A Novel Site-Specific Integration System for Genetic Modification of Aspergillus flavus

Fang Tao,*1,2 Kai Zhao,*1 Qianqian Zhao,*1 Fangzhi Xiang,* and Guomin Han†
*School of Life Sciences, Anhui Agricultural University, Hefei 230036, P. R. China and †The National Engineering Laboratory of Crop Stress Resistance Breeding, Anhui Agricultural University, Hefei, China
ORCID ID: 0000-0002-6123-2134 (F.T.)

ABSTRACT Aspergillus flavus is a fungus that produces aflatoxin B1, one of the most carcinogenic secondary metabolites. Understanding the regulation mechanism of aflatoxin biosynthesis in this fungus requires precise methods for genomic integration of mutant alleles. To avoid the disadvantage of DNA integration into the genome by non-homologous or ectopic recombination, we developed a novel strategy for site-specific integration of foreign DNA by using a carboxin-resistant sdh2R allele (His 249 Leu). Our results demonstrated that the transformants were generated with a high efficiency (>96%) of correct integration into the sdh2-locus of the genome of A. flavus NRRL 3357. The advantage of this method is that introduction of the eGFP expression cassette into the sdh2-locus had little effect on fungal growth and virulence while also being rapid and efficient. This system will be a valuable tool for genetic manipulation in A. flavus. To the best of our knowledge, this is the first report on the efficient site-specific integration at the sdh2-locus in the genome of Aspergillus.

Homologous recombination is a powerful tool for generating stable gene replacement mutants in fungal genetic transformation systems, however, most exogenous DNA sequences are integrated into the genome by non-homologous, ectopic recombination. Such random ectopic integration can often disrupt the structure of non-target genes resulting in unpredictable effects on gene expression. In practice, the low frequency of homologous recombination makes the construction of gene disruptants in fungi a laborious process (Takahashi et al. 2010; Koh et al. 2014). However, while use of such ku70/ku80 deleted mutants as recipient strains increases gene-targeting frequency, a second round of complementation with native ku70/ku80 genes is needed before investigation of the deletion phenotype for the gene of interest. This additional transformation step is costly and time consuming, a disadvantage that can be surmounted by site-specific integration of genetic constructs without disrupting other regions of fungal genomic DNA (Weld et al. 2006).

Mitochondrial succinate dehydrogenase (SDH) is a key enzyme in the tricarboxylic acid cycle. Another name of the enzyme is succinate:ubiquinone reductase (SQR) as it catalyzes the coupled reactions of succinate oxidation to fumarate and the reduction of ubiquinone to ubiquinol. The SDH complex normally consists of a soluble catalytic heterodimer containing a flavoprotein subunit (SdhA/SdhFp), an iron-sulfur protein subunit (SdhB/Sdi1/Sdh2/Ip), and two hydrophobic polypeptides (SdhC and SdhD). Structural analyses have revealed that the ubiquinone binding site is composed of residues that are contributed from each of the SdhB, SdhC and SdhD subunits. The systemic fungicide carboxin inhibits the enzyme activity of succinate dehydrogenase by binding to the same site as ubiquinone (Horsefield et al. 2006; Huang et al. 2006). A single histidine-to-leucine point mutation in the third cysteine-rich cluster of the SdhB subunit confers resistance to carboxin and was first used as dominant selectable marker in Ustilago maydis (Broomfield and Hargreaves 1992; Keon et al. 1991). This valuable selection marker was then used in several other fungi, such as Zymoseptoria tritici (Kilaru et al. 2015), Magnaporthe oryzae (Guo et al. 2016), Mortierella alpinae (Ando et al. 2009), Aspergillus oryzae (Shima et al. 2009), and a few mushrooms (Herzog et al. 2019; Shang et al. 2018). Although point mutations in either SdhC or SdhD were also shown to confer carboxin resistance (Ito et al. 2004), the SdhB-type mutations exhibited the strongest resistance (Shima et al. 2009).
Table 1 Primers used in this study

