. As a result, the transformants Amy15Ai showed no significant differences on colony diameter and conidiation ability when compared to A11 on glucose or cellulose **(Figure 6).**

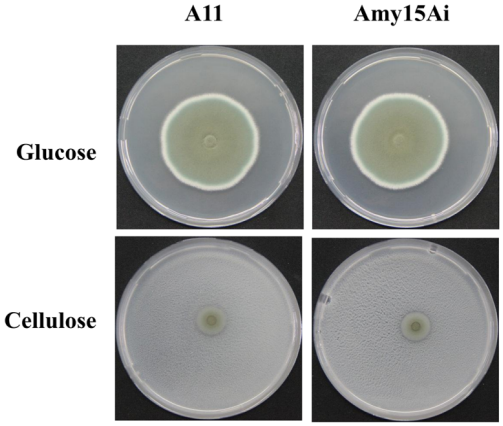

Figure 6. Growth of A11 and Amyi on different carbon sources. The plates were incubated at 30°C for 5 days.

In order to prove that the expression of amylase was down-regulated, we have detected the amylase activity of those strains. Significant decreases in amylase activity were observed when mutants Amy15Ai were cultured on starch or cellulose **(Figure 7).**

To confirm the silencing *amy15A* in *P. oxalicum* A11, the expression level of *amy15A* were further detected by RT-qPCR at different time points(12 h, 24 h, 36 h) on different carbon sources(starch or cellulose). To illustrate the stability of interference for gene *amy15A* in the recombinant strains, *Amy15A*i-1, *Amy15A*i-2, *Amy15A*i-3, and *Amy15A*i-4 were subcultured for five rounds (one round lasted for 5 days) on a solid medium that contained hygromycin B, and the transformants were further cultured in shake flasks with starch or cellulose as a sole carbon source for RNA extraction. As shown in **Figure 8**, the recombinant strains showed much lower expression levels when compared to the strain A11 under starch or cellulose inducting condition. We had designed two detection sites on a fragment near 5'-terminal or 3'-terminal of mRNA on *amy15A* gene (*amy15A*1 and *amy15A*2), similar RT-qPCR analysis results about the two detection sites indicated that the RNAi destroyed the entire mRNA of gene *amy15A*. These results suggested that the silencing of *amy15A* was easily induced and RNA-mediated gene silencing was stable, lasting and high-efficiency.

DISCUSSION

RNAi had been proven to be an effective tool for gene silencing in various fungi. Some RNAi vectors for dsRNA generation had been reported, such as pSilent-1, which had been applied in Aspergillus. However, pSilent-1 was unstable when it was applied in *Penicillium* sp. might due to its multi-copy functional elements on plasmid which led to self-recombination. pHX-RNAi as a RNAi

vector constructed in this study, showed an efficient and stable effect when it was used to target *amy15A*. Particularly, silencing gene by pHX-RNAi in *P. oxalicum* exhibited high transformation efficiency, which was preliminarily supported by the observation that *amy15A* expression showed significant down-regulation in all of the selected transformants, and this feature would give more convenience in transformants selection.

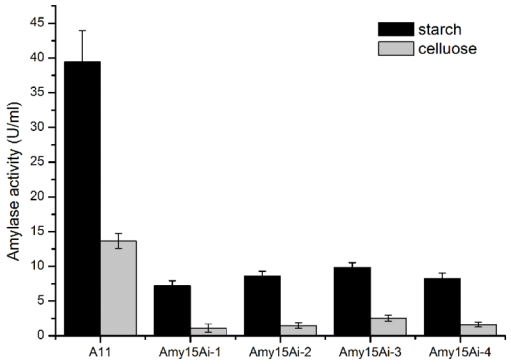

Figure 7. Amylase activity of A11 and mutants on starch or cellulose. Error bars indicated standard deviations of three independent cultivations.

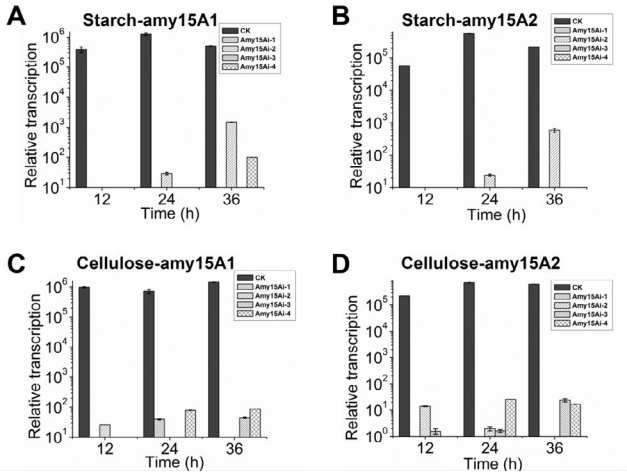

Figure 8. RT-qPCR analysis of the amy15A transcription levels for the Amy15Ai transformants at 12 h, 24 h, and 36 h on starch(A,B) or cellulose (C,D); amy15A1, amy15A2 presented the detection positons near 5'-terminal and 3'-terminal of mRNA, respectiively . Relative transcription values represent the transcript copy numbers of each gene per 105 copies of actin gene transcript. Error bars indicated standard deviations of three independent cultivations.

