Fast gene disruption in *Trichoderma reesei* using in vitro assembled Cas9/gRNA complex

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**Abstract**

**Background:** CRISPR/Cas9 has wide application potentials in a variety of biological species including *Trichoderma reesei*, a filamentous fungus workhorse for cellulase production. However, expression of Cas9 heterologously in the host cell could be time-consuming and sometimes even troublesome.

**Results:** We tested two gene disruption methods in *T. reesei* using CRISPR/Cas9 in this study. The intracellularly expressed Cas9 led to unexpected off-target gene disruption in *T. reesei* QM9414, favoring inserting 9- or 12-bp at 70- and 100-bp downstream of the targeted *ura5*. An alternative method was, therefore, established by assembling Cas9 and gRNA in vitro, followed by transformation of the ribonucleoprotein complex with a plasmid containing the *pyr4* marker gene into *T. reesei* TU-6. When the gRNA targeting *cbh1* was used, eight among the twenty seven transformants were found to lose the ability to express CBH1, indicative of successful *cbh1* disruption through genome editing. Large DNA fragments including the co-transformed plasmid, chromosomal genes, or a mixture of these nucleotides, were inserted in the disrupted *cbh1* locus.

**Conclusions:** Direct transformation of Cas9/gRNA complex into the cell is a fast means to disrupt a gene in *T. reesei* and may find wide applications in strain improvement and functional genomics study.

**Keywords:** CRISPR/Cas9, *Trichoderma reesei*, Gene disruption, Ribonucleoprotein
In the present study, it was first found that the intracellularly expressed Cas9 led to unexpected off-target gene disruption in T. reesei QM9414. An alternative means, i.e. direct transformation of the in vitro assembled Cas9/gRNA, was evaluated for its efficacy in gene disruption using the cellobiohydrolase I (cbh1) gene as a model. The sequences of the disrupted gene were analyzed, identifying insertion of large chromosomal DNAs or plasmid fragments.

Results
Intracellularly expressed Cas9 led to unexpected off-target genome editing

The cas9 gene has been expressed constitutively or induced successfully in T. reesei, in both cases which can be used in successful genome editing in QM6a and RUT-C30 [7]. The codon-optimized cas9 gene [7] was synthesized, ligated downstream of the strong, constitutive pdc1 promoter, and transformed into T. reesei QM9414. Nine positive transformants were obtained, among which strain C5 had the highest cas9 transcript level (determined by RT-qPCR, data not shown) and was therefore selected for subsequent gene disruption.

The ura5 gene has been used as the target when the CRISPR/Cas9 technology was established in T. reesei [7]. The in vitro transcribed gRNA targeting the same ura5 locus was transformed into C5. Nineteen 5-fluoroorotic acid (5-FOA) resistant transformants were obtained. While no mutation was found in the expected editing locus for all these transformants, seven had an unexpected insertion of 12-bp at the 100-bp downstream of the expected editing locus (Fig. 1, Additional file 1: FigureS1). One (QM9414-C5/T18) had an insertion of 9-bp, while another (QM9414-C5/T13) had a deletion of this 9-bp fragment, at the 70-bp downstream of the expected editing locus. Interestingly, the inserted short DNA fragments were a direct repeat of the nucleotides downstream of the insertion site. Moreover, two direct repeats (7- and 11-bp, respectively) were found in both sites (Fig. 1), suggestive of biased off-target editing.

Designing gRNA for cbh1 gene disruption

Direct transformation of Cas9/gRNA complex may be an alternative for genome editing. Indeed, this technique has already been successfully used for gene disruption in various filamentous fungi including Mucor circinelloides [9], Aspergillus fumigatus [10], and Fusarium oxysporum [11]. For those filamentous fungi that are difficult to manipulate, the transformation of Cas9/gRNA complex provides a means to speed up genome editing. With this advantage, nevertheless, there was no report of using such a technique in T. reesei. CBH1 is the major cellulase secreted by T. reesei, accounting for above 50–60% in the fermentation broth [12]. Disruption of cbh1 will result in loss of the major band on SDS-PAGE gel, facilitating identification of the strains with successful genome editing. Therefore, for ease of verification, cbh1 was selected as the target gene. Three different gRNAs on cbh1 were designed and synthesized by in vitro transcription. Cas9 and a certain gRNA was incubated with the BcuI-linearized pT3cbh1 plasmid (4717-bp) containing the cbh1 gene. gRNA-3 was the best one to guide Cas9-mediated digestion of pT3cbh1 into two DNA fragments with expected sizes (Fig. 2).
Further sequence analyses indicated that there were gene editing events at the expected cbh1 locus. Deletion of 91–705-bp in cbh1 was observed in T2, T7, and T9 (Fig. 3D). The inserted fragments varied in length and sequence by containing: i) the transformed plasmid (T2, T7, and T8); ii) a chromosome fragment (T1); or iii) mixed chromosome and plasmid sequences (T9, Fig. 3C&D). Although T2 and T7 had different deletions, they had the same inserts. It seemed that the pSKpyr4 DNA fragments starting from nucleotide 23 were favored in these gene rearrangement events (Fig. 3D). The underlying reason for this is not known. All five had another fragment (“other insert” in Fig. 3C&D) which was not from pSKpyr4 or T. reesei chromosome but from E. coli chromosome (T1) or an unidentified plasmid(s) (T2, T7, T8, and T9), which was likely contaminants from pSKpyr4 preparation.