| Primer name | Primer sequences (5' → 3') | Remark |
|-------------|-----------------------------|--------|
| P101        | TGGCAGGATATTTTGTGTAACAAATTAGGTATCTGTTGGAACCTGTG | sdh2 left flank 592 bp sdh2 |
| P102        | CAGTTAAGATGTTGAGGCAACG | coding sequence |
| P103        | CTACGGTTGCTCCACATTTC | 103 bp of 3' end sdh2 gene |
| P104        | CAGTTACGGAACAAAGGTC | and 335 bp downstream of the sdh2 gene |
| P21         | CCGATTCTTCGACCTTTTCACTGTGTTTGGCCGATACTGTTGCCCTCTCCCTAAG | tel1 promoter |
| P22         | GTGTTAAGCCTTCCGCCTGGCTCATTTGGAAGTTGCTGAAGCAGTACAAAAAGGTTACCTAATTGACTG | eGFP |
| P31         | ATGGTGACGAAGGGACGGAAG | trpC terminator |
| P32         | TTACCTTGACAGCTGCTCGATG | right flank of sdh2 |
| P41         | AACTCTCGGACCTGACGGGACTGTAACAITGAAAGAGCTCACATACTACTGACTG | URA3-2micron (μ) origin |
| P42         | TCCGGGGCTCCATCAACTTCTCACATGTCGATGGAATGATGGAAGTG | fragment amplification |
| P43         | CATGTAAGGTATTAGTCG | Genomic PCR amplification |
| P44         | P45         | | DNA hybridization probes |
| P46         | | | amplification |

^Italics indicate part of the primer that is complementary with another DNA fragment, to be ligated by homologous recombination in S. cerevisiae. Underlined letters indicated enzyme digestion site of Sac II.

A. flavus often produce aflatoxins with highly severe toxicity to human and animals (Han et al. 2019). Genetic manipulation, via gene knock-in or knock-out schemes, is an effective method to understand the regulation mechanisms of aflatoxin biosynthesis. Established methods for the transformation of A. flavus NRRL 3357 rely on either Polyethylene glycol (PEG)-mediated protoplast transformation or Agrobacterium tumefaciens-mediated transformation (ATMT) (Han et al. 2018; He et al. 2007). Although the recently established ATMT is more efficient than the PEG-mediated transformation method, foreign DNA sequences are still integrated randomly into the genome (Han et al. 2018). In the present study, based on the ATMT system, we used the sdh2 gene to create a specific locus as a "soft-landing" site for single copy insertions into the A. flavus genome. The pFC-eGFP vector designed for site-specific homologous recombination and harboring the sdh2 mutant was established in A. flavus via ATMT. The coding region for eGFP was used as the foreign DNA sequence and carboxin as selection marker in this system. Here, we also provide a yeast recombination cloning strategy to assemble DNA fragments in a single step for high throughput vector generation.

To our knowledge, this is the first report utilizing the robustness of the yeast recombination cloning approach in combination with efficient site-specific integration into the genome of an Aspergillus species. This system will be a powerful tool for high throughput functional genomics studies in A. flavus.

MATERIALS AND METHODS

Strains and culture conditions

Escherichia coli strain DH5α was used for vector cloning and plasmid maintenance. A. tumefaciens strain AGL-1 was used for A. flavus transformation. E. coli and A. tumefaciens strains were grown in DYT media (tryptone, 16 g/l; yeast extract, 10 g/l; NaCl, 5 g/l; with 15 g/l agar added for preparing the plates) at 37° and 28° respectively.

Saccharomyces cerevisiae strain FY834 (MATα; his3Δ100; ura3-52; leu2Δ1; lys2Δ202) was used for recombination-based cloning (Kilaru et al. 2015). The strain was refreshed on YPD agar medium (yeast extract, 10 g/l; glucose, 20 g/l; peptone, 20 g/l) at 28° for 48 h, and then used for preparing the plates) at 37° and 28° respectively.

