INTRODUCTION

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.

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.
fungi. However, pSilent-1 was unstable when it was applied in *Penicillium* sp. may due to its multi-copy functional elements (two copies of *trpC* 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 *ace1* gene in *Trichoderma reesei* [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-celullose [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 activating 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 genetic 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 HindIII/SphI fragment containing *hph* ORF region with several restriction enzyme cutting sites was synthesized using the *hph* from the plasmid pSilent-1 as reference. Then the *hph* fragment was amplified with primer pairs Hphs-F (HindIII) and Hphs-R (SphI). The *hph* fragment digested by restriction enzymes was inserted into the HindIII/SphI sites of the original plasmid pUC19.

Construction of expression vector *K*-*hph*-Pgmc: the 470 bp terminator of *trpC* fragment containing EcoRI/SalI was amplified by PCR from the plasmid pSilent-1 using primer pairs Trpc-F (SalI) and Trpc-R (EcoRI). Then, a 879 bp promoter of pgmC containing SphI/PstI was amplified from the *P. oxalicum* 114-2 genomic DNA by using primers Pgm-F (SphI) and Pgm-R (PstI). Two DNA fragments (Trpc and PgmC) digested by restriction enzymes were inserted into the plasmid K-*hph* through SalI/EcoRI and SphI/PstI respectively. The resulting expression vector was designated as *K*-*hph*-PgmC.

Construction of RNAi expression vector PHX-RNAi: a 134 bp synthetic intron fragment of the *P. oxalicum* PGA3 gene that contains PstI, Sall, BamHI and Xbal, KpnI, Sall was inserted into the PstI/SalI sites of the K-*hph*-PgmC 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 (SacI/iamyR2(XbaI)) and iamyF1(PstI)/iamyR1(BamHI), respectively. And then *amy15A* - and *amy15A*+ were inversely inserted into the RNAi expression vector PHX-RNAi by using the restriction enzymes SacI/XbaI and PstI/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.
Table 1. Primers used in this study; The restriction site sequences are underlined.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’-3’)</th>
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<tbody>
<tr>
<td>Hphs-F(HindIII)</td>
<td>CCCAAGCTTCGACCTTAACTGATATTGAA</td>
</tr>
<tr>
<td>Hphs-R(SphI)</td>
<td>ACATGCATGCAACCAGGGCTTGAGCAGG</td>
</tr>
<tr>
<td>TrpC-F(SacI)</td>
<td>CGGAGCTCACTTAACTGATATTGAA</td>
</tr>
<tr>
<td>TrpC-R(EcoRI)</td>
<td>CGGAATTCCTAGCGCAGGCGAACCAGCAG</td>
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<tr>
<td>Pgm-F(SphI)</td>
<td>ACATGCATGCAACCAGGGCTTGAGCAGG</td>
</tr>
<tr>
<td>Pgm-R(PstI)</td>
<td>AACTGCAGGAGGTGAGTGAAGTGGATTGG</td>
</tr>
<tr>
<td>iamyF1(PstI)</td>
<td>AACTGCAGTGAACATGCTCCTAATGAA</td>
</tr>
<tr>
<td>iamyR1(BamHI)</td>
<td>CCGGAATCCAGCAACGCGTGAAGGCGG</td>
</tr>
<tr>
<td>iamyR2(SacI)</td>
<td>CCGGAATCCAGCAACGCGTGAAGGCGG</td>
</tr>
<tr>
<td>amy15Ai-qF1</td>
<td>TACCTCAAGCCCTGGGTGGAT</td>
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<tr>
<td>amy15Ai-qR1</td>
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</tr>
<tr>
<td>amy15Ai-qF2</td>
<td>GGTCTGAGTTCTAATGCTG</td>
</tr>
<tr>
<td>amy15Ai-qR2</td>
<td>ACTTGAGCAGGAGGTGAGG</td>
</tr>
<tr>
<td>act-qF</td>
<td>CTCCATCCAGGCGTCTTCTC</td>
</tr>
<tr>
<td>act-qR</td>
<td>CATGAGGATGTCGCGAAGTCA</td>
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</table>

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°C and 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 TrpC and PgmC were inserted into the K-hph to construct an expression vector K-hph-PgmC (Figures 2C and 2D). The intron fragment from G protein subunit PGA3 gene was inserted into the K-hph-PgmC 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-pgmC 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 Amy15Ai-1, Amy15Ai-2, Amy15Ai-3, Amy15Ai-4, Amy15Ai-5, Amy15Ai-6, and Amy15Ai-7.
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/SphI; Lane 2: K-hph-ter digested by EcoRI/Sacl; Lane 3 was K-hph-P_{pgm}C digested by PstI/SphI; Lane 4: PHX-RNAi digested by PstI/Sacl; Lane 5: PHX-Amyi(-) digested by XbaI/Sacl; Lane 6 and 7: PHX-Amyi digested by PstI/BamHI and BamHI/XbaI, respectively; Lane 8-10: agarose gel electrophoresis analysis of plasmids PHX-RNAi, PHX-Amyi(-), and PHX-Amyi, respectively. Lane M: the DNA marker.
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, Amy15Ai-1, Amy15Ai-2, Amy15Ai-3, and Amy15Ai-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 (amy15A1 and amy15A2), 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 positions near 5’-terminal and 3’-terminal of mRNA, respectively. 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 [33,34]. 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].
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


