A highly efficient identification of mutants generated by CRISPR/Cas9 using the non-functional DsRed assisted selection in *Aspergillus oryzae*

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Received: 2 February 2021 / Accepted: 18 June 2021 / Published online: 9 July 2021
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Abstract
The CRISPR/Cas9 system has become a great tool for target gene knock-out in filamentous fungi. It is laborious and time-consuming that identification mutants from a large number of transformants through PCR or enzyme-cut method. Here, we first developed a CRISPR/Cas9 system in *Aspergillus oryzae* using AMA1-based autonomously replicating plasmid and Cas9 under the control of the *Aspergillus nidulans gpdA* promoter. By the genome editing technique, we successfully obtained mutations within each target gene in *Aspergillus oryzae*. Then, we put the protospacer sequence of a target gene and its protospacer adjacent motif (PAM) behind the start codon “ATG” of DsRed, yielding the non-functional DsRed (nDsRed) reporter gene, and the nDsRed reporter gene could be rescued after successful targeted editing. Moreover, this method was also applied by targeting the kojic acid synthesis gene *kojA*, and the transformants with DsRed activity were found to harbor targeted mutations in *kojA*. These results suggest that the nDsRed can be used as a powerful tool to facilitate the identification of mutants generated by CRISPR/Cas9 in *Aspergillus oryzae*.

Keywords CRISPR/Cas9 · Identification mutant · DsRed · *Aspergillus oryzae*

Introduction
*Aspergillus oryzae* is used widely for food fermentation and industrial production of enzymes and secondary metabolites (Kobayashi et al. 2007; Machida et al. 2005). The development of genome engineering technology allows for improved quality and yield of production in *A. oryzae*. The clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system provides an overwhelming genome engineering tool for genetic modification of *A. oryzae* (Chutrakul et al. 2019; Fan et al. 2020; Katayama et al. 2019, 2016). The CRISPR/Cas9 system contains two components: the Cas9 nuclease and a single guide RNA (sgRNA) (Donohoue et al. 2018; Jiang and Doudna 2017; Lander 2016). sgRNA can guide the Cas9 nuclease to recognize and cut the target sequence that is just upstream of a protospacer adjacent motif (PAM) (Jiang and Doudna 2017). When DNA double-strand breaks (DSB) generated by Cas9 are repaired through non-homologous end joining (NHEJ) or homology-directed repair (HDR) mechanisms, random insertions or deletions of nucleotides (InDels) are introduced at the target sites (Wang and Coleman 2019). The CRISPR/Cas9 system has been applied to various filamentous fungi, such as *A. oryzae*, *A. niger*, *A. aculeatus* (Kadooka et al. 2020; Nodvig et al. 2018, 2015; Song et al. 2018; Song, 2019; Weyda et al. 2017; Zhang et al. 2016). The fungi modified by CRISPR/Cas9 system need to be screened rapidly to meet the need of industrial production and functional study.

The methods for mutant identification have been developed based on the PCR, restriction enzyme and sequencing. For example, the PCR/restriction enzyme (PCR/RE) assay requires the amplification and digestion of available restriction enzyme sites in the target sites. Once the restriction enzyme sites in the target sites are destroyed by editing,
the mutants are identified due to the indigestion of the target sites (Shan et al. 2014). Similarly, the T7EI assay takes advantages of the bacteriophage resolvase T7E1 that has the greater cleavage activity on mismatches of the target sites introduced by editing (Vouillot et al. 2015). Although sequencing can accurately display the mutation types of mutants, it is costly and laborious for batch screening of mutants. One good way to rapidly identify mutants is to directly observe the fluorescence from materials. It has been reported that the green fluorescence protein (GFP) reporter gene is fused to Cas9 coding gene to obtain transformed materials (Osakabe et al. 2018), but it is impossible to distinguish the transformed materials from a large number of materials just on the basis of the fluorescence. Therefore, it is very necessary to develop a highly efficient method to identify mutants generated by CRISPR/Cas9.