Construction of a yeast-escherichia-agrobacterium shuttle vector pUM

The plasmid pUM is a vector built on the framework of the binary shuttle vector pUM2 (Chang et al. 2017) was used for observation of sclerotium formation. Aflatoxin analysis was carried out on strains grown on YES medium (20 g/l yeast extract, 150 g/l sucrose, 15 g/l agar). For the carboxin sensitivity test, fungal spores were point inoculated onto MM (Czapec-Dox Broth (Difco) + 1.5% agar) plates with different concentrations of carboxin and cultured at 30° for 3 days. Wickerham medium (Chang et al. 2017) for 3 days. Wickerham medium (Chang et al. 2017) was used for construction of targeted ectopic integration vector pFC-eGFP

The pFC-eGFP vector was generated by in vivo recombination in the yeast strain FY834 following published procedures (Guo et al. 2016). To facilitate assembly of the DNA fragments in yeast, primers were designed with 30 bp DNA sequences homologous to the upstream and downstream of regions of the adjacent fragments to be combined together. The resulting pFC-eGFP vector contains eGFP under the control of the A. flavus tel1 promoter and the A. nidulans trpC transcription terminator for integration into the sdh2 locus by using carboxin as a selection agent. A 9735 bp fragment of pUM (digested with Xho I and EcoR I), a 592 bp region of the sdh2 coding sequence (amplified with P101/P102), a point-mutated (H249L) 438 bp fragment

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contains 103 bp of the 3’ end of the *sdh2* gene, and a 335 bp region downstream of the *sdh2* gene (amplified with P103/P104), a 838 bp portion of the *tefl* promoter (amplified with P21/P22), a 720 bp coding sequence of eGFP (amplified with P31/P32), a 771 bp *trpC* terminator (amplified with P41/P42), and a 1074 bp fragment covering the right flank of the *sdh2* gene (amplified with P105/P106) were designed to have overlapping homologous sequences and assembled according to Figure 3A. All above DNA fragments were transformed into the FY384 competent yeast cells following a small-scale yeast transformation protocol in the pYES2 user manual (Invitrogen, USA) and selected on Sc-U medium. The homologous recombination plasmid products were purified using the TIANprep yeast plasmid DNA kit (DP112, Tiangen Biotech, China), and then transformed into *E. coli* DH5α competent cells. The DNA sequence of the final assembled pFC-eGFP plasmid was confirmed by PCR and DNA sequencing, after which it was transformed into the AGL-1 strain using the freeze/thaw shock transformation method. Primers used in this study were listed in Table 1.

### A. flavus transformation

*A. tumefaciens*-mediated transformation of *A. flavus* was performed as described previously (Han et al. 2018), with some modification. The *A. tumefaciens* AGL-1 strain containing the pFC-eGFP plasmid was grown in 10 ml DYT medium supplemented with rifampicin (20 mg/ml) and kanamycin (50 μg/ml) overnight at 28°C with shaking at 200 rpm. The overnight culture was then diluted to an OD₆₀₀ of 0.15 with induction medium (IM) and grown at 28°C with shaking at 200 rpm until reaching an OD₆₀₀ 0.35-0.4. The *A. tumefaciens* cultures were then mixed with an equal volume of *A. flavus* conidial suspensions (2×10⁴ spores/ml) and subsequently 200 μl of the mixed cultures were plated onto cellulose nitrate membranes (0.45 μm pore, Sartorius Biotech, Goettingen, Germany) placed on co-cultivation medium and grown at 22°C for 48 h. The cellulose nitrate membranes were then transferred to selection medium containing 300 μg/ml cefotaxime (Sangon Biotech, China), 60 μg/ml streptomycin (Bomei, China) and 50 μg/ml carboxin (45371, Sigma-Aldrich, Germany) and incubated at 28°C in the dark until colonies appeared. The individual colonies were transferred to selection medium with the appropriate antibiotics, as described above, and grown at 28°C for 3-4 days.

