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A Highly Efficient Identification of Mutants Generated by CRISPR/Cas9 Using the Non-Functional DsRed Assisted Selection in *Aspergillus Oryzae*

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Research Article

Keywords: CRISPR/Cas9, Identification mutant, DsRed, Aspergillus oryzae

Posted Date: February 18th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-202275/v1

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Version of Record: A version of this preprint was published at World Journal of Microbiology and Biotechnology on July 9th, 2021. See the published version at https://doi.org/10.1007/s11274-021-03100-8.

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*.

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; Katayama et al. 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; Nodvig et al. 2015; Song et al. 2018; Song et al. 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 destructed 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 identity 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*, pPRT-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-transformated into the uridine/uracil auxotrophic *A. oryzae* strain 3.042. Among the transformed strains with DsRed fluorescence, the transformants with DsRed signal were identified as desired mutants. These results demonstrated that the CRISPR/Cas9/nDsRed system can be used as an overwhelming screening tool to help identify mutants generated by CRISPR/Cas9 in *A. oryzae* during the mutant screening procedure.

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. Czapek-Dox (CD) medium (2% maltose, 0.2% $NaNO_3$, 0.1% KH_2PO_4, 0.05% $MgSO_4$, 0.05% KCl, 0.05% NaCl, 0.002% $FeSO_4$, pH 5.5) was used to culture fungal strains. M+Met medium (0.2% NH_4Cl, 0.1% $(NH_4)_2SO_4$, 0.05% KCl, 0.05% NaCl, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.002% $FeSO_4 \cdot 7H_2O$, 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-R 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. nidulansgpdA* promoter (P*gpdA*) and the pyrithiamine resistance marker ptrA, yielding pPTRII-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 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 (P*amyB*), generating the plasmid pEX2B-nDsRed-kojA. The resulting plasmids, pPTRII-Cas9-kojA and pEX2B-nDsRed-kojA were co-transformed into the uridine/uracil auxotrophic 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. Briefly, the strains were grown in DPY liquid medium (2% dextrin, 1% polypeptone, 0.5% yeast extract, 0.5% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 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 $(NH_4)_2SO_4$, 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 $CaCl_2 \cdot 2H_2O$, 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 $CaCl_2 \cdot 2H_2O$, 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 1,000 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

CD medium was used to culture mutants of kojic acid synthesis genes for seven days. Red color formation was observed and the concentration of kojic acid was measured by the colorimetric method. 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. The transcription factor kojR is presumed to be involved in kojic acid production through regulating *kojA* and *kojT*. 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 transformated 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). 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 as described above (Fig. 3A). These two vectors were cotransformated 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 ony 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 cotransformated into *A. oryzae* 3.042 strain. After transformation, four transformants were selected to identify mutants through the

recovery of DsRed. Among the four candidates, two candidates with pEX2B-nDsRed-kojA construct were detected with DsRed signal (Fig. 4A). The four transformants were further analyzed through sequencing the target site within nDsRed. The two mutants (Δ*kojA*-1 and Δ*kojA*-2) with DsRed fluorescence exhibited two different types of mutations in nDsRed, Δ*kojA*-1 had 6-bp insertion before the translation start codon and 23-bp fragment deletion, Δ*kojA*-1 exhibited 1-bp insertion (Fig. 4B). To further confirm whether *kojA* was edited in Δ*kojA*-1 and Δ*kojA*-2, the two candidates were also sequenced in *kojA*. The results showed that Δ*kojA*-1 and Δ*kojA*-2 exhibited 1-bp insertion and 51-bp fragment deletion, respectively, resulting in frameshift mutation of *kojA* (Fig. 4C). These results indicate that the rescued DsRed fluorescence could be regarded as an indicator of mutant identification.

Discussion

In this study, we have constructed an autonomous replicating genome-editing vector for *A. oryzae* (Figs. 1 and 2). The remarkable features of this vector are that it doesn't be integrated into the recipient genome by the application of the *AMA1* gene and it doesn't be 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; Katayama et al. 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 (Fig. 4). 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). These effect factors also affect the system of identification mutant through the recovered DsRed activity. 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 doesn't be edited, the candidate is false-positive. 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 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 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*.

