Examination of Cyp51A-mediated azole resistance in *Aspergillus lentulus* using CRISPR/Cas9 genome editing

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Key words

Aspergillus lentulus, Azole resistance, Cas9/CRISPR, Cyp51A

Abstract

Aspergillus lentulus was first reported in 2005 as a cryptic species of *Aspergillus fumigatus*, and its resistance to azole drugs and the high mortality rate of infected individuals have emerged as problems. Although it has been reported that P450 14-α sterol demethylase (Cyp51) is involved in azole resistance in *A. lentulus*, the specific resistance mechanism has not been elucidated. In this study, we successfully introduced the entire *A. fumigatus cyp51A* gene into the *cyp51A* locus in *A. lentulus* using the CRISPR/Cas9 genome-editing system. The *A. lentulus* strains harboring *A. fumigatus cyp51A* showed reduced MICs for itraconazole and voriconazole compared with those of the parent strain. This finding suggests that Cyp51A is involved in azole resistance in *A. lentulus* and may contribute to the elucidation of Cyp51A's mechanism of resistance to azole drugs and to the development of new antifungal drugs. In addition, our successful application of the CRISPR/Cas9 system to *A. lentulus* opens the door to examination of other gene functions in this fungus.

Introduction

Aspergillosis is one of the important deep-seated mycoses caused by *Aspergillus* spp., the most ubiquitous group of molds. The major causative agents are *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus terreus*, of which *A. fumigatus* is the most prevalent. Until now, the identification of *Aspergillus* species has been done by morphological observations, but recent molecular genetic analyses have revealed that some of the species that were previously considered as morphologically resembling the representative species are completely different species, or so-called cryptic species. It has been reported that 10~12% of the isolates from patients with aspergillosis are cryptic species[1,2]. *Aspergillus lentulus* is a member of *Aspergillus* section *Fumigati*, and was first reported as a human clinical isolate in 2005[2]. Cases caused by *A. lentulus* has been reported in many countries, including Japan[3,4], and is the most frequently isolated of the *Fumigati* cryptic species, with drug resistance and high mortality rates being problems.

The main therapeutic agents targeting aspergillosis in Japan[5] are liposomal amphotericin B (L-AMB), itraconazole (ITCZ), and voriconazole (VRCZ), which act as fungicides, and micafungin (MCFG) and caspofungin (CPFG), which act as fungistatic agents. *A. fumigatus* is the most frequently isolated causative fungus of invasive pulmonary aspergillosis (IPA), and according to the guidelines, VRCZ and L-AMB are the first-line drugs for treatment, while CPFG, MCFG, and ITCZ are used as alternatives. However, some IPAs morphologically thought to be caused by *A. fumigatus* were found to be caused by *A. lentulus*, which as a cryptic species would likely require a different treatment strategy than that for *A. fumigatus*[4,6].

The mechanism of azole resistance in *A. fumigatus* is known to involve differences in the uptake of azole drugs, differences in intracellular azole levels in *A.*

fumigatus caused by increased expression of efflux pumps such as ABC transporters, and partial mutations in target proteins of azole drugs such as P450 14-α-sterol demethylase (Cyp51)[7-9]. It has been reported that both Cyp51A and Cyp51B exist in *A. fumigatus* and *A. lentulus*, and that Cyp51A is mainly responsible for azole resistance in both species[10-13]. Alcazar-Fuoli[6] et al. succeeded in heterologous expression of the *cyp51A* region of *A. fumigatus* or *A. lentulus* in *Saccharomyces cerevisiae*, and conducted drug susceptibility testing. *S. cerevisiae* harboring *cyp51A* from *A. lentulus* had significantly increased MIC values for ITCZ, VRCZ, and posaconazole (PSCZ) compared to *S. cerevisiae* harboring *cyp51A* from *A. fumigatus*. Mellado[12] et al. reported that introduction of *A. lentulus cyp51A* into *A. fumigatus* resulted in azole resistance and that the strain partially lacking *A. lentulus* Cyp51A became susceptible to azole drugs. However, the number of reports on azole resistance in *A. lentulus* is small, and further investigation is needed to determine the role of Cyp51A in the azole resistance of this species.

In recent years, genetic recombination using CRISPR/Cas9 has been reported for various fungal species[14-16], but there has been no report in *A. lentulus*. We previously generated a Cyp51A mutant strain of *A. fumigatus* using the CRISPR/Cas9 system[17]. Based on this earlier experience, we reasoned that establishing the protocol for the CRISPR/Cas9 system in *A. lentulus* would allow the design of arbitrary recombinant strains, which would contribute to the elucidation of its mechanism of resistance to azole drugs and the development of new antifungal drugs. In this study, therefore, we aimed to establish a protocol for the introduction of *A. fumigatus cyp51A* into *A. lentulus* using the CRISPR/Cas9 system, and to elucidate the involvement of Cyp51A in azole resistance in *A. lentulus* by studying the changes in drug susceptibility of the recombinant strains.