### Molecular analysis of transformants

Isolation of single spores from each transformant was achieved by spreading the diluted spore suspension onto PDA plates and incubating at 30°C for 1-2 days. Individual conidia were then transferred to new PDA plates and incubated at 30°C for a further 7 days. Genomic DNA was obtained by using a modified CTAB method (Han et al. 2018) and used for PCR amplification performed using primers P100/P32 to validate the specific integration of the vector pFC-eGFP into the *sdh2* locus of *A. flavus*.

For Southern blot detection, genomic DNA isolated from the transformants was digested with the Hind III and separated by electrophoresis in a 1.0% agarose gel and capillary transferred onto a Hybond-N* membrane (GE Healthcare). A specific probe was generated with PCR with primers P105/107, labeled with digoxigenin-dUTP, and hybridized with the membrane according to the instruction (1185614910, Roche, Germany). The wild-type *A. flavus* NRRL 3357 genomic DNA was used as the negative control.

### Microscopy

The expression of GFP in *A. flavus* transformants was analyzed using a Leica DM5000 B fluorescence microscope (Leica, Germany). The selected transformants were incubated on PDA plates at 30°C for 2-5 days after which spores, mycelia and conidiophores were collected for fluorescence analysis. The wild-type strain NRRL 3357 was used as negative control.

### Conidial and sclerotial production

For quantitative comparison of the production of conidia, an aliquot of conidial suspension (10⁴ spores) was point inoculated onto the center of a PDA plate. Cultures were grown at 30°C for 5 days. Conidia were washed off the agar plates using 0.01% Triton X-100 solution and counted in a hemocytometer.

For sclerotial analysis, conidial suspensions (10⁴ spores) were spread on Wickerham medium agar plates and incubated in darkness at 37°C for 10 days. Conidia were washed off the plates using 0.01% Triton X-100 solution, and remaining sclerotia were counted under a microscope.

### Quantitative determination of aflatoxin B1 production by HPLC

The method for quantitative comparison of the production of aflatoxin B1 was conducted as previously described with some modification (Han et al. 2018). A conidial suspension (2×10⁴ spores) was seeded centrally onto sterile cellophane sheets that were placed over a YES agar plate and incubated at 30°C for 5 days. The fungal biomass was scraped from the plates, and extracted by incubation with 5 ml of methanol at room temperature with shaking at 150 rpm for 30 min. The supernatant was collected by centrifugation at 3,000 g and filtered through a syringe filter (RC 0.22 μm, Alltech, Deerfield, IL). The presence of aflatoxin B1 was determined by HPLC with fluorescence detection, using a Waters 600 series HPLC equipped with a 600 pump, a 2707 autosampler and a 600 column thermostat set at 30°C. Detection was performed using a 2475 Multi A fluorescence detector set at 365 nm (λex) and 465 nm (λem), with a Waters Empower Windows xp operating system (Waters, Milford, MA, USA). The analytical column was a Luna 3u C18 (2) (150×4.6 mm, 3 μm) (Phenomenex, Torrance, CA, USA) preceded by a SecurityGuard TM precolumn (C18, 4×3.0 mm, Phenomenex). The mobile phase consisted of methanol: water (55:45), eluted at a flow rate of 0.6 ml/min, with 20 μl of filtered extract injected into the HPLC per run. Aflatoxin B1 production was measured in μg/g of mycelia.

### Kernel infection assays

Conidia of the *A. flavus* transformants were harvested from PDA plates and suspended in water and adjusted to a cell density of 4×10⁶/ml. Undamaged maize seeds were surface-sterilized with 70% ethanol. Ten μl of spore suspension was dripped onto the embryo of the kernel which were then placed in 3.5 cm petri dishes and incubated in the dark at 30°C for 7 days. High humidity (> 95% RH) was maintained.

### Data availability

Strains and plasmids used in this study are available upon request. The authors state that all data necessary for confirming the conclusions of the article are present within the article.