Declarations

Acknowledgements

We thank Dr. Van-Tuan Tran for kindly providing the plasmids pEX1 and pEX2B. This work was supported by Science and Technology Research Project of Jiangxi Provincial Department of Education (GJJ190611), Jiangxi Provincial Natural Science Foundation (20202BABL213042), Natural Science Foundation of China (32000049) and Doctoral Scientific Research Foundation of Jiangxi Science and Technology Normal University (2018BSQD030).

Compliance with ethical standards

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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Figures

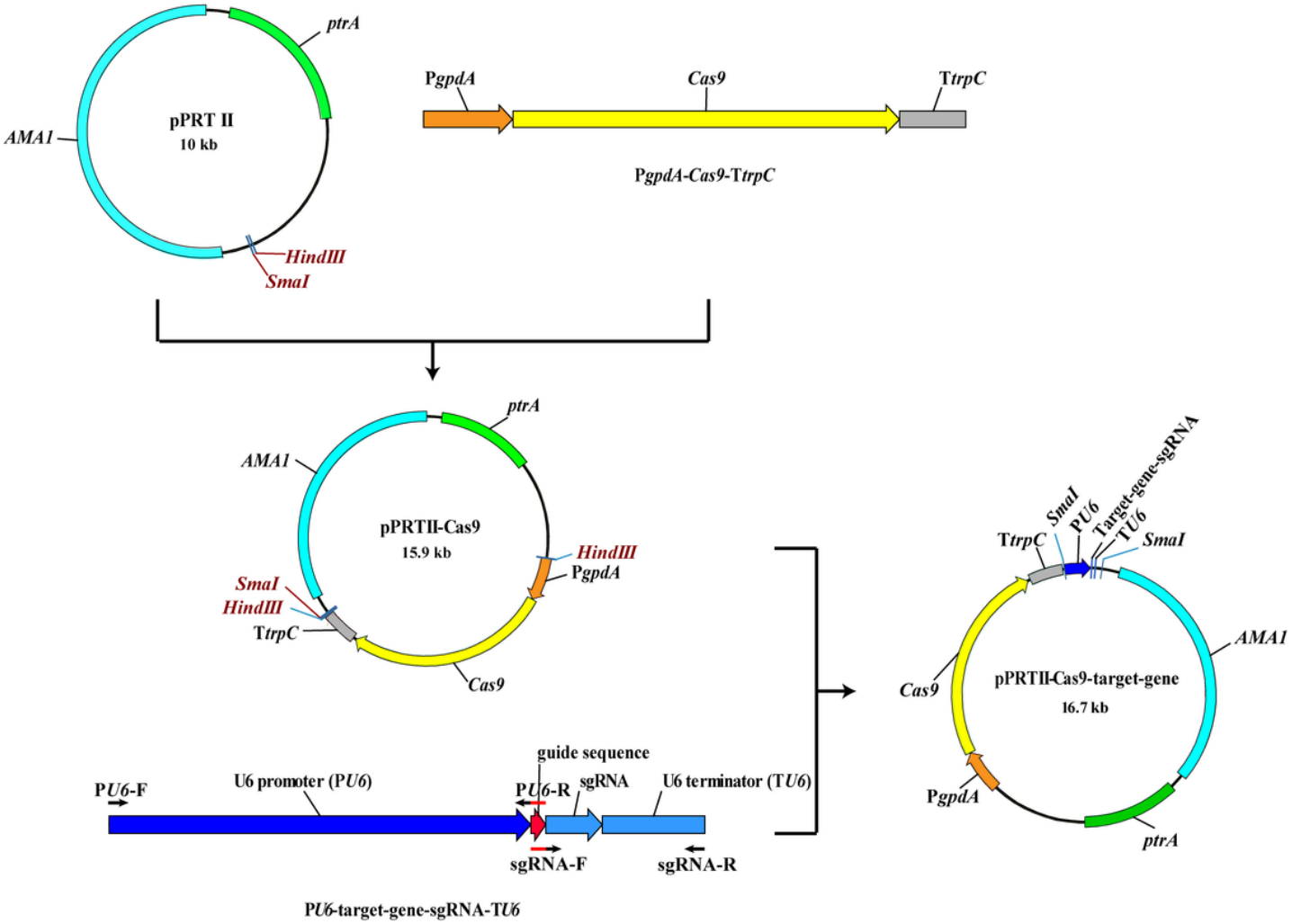

Figure 1

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 SmaI-digested pPRTII-Cas9 by fusion reaction, yielding the AMA1-based genome-editing plasmid pPRTII-Cas9-target_gene.