Discussion

The intracellularly expressed Cas9 successfully disrupts the ura5 gene in the T. reesei QM6a and RUT-C30 strains by creating small deletions [7]. However, disruption of the same gene was quite different in QM9414 in this study since no such small deletions were observed in the 5-FOA-resistant transformants. In contrast, insertion (or less frequently, deletion) of small DNA fragments was found downstream of the genomic locus expected for editing. These mutations resulted in 3–4 amino acids duplication (or 3 amino acids deletion) in the ura5-encoded orotate phosphoribosyl transferase, which might interfere with the normal function of this enzyme, thereby enabling the transformants to resist the toxicity of 5-FOA. Since the sequence of gRNA was the same as that used in [7], the off-target genome editing was unexpected. In the previous report [7], QM6a and RUT-C30 were used as the cas9-recipient strains. Being cellulase hyperproducer derivatives of QM6a, RUT-C30 and QM9414 are both created by chemical and/or physical mutations but belong to different lineages [15]. It is conceivable that the genetic backgrounds of QM9414 and the two strains (QM6a and RUT-C30) are thus quite different. It is also well-known that cellular factors involved in DNA damage response [16], NHEJ, and HDR [17] affect the CRISPR/Cas9-mediated genome editing. Therefore, it is possible that the much different genetic backgrounds may account for the unexpected genome editing.

In contrast, direct transformation of the Cas9-gRNA complex into T. reesei TU-6, a uridine auxotrophic mutant of QM9414, led to genome editing in the predicted cbh1 locus. However, large DNA fragments including the co-transformed plasmid, chromosomal DNA, or their hybrid form, were found to be inserted in the targeted cbh1 gene. Insertion of large DNA fragments was involved insertion of large DNA fragments

Twenty-seven transformants were obtained in two transformations of Cas9/gRNA with pSKpyr4 in TU-6. Through SDS-PAGE analysis, it was suggested that eight among the 27 transformants could have the CBH1 disappeared (Fig. 3A). Interestingly, in these strains, expression of other proteins apparently increased, similar to improved secretion of a heterologous lipase in a T. reesei strain [13]. Using the primers specific for cbh1-silenced T. reesei strain [7], QM6a and RUT-C30 were used as the cas9-recipient strains. Being cellulase hyperproducer derivatives of QM6a and QM9414 were found to be inserted in the tar-
| Transformants | Deletion | Insert segment number | Insert segment | Chromosome of T. reesei |
|---------------|----------|-----------------------|----------------|------------------------|
| T1            | None     | 1                     | Gene ID: 122824 (1072 - 1192) | Chromosome VI         |
|               |          | 2                     | Other insert   |                        |
|               |          | 3                     | Gene ID: 65223 (447 - 507)   |                        |
| T2            | 676-bp (cbh1: 687-1362) | 1                     | pSKpyr4 (345 - 23)   |                        |
|               |          | 2                     | Other insert    |                        |
|               |          | 3                     | pSKpyr4 (5752 - 4648)  | Chromosome VI         |
| T7            | 705-bp (cbh1: 657-1362) | 1                     | pSKpyr4 (345 - 23)   |                        |
|               |          | 2                     | Other insert    |                        |
|               |          | 3                     | pSKpyr4 (5752 - 4648)  |                        |
| T8            | None     | 1                     | pSKpyr4 (5149 - 5752)  |                        |
|               |          | 2                     | Other insert    |                        |
|               |          | 3                     | pSKpyr4 (23 - 101)    |                        |
| T9            | 91-bp (cbh1: 1293-1383) | 1                     | pSKpyr4 (3584 - 23)  |                        |
|               |          | 2                     | Other insert    |                        |
|               |          | 1'                    | pSKpyr4 (4343 - 3697)  |                        |
|               |          | 3                     | Gene ID: 75230 (-261 - 640) | Chromosome VII        |