Materials and Methods

Strains, media, and growth conditions

The strains used are listed in Table 1. Afs35 was used as the parental strain of *A. fumigatus*. NIID0096[3] was used as the parental strain of *A. lentulus*. The culture conditions for *A. fumigatus* were set based on the methods of Szewczyk et al.[18] and Umeyama et al.[17]. *A. fumigatus* strains were routinely grown in *Aspergillus* minimal medium (AMM: 10 g glucose, 0.516 g KCl, 0.516 g $MgSO_4 \cdot 7H_2O$, 1.516 g KH_2PO_4, 1.516 g $Mg(NO_3)_2 \cdot 6H_2O$, 1 ml trace elements[19] in 1 l distilled water), Czapek-Dox medium (CD: BD Difco Laboratories Inc., Franklin Lakes, NJ), YG medium (5 g yeast extract (DIFCO), 20 g D-glucose and 400 µl trace elements[19] in 1:l distilled water), or potato dextrose agar medium (PDA: BD Difco). For solid medium, 1.5% (w/v) agar was added. *A. fumigatus* conidia were obtained from mycelia cultured on AMM or PDA at 30°C for 3–7 days, harvested with PBS containing 0.05% (v/v) Tween 20 and 20% (v/v) glycerol, and filtered through a 40-µm nylon cell strainer (Greiner Bio-One, Germany). *A. lentulus* was cultured for 14–18 days so that the growth stages would be comparable using the same medium and culture temperature as used for *A. fumigatus*.

DNA extraction, PCR, and sequencing

Genomic DNA extractions and purifications were performed using a DNeasy Plant Mini Kit (QIAGEN, Germany). Primers for the amplification and sequencing of *cyp51A* are listed in Table 2. PCR amplification of *cyp51A* was performed with Q5 Hot Start High-Fidelity 2× Master Mix (New England Biolabs, Ipswich, MA) using Afs35 or NIID0096 genomic DNA as a template and primers AlCyp51A-dischеck5 and

AlCyp51A-discheck3.

The DNA sequence of Cyp51A, including its surrounding regions, has been registered in DDBJ as LC649065 (*A. lentulus*) and LC649066 (*A. fumigatus*).

sgRNA in vitro synthesis

We manually searched for target sequences consisting of G(N)15(A/T)(N)3NGG near the N-terminus (for sgRNA1) and C-terminus (for sgRNA2) as sgRNA target sequences and synthesized two oligonucleotides (T7-sgRNA1 and T7-sgRNA2; Table 2) consisting of the T7 promoter, sgRNA target sequence, and overlap sequence with the Cas9 scaffold. These oligonucleotides were used for sgRNA synthesis with an EnGen® sgRNA Synthesis Kit, S. pyogenes (New England Biolabs). The synthesized sgRNAs were purified using an RNA Clean & Concentrator-25 (Zymo Research, Irvine, CA), quantified using a QuantiFluor RNA system (Promega, Madison, WI) and Quantus Fluorometer (Promega), and used for ribonucleoprotein formation with Cas9.

Repair templates

Primers for the repair template construction are listed in Table 2. A region from 800-bp upstream to 1600-bp downstream of the *cyp51A* coding region was used for repair templates. To prevent double-strand DNA breaks (DSBs) in the recombinant strains, the primers were designed to contain silent mutations at residues 8, 9, and 10 and residues 500, 501, and 502 of the Cyp51A region. The mutations and *hph* marker

were introduced via PCR-sewing or overlap extension PCR. The hph marker from the plasmid pHph[17] for selection of transformants was inserted between nucleotides 500 and 501 downstream of the *cyp51A* stop codon. Q5 Hot Start High-Fidelity 2× Master Mix (New England Biolabs) was used for PCR amplification. The primer combinations for overlap extension PCR are listed in Table 3. Briefly, Afs35 or NIID0096 genomic DNA was used as a template to generate overlapping PCR products with the corresponding site-specific mutations or junctions between *cyp51A* and the *hph* marker. The overlapping PCR products were mixed and used as a template in the PCR-sewing step using the primers Al-LFH1 and Al-LFH4. Overlapping PCR product combinations are listed in Table 4. The fused PCR products were purified using a NucleoSpin® Gel and PCR Cleanup kit (Takara Bio, Shiga, Japan) and used for *A. lentulus* protoplast transformation.