Here, we firstly constructed the AMA1-based genome-editing system and evaluated the feasibility of the CRISPR/Cas9 system using kojic acid synthesis genes (kojA, kojR and kojT). Then we developed the non-functional DsRed (nDsRed) by putting the sgRNA and the PAM sequences after the start codon of DsRed and used it as the reporter to help screen mutants edited by CRISPR/Cas9 in A. oryzae. Loss of function of kojA that functions in kojic acid synthesis results in the inhibition of kojic acid production in A. oryzae (Terabayashi et al. 2010). In this study, the kojA gene as the target to test our method in A. oryzae, we constructed AMA1-based genome-editing plasmids for the mutagenesis of kojA, pPRTII-Cas9-kojA, which allowed for autonomous plasmid replication and not being integrated into the genome of A. oryzae. Meanwhile, the nDsRed containing the sgRNA and the PAM sequences of kojA was inserted into pEX2B vector to generate pEX2B-kojA-nDsRed plasmid. The plasmids were co-transformed into the uridine/uracil auxotrophic mutant of 3.042 through the PEG-protoplast method. The fungal strains were cultured at 30 °C.

**Materials and methods**

**Microorganisms and cultivations**

The A. oryzae 3.042 strain (CICC 40092) was used as wild-type strain. The recipient strain for fungal transformation was from the A. oryzae 3.042 strain with the destruction of the pyrG gene (Fan et al. 2020). Czapek-Dox (CD) medium (2% soluble starch, 0.2% NaNO₃, 0.1% KH₂PO₄, 0.05% MgSO₄, 0.05% KCl, 0.05% NaCl, 0.002% FeSO₄, pH 5.5) was used to culture fungal strains. M + Met medium (0.2% NH₄Cl, 0.1% (NH₄)₂SO₄, 0.05% KCl, 0.05% NaCl, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, 0.002% FeSO₄·7H₂O, 2% glucose, 0.15% methionine, pH 5.5) was used for fungal transformation. The fungal strains were cultured at 30 °C.

**Plasmid construction**

For the CRISPR/Cas9 vector, the U6 promoter (PU6) was amplified using the genomic DNA of the A. oryzae 3.042 strain as the template with the forward primer PU6-F and reverse primer PU6-kojA/kojR/kojT-containing the protospacer sequences targeting for kojA/kojR/kojT, generating PU6-kojA/kojR/kojT. The U6 terminator and sgRNA sequence (sgRNA-TU6) were synthesized by GenScript gene synthesis, and then used as the template to amplify with the primers, kojA/kojR/kojT-TU6-F and TU6-R to attach the protospacer sequence of kojA/kojR/kojT at the N-terminal end of sgRNA-TU6, generating kojA/kojR/kojT-sgRNA-TU6. The above two fragments, PU6-kojA/kojR/kojT and kojA/kojR/kojT-sgRNA-TU6, both including the protospacer sequence of kojA/kojR/kojT, were fused and then cloned into the AMA1-based pPTRII-Cas9 vector containing the Cas9 under the control of the A. nidulans gpdA promoter (PgdA) and the pyrithiamine resistance marker ptrA, yielding pPRTII-Cas9-kojA/kojR/kojT. To construct the nDsRed reporter vector, the nDsRed reporter gene where the protospacer sequence of kojA (20 bp) and its protospacer adjacent motif (PAM) were inserted just downstream of the start codon of DsRed was amplified by PCR with its specific primers, nDsRed-kojA-F and nDsRed-kojA-R at the 5' end of which the protospacer sequence of kojA and its PAM were added. The fragment nDsRed-kojA was cloned into the linearized vector pEX2B with the A. oryzae amyB promoter (AmyB) (Nguyen et al. 2017), generating the plasmid pEX2B-nDsRed-kojA. The resulting plasmids, pPRTII-Cas9-kojA and pEX2B-nDsRed-kojA were co-transformed into the uridine/uracil auxotropic mutant of 3.042 through the PEG-protoplast method.

**PEG-mediated transformation**

Transformation of A. oryzae was performed using the PEG-protoplast method previously described (Maruyama and Kitamoto 2011). Briefly, the strains were grown in DPY liquid medium (2% dextrin, 1% polypeptone, 0.5% yeast extract, 0.5% KH₂PO₄, 0.05% MgSO₄·7H₂O, pH 5.5) for 16–20 h. Then samples of the mycelium were collected and washed with the enzyme buffer (50 mM maleic acid, 0.6 M (NH₄)₂SO₄, pH 5.5). Protoplasts were separated from the harvested mycelium by treatment with 1% Yatalase (TaKaRa) and 1.5% lysing enzymes (Sigma). The obtained protoplasts were resuspended with the wash
buffer (1.2 M sorbitol, 50 mM CaCl₂·2H₂O, 35 mM NaCl, 10 mM Tris–HCl, pH 7.5), followed by mixing with the plasmids (10 μg) in the PEG buffer (60% PEG 4000, 50 mM CaCl₂·2H₂O, 10 mM Tris–HCl, pH 7.5). After incubation at room temperature for 10–20 min, the PEG-treated protoplasts were diluted with the wash buffer and pelleted by centrifugation at 1000 rpm for 8 min at 4 °C. The PEG-treated protoplasts were suspended in M+Met medium containing 1.2 M sorbitol and 0.5% agar. The soft agar suspension (10 mL) was laid over a M+Met agar plate containing 1.2 M sorbitol and 1.5% agar. The plate was incubated at 30 °C for 3–5 days.