Fig. 3 (See legend on next page.)
not observed in *Mucor circinelloides* [9] and *Fusarium oxysporum* [11] when direct transformation of Cas9/gRNA was employed. For these two fungi, only the Cas9/gRNA complex, but none exogenous DNA, was used in the transformation. In *Nodulisporium* sp., similar insertion of large DNA fragment was also observed. However, in that case an exogenous plasmid containing the selection marker was co-transformed with the in vitro transcribed gRNA into a recipient strain with in vivo expressed Cas9. Note, however, unlike the discovery reported herein, only the exogenous plasmid sequence was found in the inserted fragments in *Nodulisporium* sp. The promiscuity of the inserted fragments in *T. reesei* suggested complex DNA-repair events in the target locus.

With the success in *cbh1* disruption, direct transformation of Cas9/gRNA complex provides a fast means for disruption of genes of interest in *T. reesei*. This method may also be used for HDR-mediated gene replacement. In a preliminary experiment we have observed gene replacement at the cel3c locus, albeit at a low frequency (5 out of 143 transformants), by co-transformation of Cas9/gRNA (targeting *cel3c* [18]) with a donor DNA in *T. reesei* (data not shown). Gene replacement efficiency can be improved by optimizing the amounts, nature and ratio of Cas9 and gRNA or using a strain with dysfunctional NHEJ DNA repair pathway.

**Conclusions**

The intracellularly expressed Cas9 led to biased off-target editing in *ura5*, which might be explained by the different strains used in our study and by other researchers. Direct transformation of Cas9/gRNA complex allowed disruption of *cbh1* in *T. reesei* with large DNA inserts found in the edited locus. Compared with the previous report, our method avoids the time and labor needed for introducing the cas9 gene into *T. reesei* and the uncertainty that may occur with its expression. In our hands, both in vivo expressed Cas9 and in vitro assembled Cas9/gRNA led to insertion of small or large DNA fragments during genome editing. This might be explained by the robust NHEJ process, which is the dominant DNA repair mechanism in *T. reesei*.

**Methods**

**Strains, plasmids and culture conditions**

The *Escherichia coli* Trans 1-T1 strain (Transgen, Beijing, China) was used as the host for plasmid construction and propagation throughout the study. The *Saccharomyces cerevisiae* AH109 strain (Clontech, San Francisco, CA) was used for construction of the cas9-expressing plasmid. The pPdc1-Cas9 plasmid for expressing the codon-optimized *Streptococcus pyogenes* cas9 [7] was obtained by assembling *cas9* between the strong, constitutive *pdc1* promoter and *pdc1* terminator via DNA assembler using pRS424 (New England Biolabs, Beverly, MA) as a backbone plasmid [19]. The *T. reesei* QM9414 and its uridine auxotrophic strain TU-6 were maintained in our lab. *T. reesei* was grown in the minimal medium (MM), containing *(NH₄)₂SO₄, 5.0 g/L; KH₂PO₄, 15 g/L; MgSO₄, 0.6 g/L; CaCl₂, 0.6 g/L; FeSO₄·7H₂O, 0.005 g/L; MnSO₄·H₂O, 0.0016 g/L; ZnSO₄·7H₂O, 0.0014 g/L; CoCl₂, 0.002 g/L) supplemented with a certain kind of carbohydrate (2% glucose for mycelial growth and 2% Avicel cellulose for cellulase induction) as the sole carbon source. For sporulation, *T. reesei* was grown on potato dextrose agar (PDA) plates at 28°C.