RNA silencing system had been established in some filamentous fungi, such as *T. koningi*, *T. reesei* and *Botrytis cinerea* [27-29]. In those studies, heterologous fluorescent protein was used as the reporter. In contrast, endogenous amylase was developed as the reporter in our work, and the transformants could be used to investigate the effect of the impairment of amylase on the cellulase synthesis. Wang et al. improved the cellulase production by silencing the regulator Cre1 or ACE1. And Qin et al. enhanced the lipase by silencing the expression of major endogenic gene [11]. All the above reports suggested that it was potential to improve cellulase synthesis by silencing the amylase genes, or other high-expression genes in cellulolytic fungus *P. oxalicum*.

Homologous recombination occurs at a low frequency in filamentous fungi and multiple genetic manipulations are difficult due to the limitation of screening marker. Thereby, using RNAi to regulating gene expression gives a way to the research of those interesting but lethal genes, what's more, it provides a possible method for multiple genes modification [30]. Moreover, we can control the degree of the gene silencing by changing the promoters with different intensities. The expression of dsRNA can be induced by the inducible promoters, such as constitutive stronger/weak promoters, condition-dependent promoters, which can switch on the target gene silencing by adding the inducer. Thus, the target genes could be silent at the proper time and analyze their functions at the specific period of the growth [31].

RNAi also have irreplaceable advantages in the construction of protein expression system for filamentous fungi. Recently, the synthesis and secretion processes of proteins have been studied widely in filamentous fungi. However, whether the protein interactions in the secretory pathway affect their secretion is not clear [32,33]. Strains with strong ability of protein secretion are usually chosen as the host for heterologous proteins expression. But the high level of target protein always can't be achieved even with a stronger promoter because of the pressure in intracellular protein folding and modification in the host strains, such as CBHI in *T. reesei* cellulase and amylase in *A. oryzae* [11,34]. Considering highly expressed host genes might lead to saturation of the machinery involved in protein folding and assembly in secretion of proteins, down regulating the highly expressed host gene by RNAi could enhance the target protein secretion. Moreover, it could avoid the disabled utilization of substrate that caused by gene deletion, such as CBHI in *T. reesei* [35].

e-ISSN:2320-3528
p-ISSN:2347-2286

CONCLUSION

In this study, the amylase gene *amy15A* was proved to be successfully interfered by constructing plasmid pHX-RNAi and RNAi produced marked and stable effects at mRNA level. The silencing of gene *amy15A* in this experiment showed that the pHX-RNAi could play an important role in the gene modification and gene function analysis in *Penicillium oxalicum*. Considering the conservatism of RNAi machinery in fungi, pHX-RNAi could be applied to other filamentous fungi.

ACKNOWLEDGEMENTS

This study was financially supported by National Basic Research Program of China (2011CB707403), National Natural Sciences Foundation of China (31030001, 30970052 and 31270089), National Key Technology R&D Program (2011BAC02B04), and National High Technology Research and Development Program of China (2012AA022203B).