Detection of mutations

Conidial suspension (10⁸/ml) of mutants of kojic acid synthesis genes and the control wild-type strain was inoculated in CD liquid medium to detect the content of kojic acid. After cultivation for seven days, the supernatant was collected by the centrifugation at 10,000 rpm for 5 min and the concentration of kojic acid was measured by the colorimetric method (Terabayashi et al. 2010). The presence of red color formed by chelate reaction between kojic acid and FeCl₃ indicated the production of kojic acid, which also was used to observe kojic acid. The target genes were amplified using the primers listed in Table S1 to detect targeted mutations in each gene. The primers pEX2B-Sequence-F/pEX2B-Sequence-R were used to identify the mutations of target sites in nDsRed genes.

Detection of DsRed

The DsRed fluorescence of transformants was detected using ZOE™ Fluorescent Cell Imager (BIO-RAD) with excitation at 556 ± 20 nm, emission at 615 ± 61 nm.

Results

Construction of AMA1-based genome-editing system

The CRISPR/Cas9 system included a CRISPR-associated Cas9 endonuclease and a guide RNA (gRNA). Our strategy was to integrate the Cas9 and gRNA into an AMA1-based autonomously replicating plasmid that was expressed in A. oryzae. Firstly, the optimized Cas9 was synthesized and inserted between the gpdA promoter and trpC terminator in pEX1, yielding pEX1-Cas9 vector (Fig. S1). Then the Cas9-expression cassette was extended by PCR using primers pEX1-Cas9-F and pEX1-Cas9-R, which contained gpdA promoter, Cas9, and trpC terminator, and the amplicon was inserted into the HindIII site of the plasmid pPTRII (Takara), generating pPTRII-Cas9 plasmid (Fig. 1). The sgRNA sequence including protospacer sequences of a target gene was fused with the U6 promoter and terminator and then inserted into pPTRII-Cas9, producing pPTRII-Cas9-target-gene vector (Fig. 1).

Assessment of AMA1-based genome-editing system

To assess the feasibility of the CRISPR/Cas9 system, we selected to mutate the kojic acid synthesis genes (kojA, kojR and kojT), mutagenesis of which caused a sharp decrease in kojic acid production that was visualized by the color reaction. kojA and kojT encode a FAD-dependent oxidoreductase and a major facilitator superfamily transporter, respectively (Terabayashi et al. 2010). The transcription factor kojR is presumed to be involved in kojic acid production through regulating kojA and kojT (Marui anf Kitamoto 2011). The protospacer sequences from kojA, kojR and kojT were introduced into pPTRII-Cas9, producing the plasmids pPTRII-Cas9-kojA, pPTRII-Cas9-kojR and pPTRII-Cas9-kojT, respectively. They were transformed into A. oryzae strain 3.042. Transformants were selected on CD agar medium with 0.1 μg/ml pyrithiamine. The transformants exhibited the expected phenotypes that the successfully edited strains all had a decrease in the production of kojic acid (Fig. 2D, E). Nucleotide sequencing of transformants revealed that deletion or insertion mutations occurred near the PAM sequences and seven types of mutations were found in the total of 12 sequenced transformants (Fig. 2A–C). Based on the sequencing of four transformants for each target gene, mutational rates were 50%, 75% and 50% for kojA, kojR and kojT, respectively.