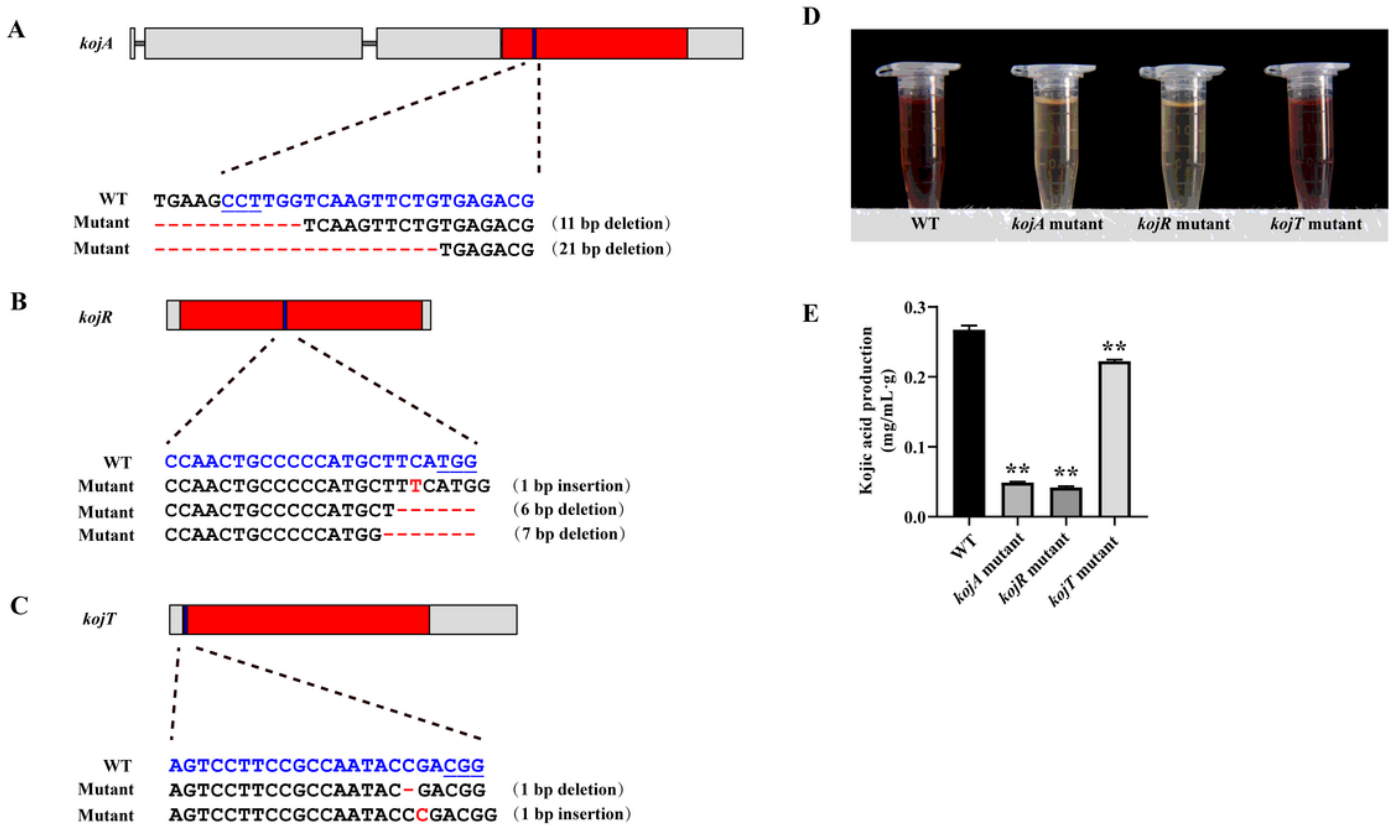

Figure 2

Mutagenesis of kojic acid synthesis genes (kojA, kojR and kojT) of A. oryzae strain 3.042. Nucleotide sequences of target region of mutants of kojA (A), kojR (B) and kojT (C). (D, E) Color reaction and quantification of kojic acid in wild-type (WT) and mutants of kojA, kojR and kojT.