A. lentulus **transformation**

A. lentulus protoplasts were generated and fungal transformation was performed as previously described[18], with slight modifications. Briefly, conidia were incubated in YG medium for 6.5 h at 37°C. Following incubation, the cell walls of germlings were digested with 0.2 g/ml VinoTaste Pro (Novozymes, Denmark) for 1 h at 30°C. Then, 20 pmol Cas9-NLS protein (Fasmac, Japan) or Alt-R® S.p. Cas9 Nuclease V3 (Integrated DNA Technologies, Japan) and 10 pmol each of *in vitro*-synthesized sgRNA1 and sgRNA2 were mixed and incubated for 25 min, generating ribonucleoproteins (RNPs). Protoplasts were transformed with 2–3 μg of repair templates and RNPs and plated onto CD supplemented with 1 M sucrose. Using NIID0096 clinical isolate as a host, repair templates Al-AfCyp51A and Al-AlCyp51A

were used to generate strains NIID0096AF and NIID0096AL, respectively. Following a 15 h incubation at 37°C, plates were overlaid with 10 ml CD top agar containing 500 μg/ml hygromycin. Positive colonies were verified by direct colony PCR using KOD FX Neo DNA polymerase (TOYOBO, Japan) with the primers AlCyp51A-discheck5 and AlCyp51A-discheck3 (designed at the region outside the repair template sequence), followed by nucleotide sequencing of *cyp51A*, including the promoter region.

Phenotypic changes in the recombinant strains

To confirm the phenotypic changes of the parental strains and the mutant strains, the culture was carried out by changing the medium, incubation time and temperature. The conidia of each strain were diluted with PBS-glycerol-Tween 80 to 10^6 /ml, and 2 μl droplets were spotted on AMM and PDA media and incubated at 30°C or 37°C. Visual observation was made after 24, 48, and 72 h of incubation.

Antifungal susceptibility testing

The conidia of the parental strains and the resulting recombinant strains were adjusted to $2.5x10^6$ /ml (range $0.5–5x10^6$ /ml) with PBS-glycerol-Tween80. Drug susceptibility testing by the micro liquid dilution method was performed using fungal FP 'Eiken' for yeast (Eiken Chemical Co., Ltd.). The fungi were incubated at 35°C, and the MECs of MCFG and CPFG were visually measured after 24 h (21–26 h). The MICs of AMPH-B, ITCZ, VRCZ, miconazole (MCZ), and the IC_{50} of 5-fluorocytosine (5-FC) and fluconazole (FLCZ) were determined after 48 h (46-50 h). The Clinical and Laboratory Standards Institute (CLSI) method M38-A2 was used as a reference to

182 determine the degree of resistance to each drug[20].

183

184 ## Results

185 **Cyp51A gene sequence of *A. fumigatus* and *A. lentulus***

186 The lengths of the *cyp51A* sequences of *A. fumigatus* and *A. lentulus* species
187 were found to be equivalent, with 515 coding amino acids. There was a 95% identity
188 between *A. lentulus cyp51A* (Al*cyp51A*) and *A. fumigatus cyp51A* (Af*cyp51A*). We
189 observed substitutions at the 25 positions shown in Table 5. The amino acids reported to
190 be azole-resistant in *A. fumigatus*, such as L98H and G448S, were identical to those in
191 azole-sensitive *A. fumigatus*[7,21]. In addition, there was little difference among the
192 Cyp51A sequences registered in NCBI for the azole-resistant *A. lentulus* strains and
193 NIID0096 (Table 5).

194 A comparison of the amino acids in the Cyp51A region of the *A. lentulus* CM-
195 1290 strain used by Mellado[12] et al. and the NIID0096 strain used in this study revealed
196 four differences: M11T(ACG)/M11(ATG), F29(TTC)/F29Y(TAC),
197 H352Q(CAG)/H352(CAT), and T513(ACA)/T513S(TCA) (CM-1290/NIID0096).

198

199 **Characteristics of recombinants**

200 We used Umeyama's[17] method for genetic recombination using CRISPR/Cas9
201 (Fig.1). The incubation time in YG medium for the preparation of protoplasts against *A.*
202 *fumigatus* was set at 6 h, but sufficient recombinant strains for *A. lentulus* could not be
203 obtained. *A. lentulus* grows more slowly than *A. fumigatus*, so the recombination
204 efficiency was increased by extending the incubation time to 6.5 h. The final
205 concentration of protoplasts was reduced by half so that the number of enzymes and

repair templates added by CRISPR/Cas9 recombination to protoplasts would be doubled. When the concentration of hygromycin was increased from 400 to 500 μg/ml, selection of the desired recombinant strains containing the Hph cassette could be efficiently performed.