Application of the nDsRed reporter gene and AMA1-based genome-editing system in A. oryzae

To examine the feasibility of nDsRed that can be recovered by targeted editing, we used the kojic acid synthesis gene kojA to test the nDsRed reporter system. The genome-editing plasmid pPTRII-Cas9-kojA and the reporter vector pEX2B-nDsRed-kojA were constructed using the basic steps shown in Fig. 3A. These two vectors were cotransformed into the uridine/uracil auxotrophic mutant of 3.042. The DsRed signals of the transformants were detected, whereas no DsRed fluorescence was found in the transformants only with pEX2B-nDsRed-kojA vector (Fig. 3B), indicating that the recovery of nDsRed activity could be regarded as an indicator of successful genome editing.
Identification of mutants according to the DsRed fluorescence in *A. oryzae*

The pEX2B-nDsRed-kojA reporter vector and pPTRII-Cas9-kojA vector were cotransformed into *A. oryzae* 3.042 strain. After transformation, fourteen candidate transformant strains were selected to identify mutants through the recovery of DsRed. Among the fourteen candidates, eight candidates with pEX2B-nDsRed-kojA construct were detected with DsRed signal (Fig. 4). All candidate transformants were further analyzed through sequencing the target site within nDsRed. The eight candidate transformant strains with DsRed fluorescence exhibited two different types of mutations in nDsRed, kojA-1 had 6-bp insertion before the translation start codon and 23-bp fragment deletion while kojA-2, kojA-3, kojA-4, kojA-5, kojA-9, kojA-10 and kojA-12 exhibited 1-bp insertion (Fig. 5A). Meanwhile, the six candidates without DsRed fluorescence were also sequenced. The sequencing results showed that the nDsRed in the four transformants (kojA-6, kojA-7, kojA-8 and kojA-11) contained 6-bp insertion, 23-bp fragment deletion and 2-bp insertion occurred, leading to the termination of translation, and the remaining two candidates kojA-13 and kojA-14 had no mutations (Fig. 5A). To further confirm whether *kojA* was edited in all fourteen transformants, the candidates were sequenced in *kojA*. The sequencing results displayed that all candidates had mutations except for kojA-3, kojA-12, kojA-13 and kojA-14 transformants (Fig. 5B). Various mutation patterns in *kojA* were detected, including 1-bp insertion or deletion, short deletions of up to 51 bps, and 64 bps insertion (Fig. 5B). These results indicate that the rescued DsRed fluorescence could be regarded as an indicator of mutant identification.

**Fig. 1** Construction of AMA1-based genome-editing plasmid. The Cas9 expression cassette (PgpdA-Cas9-TtrpC) was amplified from the intermediate vector pEX1-Cas9 and inserted into the HindIII site of pPRTII, producing pPRTII-Cas9 plasmid. Then the fragment of U6 promoter and terminator with the sgRNA sequence of a target gene was ligated with the Smal-digested pPRTII-Cas9 by fusion reaction, yielding the AMA1-based genome-editing plasmid pPRTII-Cas9-target-gene.
Discussion

In this study, we have constructed an autonomous replicating genome-editing vector for *A. oryzae* (Figs. 1, 2). The remarkable features of this vector are that it isn’t integrated into the recipient genome by the application of the *AMA1* gene and it isn’t affected by carbon source because of Cas9 controlled by the *gpdA* promoter, which has advantageous to the expression of Cas9 and sgRNA of the target gene and reduces the influence of culture condition, respectively, distinct with the previous reports on genome-editing in *A. oryzae* (Katayama et al. 2019, 2016). The successful mutagenesis of kojic acid synthesis genes by the genome-editing system proves the feasibility of the scheme (Fig. 2).

Give that it is laborious and time-consuming to screen mutants from a large number of transformants in *A. oryzae* by Sanger sequencing, the scheme is set up to help the rapid identification of mutants through the recovered DsRed fluorescence (Fig. 3). Based on the results, it is easy to obtain desired mutants by detecting DsRed signal in the transformants (Figs. 4, 5). These results indicate that the rescued fluorescence is an effective method for mutant identification in *A. oryzae*. The notable characteristic of the method is simple, only containing the genome-editing vector and reporter vector (Fig. 3). It doesn’t need to construct complex vectors, and among the two vectors, the reporter vector can be from any expression vectors with a reporter gene, such as *GFP*, *LUC* and *GUS*. Therefore, this method is also able to be widely applied in other species. Additionally, due to *Aspergillus* having a large number of dispersed spores, it often happens that transformants were contaminated by other spores, resulting in the failure of transformation. The recovered DsRed signal allows us to guard against other conidia contamination.