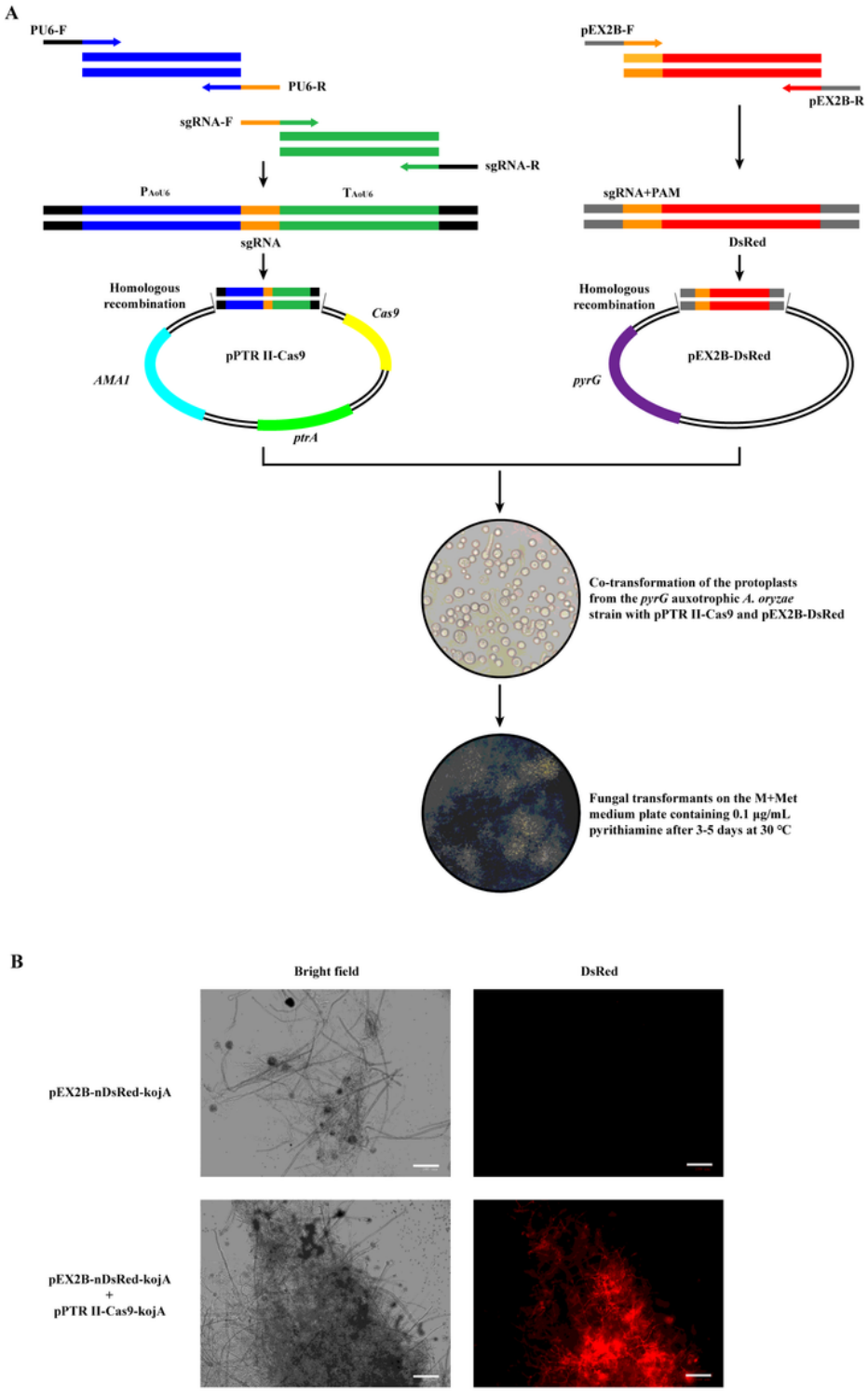

Figure 3

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 used for co-transformation in 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.