### RESULTS

**Sensitivity of conidia germination to carboxin**

In order to find a suitable concentration of antibiotic for screening transformants, the sensitivity of *A. flavus* wild type strain NRRL 3357 to carboxin was analyzed. The results showed that mycelial growth was

| Antibiotic Concentration (μg/ml) | Germination Percentage |
|--------------------------------|-----------------------|
| 0                             | 100%                  |
| 5                             | 95%                   |
| 10                            | 90%                   |
| 20                            | 80%                   |
| 50                            | 50%                   |
| 100                           | 0%                    |
completely inhibited in MM medium supplemented with 150 µg/ml carboxin (Figure 1).

**Identification of Sdh2 in A. flavus**

In *A. flavus* NRRL 3357, the iron-sulfur containing subunit of the succinate dehydrogenase enzyme encoded by the *sdh2* gene is a 278 amino acid protein (Accession number, EED45758) which shares 54.91% and 100% sequence similarities to the corresponding sequences in *U. maydis* (Accession No., XP_011386878) and *A. oryzae* RIB40 (accession number, XP_001827486), respectively (Figure 2). It has been proven that the substitution of the His (249) residue with a Leu in *A. oryzae* confers the highest resistance to the fungicide carboxin (Shima et al. 2009). The conserved histidine (249) in *Sdh2* should play a similar role in conferring resistance to the fungicide carboxin in *Aspergillus*.

**Construction of *sdh2*R based vector**

A locus specific integration vector, pFC-eGFP, containing the mutant *sdh2* sequence was constructed as described in the methods. In this vector, RNA transcription of the eGFP gene is initiated by the *A. flavus* tef1 promoter and terminated by the *A. nidulans* trpC terminator. To integrate the foreign DNA into the wild type *sdh2* locus, the *sdh2R* left and right flanks were introduced adjacent to the eGFP cassette. The most important characteristic feature of the *sdh2*R left flank is that it is made up of 695 bp from the 3’ end of the *sdh2* gene (the full-length gene is 1032 bp) containing the H249L point mutation and 335 bp of the *sdh2* terminator sequence (Figure 3A, left flank). The right flank contains the 1074 bp downstream regions of *sdh2*. The point mutation (H249L) in the *sdh2*R left flank will mutate the endogenous *sdh2* gene, conferring resistance to carboxin after integration by homologous recombination (Figure 3B).

In addition, a yeast recombination cassette consisting of *URA3* and a 2µ ori was introduced into the vector. This method allows the multiple overlapping DNA fragments to be assembled in a single step by homologous recombination in yeast, avoiding laborious restriction/ligation-based cloning procedures.

**Targeted integration of the pFC-eGFP vector into the *sdh2* locus**

We transformed vector pFC-eGFP into the *A. flavus* strain NRRL 3357 by *A. tumefaciens*-mediated transformation to generate *sdh2*R mutants expressing eGFP (*sdh2*RG). The transformants were selected on MM medium containing 150 µg/ml carboxin. To confirm the efficiency of targeted integration of pFC-eGFP into the native *sdh2* locus of *A. flavus*, genomic DNA was extracted from five randomly selected *sdh2*RG mutants. PCR analysis showed that a 2.9kb DNA fragment was obtained after amplification with primer pair P100/P32 in all mutants but not in wild type (Figure 3D). Southern blot assays identified a single band at the expected size (4.1kb) in all transformants, while a band of 5kb in size was identified in the wild type genomic DNA (Figure 3E). We found that 96% of transformants has a single copy integration into the *sdh2* locus (in total 52 transformants), and the remaining 4% of transformants has a second integration event via qPCR (Lu et al. 2014) (data not shown). Moreover, GFP fluorescence was observed at all developmental stages, including in the spores, mycelia and conidiophores from all analyzed *sdh2*RG mutants (Figure 3C).
Phenotypes of carboxin-resistant transformants of A. flavus