The mutation efficiency is one of our focuses in CRISPR/Cas9 system (Doench et al. 2014; Haeussler et al. 2016; Hsu et al. 2013). It has been reported that editing efficiency depends on protospacer sequences (Labuhn et al. 2018; Malina et al. 2015). Also, the GC content and secondary structure of protospacer sequences affect the editing efficiency (Ma et al. 2015; Tsai et al. 2015). Herein, nDsRed of *kojA* in the twelve transformants (*kojA*-1 ~ *kojA*-12) was edited whereas *kojA* of *kojA*-3 and *kojA*-12 in the twelve candidate transformant strains wasn’t edited (Fig. 5A). Furthermore, in the fourteen transformants, the edited nDsRed only displayed three different types of mutations while seven mutation patterns were observed in *kojA*, and there was no the occurrence of identical mutations in *kojA* gene and nDsRed-kojA (Fig. 5), suggesting that the genome site of a target gene is independent on the plasmid-borne target site and they only share the same protospacer sequence for target gene that to some extent determines whether the target sequence is edited, and the mutation patterns might be affected by the sequence adjacent to protospacer sequence. There is another important factor that the unsynchronization of genome editing of the target gene and nDsRed, producing false-positive. The introduction of protospacer sequences
of a target gene into DsRed results in two target sites for genome editing, one is DsRed with protospacer sequences of the target gene, the other is the target gene. If the target site within DsRed is edited, rescuing the DsRed fluorescence, but the target gene isn’t edited, the candidate is false-positive, for example, kojA-3 and kojA-12 transformants. Additionally, the target gene and nDsRed are both edited, but nDsRed with frame-shift mutations can’t give birth to functional DsRed protein, causing that the mutants are not able to be identified. However, the deficiency of the

Fig. 3 The system of the non-functional DsRed-assisted identification of mutants generated by CRISPR/Cas9. A Schematic diagram of the construction of genome-editing vector and reporter vector pEX2B-nDsRed for target gene to co-transform into pyrG auxotrophic A. oryzae strain 3.042 with the protoplast method. B Detection of DsRed fluorescence in transformants with pEX2B-nDsRed-kojA reporter vector (serve as the control) or with pEX2B-nDsRed-kojA reporter vector and pPRTII-Cas9-kojA genome-editing vector
non-functional DsRed assisted selection is made up by the large-scale screening of mutants.

Collectively, this study provides the system of AMA1-based genome-editing and the identification mutant produced by CRISPR/Cas9 using non-functional DsRed assisted selection in *A. oryzae*. The possible mutants are rapidly isolated from an enormous number of transformants through the recovered DsRed fluorescence, and the

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**Fig. 4** Detection of DsRed signal in transformants with pEX2B-nDsRed-kojA and pPRTII-Cas9-kojA vectors. The fourteen candidate transformant strains with pEX2B-nDsRed-kojA and pPRTII-Cas9-kojA vectors (kojA-1 ~ kojA-14) were cultured on CD agar medium with 0.1 μg/mL pyrithiamine for three days at 30 °C. Then the mycelia were observed under fluorescence microscope. Scal bars = 100 μm
candidates with DsRed fluorescence can be used for further investigation. The use of our scheme will promote the large-scale screening of mutants generated by CRISPR/Cas9 in A. oryzae and accelerate the research of functional genes of A. oryzae.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11274-021-03100-8.

Acknowledgements We thank Dr. Van-Tuan Tran for kindly providing the plasmids pEX1 and pEX2B. This work was supported by Natural Science Foundation of China (32000049), Jiangxi Provincial Natural Science Foundation (20202BAB213042), Science and Technology Research Project of Jiangxi Provincial Department of Education (GJJ190611), and Doctoral Scientific Research Foundation of Jiangxi Science and Technology Normal University (2018BSQD030).

Fig. 5 Identification of mutations in nDsRed gene and kojA in the transformants with pEX2B-nDsRed-kojA and pPRTII-Cas9-kojA vectors (kojA-1 ~ kojA-14). A The schematic of nDsRed-kojA indicates in red black. The kojA-sgRNA sequence and its protospacer adjacent motif (PAM) were put behind the start codon “ATG” of DsRed, generating nDsRed-kojA. The mutations indicate in red and the numbers of mutations are shown on the right of target sequences.

The start codon “ATG” is indicated in black box. B Targeted mutation of kojA using CRISPR/Cas9 system with the nDsRed reporter gene. The target site is indicated on the schematic of kojA. The protospacer and PAM sequence for kojA indicate in blue, and PAM sequence is underlined. In the target sequences of kojA, the inserted and deleted nucleotides are indicated in red. WT, wild-type

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals experiments.
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