REFERENCES

1. Fire A, et al. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 1998; 391: 806-811.
2. Tomari Y and Zamore PD. Perspective: machines for RNAi. Genes Dev 2005; 19: 517-529.
3. Wesley SV, et al. Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J 2001; 27: 581-590.
4. Boutros M, et al. Genome-wide RNAi analysis of growth and viability in Drosophila cells. Science 2004; 303: 832-835.
5. Kennerdell JR and Carthew RW. Heritable gene silencing in Drosophila using double-stranded RNA. Nat Biotechnol 2000; 18: 896-898.
6. Svoboda P, et al. Selective reduction of dormant maternal mRNAs in mouse oocytes by RNA interference. Development 2000; 127: 4147-4156.
7. Cheng Y, et al. Genome shuffling improves production of cellulase by Penicillium decumbens JU-A10. J Appl Microbiol 2009; 107: 1837-1846.
8. Wang S, et al. Improving cellulase production in Trichoderma koningii through RNA interference on ace1 gene expression. J Microbiol Biotechnol 2012; 22: 1133-1140.
9. Whisson SC, et al. A method for double-stranded RNA-mediated transient gene silencing in Phytophthora infestans. Mol Plant Pathol 2005; 6: 153-163.
10. Nakayashiki H, et al. RNA silencing as a tool for exploring gene function in ascomycete fungi. Fungal Genet Biol 2005; 42: 275-283.
11. Qin LN, et al. Improved production of heterologous lipase in Trichoderma reesei by RNAi mediated gene silencing of an endogenic highly expressed gene. Bioresour Technol 2012; 109: 116-122.
12. Mo H, et al. Control of gas phase for enhanced cellulase production by Penicillium decumbens in solid-state culture. Process Biochemistry 2004; 39: 1293-1297.
13. Liu G, et al. Genomic and secretomic analyses reveal unique features of the lignocellulolytic enzyme system of *Penicillium decumbens*. PLoS One 2013; 8: e55185.
14. Li Z, et al. Synergistic and Dose-Controlled Regulation of Cellulase Gene Expression in *Penicillium oxalicum*. PLoS Genet 2015; 11: e1005509.
15. Yao G, et al. Redesigning the regulatory pathway to enhance cellulase production in *Penicillium oxalicum*. Biotechnol Biofuels 2015; 8: 71.
16. Liu G, et al. Long-term strain improvements accumulate mutations in regulatory elements responsible for hyper-production of cellulolytic enzymes. Sci Rep 2013; 3: 1569.
17. Hu Y, et al. Efficient production and evaluation of lignocellulolytic enzymes using a constitutive protein expression system in *Penicillium oxalicum*. J Ind Microbiol Biotechnol 2015; 42: 877-887.
18. Qu Y, et al. Screening of catabolite repressionresistant mutants of cellulase producing Penicillium spp. Acta Mycol Sinica 1984; 3:238–243.
19. Hu Y, et al. G protein-cAMP signaling pathway mediated by PGA3 plays different roles in regulating the expressions of amylases and cellulases in *Penicillium decumbens*. Fungal Genet Biol 2013; 58-59: 62-70.
20. Cheng Y, et al. Genome shuffling improves production of cellulase by *Penicillium decumbens* JU-A10. J Appl Microbiol 2009; 107: 1837-1846.
21. Vogel H. A convenient growth medium for Neurospora (medium N). Microb Genet Bull 1956; 13:2–43.

22. Gruber F, et al. The development of a heterologous transformation system for the cellulolytic fungus *Trichoderma reesei* based on a pyrG-negative mutant strain. Curr Genet 1990; 18: 71-76.

23. Li ZH, et al. Development of a highly efficient gene targeting system allowing rapid genetic manipulations in *Penicillium decumbens*. Appl Microbiol Biotechnol 2010; 87: 1065-1076.

24. Sun X, et al. The composition of basal and induced cellulase systems in *Penicillium decumbens* under induction or repression conditions. Enzyme Microb Technol 2008; 42:560–567.

25. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 1959; 31:426–442.

26. Wei X, et al. Transcription analysis of lignocellulolytic enzymes of Penicillium decumbens 114-2 and its catabolite-repression-resistant mutant. C R Biol 2011; 334: 806-811.

27. Wang S, et al. RNA interference with carbon catabolite repression in *Trichoderma koningii* for enhancing cellulase production. Enzyme Microb Technol 2013; 53: 104-109.

28. He R, et al. Construction of an efficient RNAi system in the cellulolytic fungus *Trichoderma reesei*. J Microbiol Methods 2015; 108: 70-73.

29. Espino J, et al. Efficiency of different strategies for gene silencing in *Botrytis cinerea*. Appl Microbiol Biotechnol 2014; 98: 9413-9424.

30. Liu H, et al. RNA interference in the pathogenic fungus *Cryptococcus neoformans*. Genetics 2002; 160: 463-470.

31. Bromley M, et al. The *Aspergillus fumigatus* cellobiohydrolase B (*cbhB*) promoter is tightly regulated and can be exploited for controlled protein expression and RNAi. FEMS Microbiol Lett 2006; 264: 246-254.

32. Conesa A, et al. The secretion pathway in filamentous fungi: a biotechnological view. Fungal Genet Biol 2001; 33: 155-171.

33. Yan S and Wu G. Secretory pathway of cellulase: a mini-review. Biotechnol Biofuels. 2013; 6: 177.

34. Nemoto T, et al. Improvement of heterologous protein production in *Aspergillus oryzae* by RNA interference with alpha-amylase genes. Biosci Biotechnol Biochem 2009; 73: 2370-2373.

35. Penttila M, et al. A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. Gene 61: 155-164.