To evaluate the effect of integration of pFC-eGFP into sdh2 locus on A. flavus growth and development, we compared mycelial growth, as well as conidial and sclerotial production of carboxin-resistant mutants with that of the wild-type strain. Data displayed in Figure 4 shows that there were no significant differences observed between mutant and wild type strains. In addition, level of AFB1 production was also found to be similar in the two strains, indicating that integration of the vector into sdh2 locus has little impact on aflatoxin synthesis. The infection rate of the mutants was investigated on maize kernels. No significant differences observed between mutant and wild type after integration of pFC-eGFP into the sdh2 locus. (D) The sdh2RG mutants were validated by PCR. M= DL15,000 DNA marker; W= wild type strain; 1-5= sdh2RG mutants. (E) Southern blot analysis of sdh2RG mutants. All carboxin-resistant transformants contained a single integration into the desired locus. M: DL15,000 DNA marker; W: wild type; 1-5: sdh2RG mutants.

DISCUSSION

At present, considerable efforts have been made to overcome the difficulty of using non-homologous, ectopic recombination for DNA integration into the genomes of filamentous fungi. Indeed, work has focused on improving the frequency of gene targeting by homologous recombination, such as using split-marker- or deficient-NHEJ DNA repair pathway- strategies. However, split-marker technology is based on three crossover events, significantly reducing the transformation rate compared with classical recombination strategies based on a single crossover event (Aragona and Valente 2015; Wilson et al. 2010).

Use of deficient-NHEJ repair pathway methods may generate unexpected effects in the reduction of DNA repair capacity (Emerson et al. 2018; Pannunzio et al. 2018).

Recently, a new strategy was developed based on specific integration at the site of a selectable marker gene in several filamentous fungi. Such suitable genes include pyrG in A. niger (van Hartingsveldt et al. 1987) and A. awamori (Gouka et al. 1995), ILV2 in Magnaporthe oryzae (Yang and Naqvi 2014), and sdi1 in Ustilago maydis, Zymoseptoria tritici (Kilaru et al. 2015) and M. oryzae (Guo et al. 2016). The pyrG gene encodes orotidine-5’-monophosphate (OMP) decarboxylase, a key enzyme for biosynthesis of uridine/uracil which is required for fungal survival. It’s a dominant selectable marker in many Aspergillus spp. While integration at the pyrG locus usually requires two steps. First, the pyrG gene in the recipient strain should be mutated to abrogate function, followed by integration of a wild-type pyrG gene at the mutated pyrG locus by homologous substitution (Gouka et al. 1995). However, compared with the laborious double integration method for insertion at the pyrG gene locus, integration at either ILV2 or sdi1 loci only requires one step, due to the single amino acid substitution in either gene that introduces drug resistance in recipient strain.

In this study, we devised a novel strategy for site-specific integration of foreign DNA at the sdh2 gene locus in A. flavus. The vector pFC-eGFP harboring the mutant sdh2 allele containing a single amino
acid substitution (His 249 Leu), was constructed via yeast recombination-based cloning for fungal transformation. The replacement of the native sdh2 with the sdh2R allele confers resistance to carboxin in this fungus. The resulting transformants showed a high efficiency (>96%) of correct integration into the genome of *A. flavus*, with individual transformants having no detectable variations in the intensity GFP fluorescence. Crucially, introduction of the eGFP expression cassette at the sdh2 locus did not discernably alter fungal growth and virulence. The sdh2-locus integration system will be a valuable tool not only for gene expression, but also for genetic complementation, promoter analyses, and protein cellular localization in *Aspergillus* species.

**CONCLUSION**

In this study we developed an efficient carboxin-resistance recombination strategy for sdh2 specific integration in *A. flavus*. This new strategy allows the precise integration of the DNA sequence of interest into the sdh2 locus without disturbing the expression of fungal genes. Additionally, the carboxin-resistance transformation does not influence fungal growth and virulence in any way. Thus, we have demonstrated the utility of the sdh2 locus for targeted integration as a valuable tool for mutant strain generation in *A. flavus*.

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