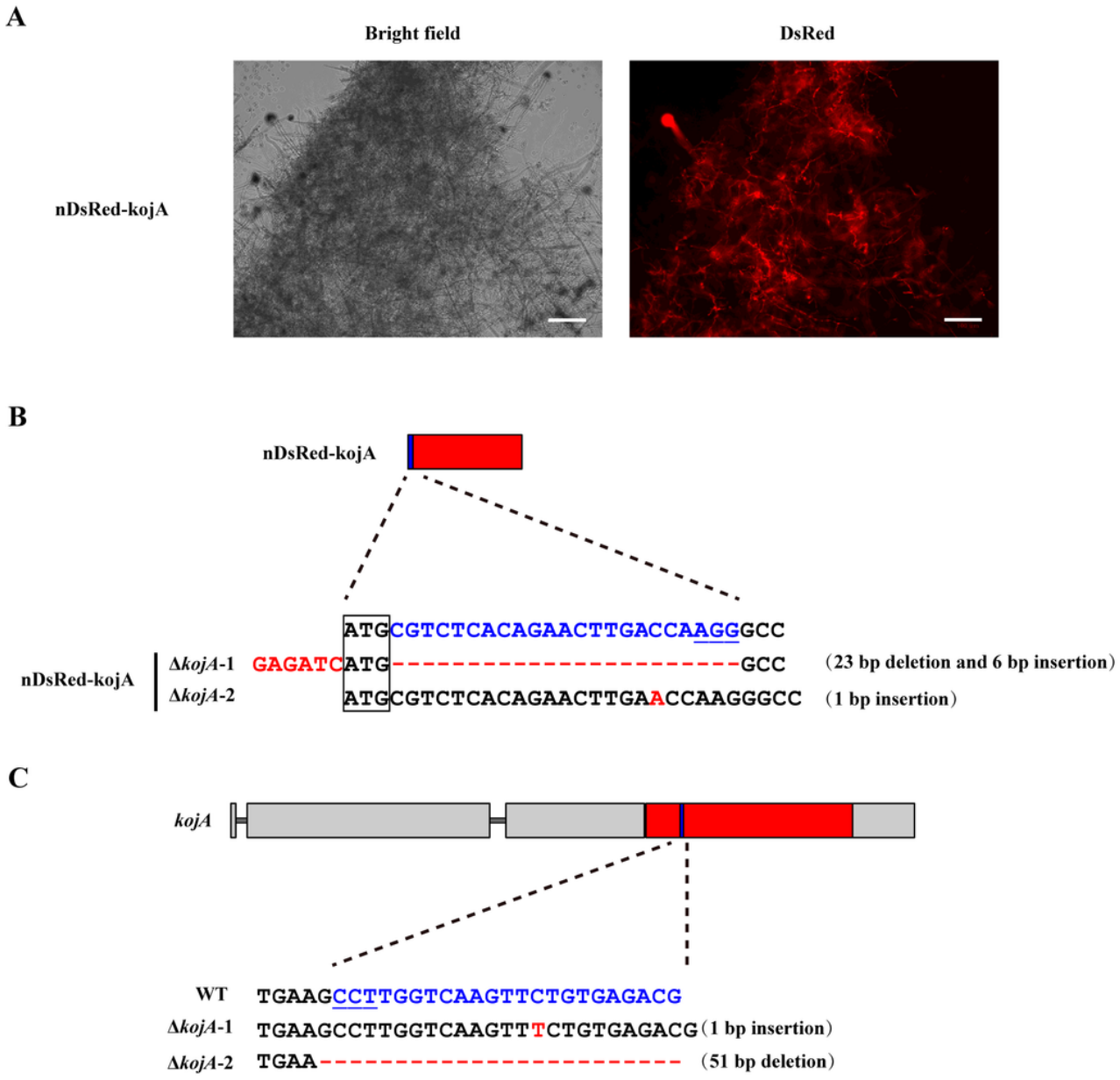

Figure 4

Targeted editing using CRISPR/Cas9 system with the nDsRed reporter gene in A. oryzae. (A) Detection of DsRed signal in mutants of A. oryzae transformed with pEX2B-nDsRed-kojA and pPRTII-Cas9-kojA vectors, Scal bars = 100 μm. (B) Identification of mutations in nEGFP gene in mutants with pEX2B-nDsRed-kojA and pPRTII-Cas9-kojA vectors (ΔkojA-1 and ΔkojA-2). The schematic of nDsRed-kojA indicates in red black. The mutations indicate in red and the number of mutations is shown on the right of target sequences. The start codon “ATG” is indicated in black box. (C) Targeted mutation of kojA using CRISPR/Cas9 system with the nDsRed reporter gene. The target site is indicated on the schematic of

kojA. Protospacer and PAM sequence 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.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarydata.docx