**Designing gRNA targeting cbh1**

The gene *cbh1* encoding the major cellulase cellobiohydrolase I (CBH1) was amplified from the genomic DNA of TU-6 by PCR using the primer pair Pcbh1F/R (Additional file 1: Table S1). It was then ligated into pEASY-T3 (Transgen, Beijing, China) to obtain pT3cbh1. Three gRNA sequences for *cbh1* were designed using the online E-CRISPR design server (http://www.e-crisp.org/E-CRISPR/). The corresponding encoding DNAs were synthesized in the form of single-stranded, complementary oligonucleotides (Additional file 1: Table S1). The T7 promoter (5’-TAATACGACTCAGAGG-3’) was designed upstream of the gRNAs. Equal amounts (1 μg) of two complementary oligonucleotides were mixed, boiled for 10 min, and cooled down at room temperature for annealing. Using the annealed double-stranded DNAs as the template, gRNAs were obtained by in vitro transcription with a MEGAscript T7 Transcription Kit (Invitrogen, Carlsbad, CA) and the RNAs
were purified by using the RNA Purification Kit (Tiangen, Beijing, China). To determine the ability to guide digestion, 1 μg of gRNA, 2 μg of recombinant Cas9 (GenScript, Nanjing, China), and 200 ng of the BclI-linearized pT3cbh1 plasmid were mixed and incubated at 37 °C for 2 h. At the end of reaction, the samples were taken out for agarose gel electrophoresis analysis.

Transformation of T. reesei
For plasmid transformation, pPdc1-Cas9 and pRLMex30 (carrying the hygromycin B phosphotransferase gene) were co-introduced into the T. reesei QM9414 strain by polyethylene glycol (PEG)-mediated chemical transformation [20]. The transformants were selected on MM-glucose plates containing 150 μg/ml of hygromycin. For transformation of gRNA targeting ura5, 6.5 μg of in vitro transcribed gRNA were similarly introduced into T. reesei through the same PEG-mediated protoplast transformation method. MM-glucose supplemented with 5-fluoroorotic acid (5-FOA, 3 mg/ml) and 10 mM uridine was used to select the transformants. Expression of wild-type, functional ura5 (encoding orotate phosphoribosyl transferase) will convert 5-FOA into toxic 5′-fluorouridine monophosphate. For Cas9/gRNA transformation, 12 μg of the Cas9 protein and 6.5 μg of gRNA were mixed and incubated at 37 °C for 30 min for in vitro assembly into the Cas9/gRNA RNP complex. The complex was then co-transformed with 6 μg of the pSKpyr4 plasmid, which contains the pyr4 marker gene expression cassette [13], into the T. reesei TU-6 protoplasts.

Analyzing cbh1 disruption in the transformants
The spores (1 × 10⁸) of TU-6 and its transformants were individually inoculated into 100 ml of liquid MM-glucose (2%) and cultured at 28 °C with shaking for 48 h. Mycelia were collected by filtering through a 200-mesh sifter, washed with MM, and transferred into the MM-Avicel cellulose (2%) medium to induce cellulase expression. The fermentation broth on the eighth day after cellulose induction was analyzed by SDS-PAGE. The genomic DNAs of TU-6 and its transformants were extracted from the harvested mycelia and were used as templates for PCR amplification of the cbh1 locus. The PCR conditions were 94 °C for 1 min for initial denaturation, then 24 cycles including 94 °C for 25 s, 61 °C for 30 s (temperature reduced by 0.2 °C per cycle), and 70 °C for 5 min.

Gene replacement of cel3c
The Cas9 (12 μg) was in vitro assembled with 6.5 μg of gRNA targeting cel3c (GenBank accession number: AY281375.1) by incubation at 37 °C for 30 min. The donor DNA was consisted of two homology arms (1.0 kb each) flanking the cel3c locus. The homology arms were intercepted by an expression cassette for Melanocarpus albomyces laccase (GenBank accession number: CAE00180, under the control of cbh1 promoter and terminator) and the pyr4 marker gene. The Cas9/gRNA complex and 3 μg of the donor DNA were co-transformed into T. reesei protoplasts and the transformants were transferred into MM-lactose plate containing 0.4 mM ABTS and 0.1 mM CuSO₄. Laccase oxidizes ABTS and blue halos form around the laccase-expressing colonies. Laccase-positive transformants were grown on PDA plates for sporulation. Replacement of cel3c in these transformants was investigated using diagnostic PCR amplifying the fragments traversing across the chromosome and the cel3c editing locus.

Additional file

Additional file 1: Table S1. Primers used in this study. Figure S1. Sequencing the ura5 gene of QM9414, CS expressing Cas9 intracellularly, and the C5-derived transformants. (DOCX 1220 kb)

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Availability of data and materials
All data generated or analyzed during this study are included in this published article (and its supplementary information files)

Authors’ contributions
XS designed the study; ZH carried out the experiments. Both authors analyzed the data, wrote the manuscript, and approved the final manuscript.

Ethics approval
Ethics approval was not needed for the study.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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