In the *cyp51A* recombinant strains, NIID0096AF and NIID0096AL, of *A. lentulus,* we observed no difference in the growth rate or phenotypic characteristics compared to the parent strain, NIID0096, from 24 to 72 h. While *A. fumigatus* strain Afs35 produced conidia on PDA at 30°C and on AMM and PDA at 37°C, in the *A. lentulus* strains NIID0096, NIID0096AF and NIID0096AL, there were no conidia in either medium at either temperature at 48 h (Fig. 2). The colony sizes of *A. lentulus* strains NIID0096, NIID0096AF and NIID0096AL were slightly larger than that of *A. fumigatus* strain Afs35 in both media at 30°C. There was also no difference in colony size between *A. fumigatus* strain Afs35 and *A. lentulus* strains NIID0096, NIID0096AF and NIID0096AL at 37°C.

Drug susceptibility of recombinants

Table 6 shows the results of drug susceptibility testing using the micro-broth dilution method. Based on the susceptibility criteria of CLSI M38-A2, the *A. fumigatus* parental strain Afs35 was susceptible to MCFG, AMPH-B, ITCZ, and VRCZ. The *A. lentulus* parental strain, NIID0096, a clinical isolate, was susceptible to MCFG and resistant to AMPH-B and azoles. Strains NIID0096AF of *A. lentulus*, the recombinant strain containing the Cyp51A region of *A. fumigatus*, showed reduced MICs for ITCZ and VRCZ when compared to the susceptibility of the host, NIID0096. On the other

hand, no change in susceptibility was observed in MCFG and AMPH-B. In the control strain NIID0096AL, the susceptibility pattern was similar to that of the host NIID0096. There was no difference in the IC_{50} and MIC values for 5-FC, FLCZ, and MCZ among all strains used in this study.

Discussion

A. lentulus is a cryptic species of *A. fumigatus* that was only recently identified due to the difficulty in its identification by phenotype and its low susceptibility to antifungals. Further research will be needed to select effective antifungal agents and the prognosis of aspergillosis by this species. CRISPR/Cas9 has been used as a genome editing system in various studies, and its application to various species of filamentous fungi has been reported. In the genus *Aspergillus*, CRISPR/Cas9 has been reported to be applied to *A. fumigatus*, *Aspergillus nidulans*, *A. niger*, and *Aspergillus novofumigatus*[14,22]. In this study we applied CRISPR/cas9 genome editing techniques to *A. lentulus* and investigated the mechanisms of azole resistance of this species via Cyp51A. It is expected that further progress will be made in elucidating the mechanisms of drug resistance and developing antifungal drugs using CRISPR/cas9 in the future.

In order to investigate the relationship between Cyp51A and resistance to azoles in *A. lentulus*, Mellado[12] et al. introduced the Al*cyp51A* gene of *A. lentulus* into the strain lacking part of the Al*cyp51A* region of *A. lentulus* and a strain lacking Af*cyp51A* of *A. fumigatus* by electroporation. In this study, we succeeded in replacing the entire *cyp51A* region of *A. fumigatus* using CRISPR/Cas9 against *A. lentulus*. In the future, the strains generated in this study will allow us to clarify the effect of Cyp51A on azole drug resistance in *A. lentulus*. Mellado et al. found that strains of *A. lentulus* in

253 which the 411–713 bp region of Al*cyp51A* was replaced became more susceptible to
254 azole drugs compared to the parental strain of *A. lentulus* strain CM-1290. They also
255 found that the Af*cyp51A*-deficient strain heterologously expressing the Al*cyp51A* gene
256 became resistant to azole drugs compared to the parental *A. fumigatus* strains. In this
257 study, when Al*cyp51A* of the *A. lentulus* azole-resistant strain was recombined with
258 Af*cyp51A*, the strain became susceptible to azoles by the micro liquid dilution method
259 based on CLSI M38-A2. By replacing the entire length of Alcyp51A with Afcyp51A,
260 we were able to prove that cyp51A is the main mechanism of azole resistance in A.
261 lentulus.

262 The amino acid sequences of AlCyp51 and AfCyp51A are about 95%
263 homologous. The amino acids G54, S52T, L98H, Y121F, G138CR, Q141H, H147Y,
264 Y431C, G434C, and G448S have been reported to be involved in the azole resistance of
265 *A. fumigatus*[23]. The differences in amino acid sequences between AlCyp51A and
266 AfCyp51A are different from those previously reported as related to azole resistance in
267 *A. fumigatus* (Table 5). This suggests that the site involved in azole resistance in
268 AlCyp51A may have a different amino acid sequence(s) from that reported for *A.*
269 *fumigatus*.

270 The azole-resistance of *A. lentulus* may be attributable to differences in the
271 conformation of the AlCyp51A protein caused by either a single amino acid difference
272 or multiple amino acid sequence differences. In addition, there may be additional factors
273 other than the interaction of Cyp51A with azole drug resistance in *A. lentulus* as
274 reported in *A. fumigatus*[8,24-27]. For example, differences in the uptake of azoles or
275 differences in intracellular azole concentrations within *A. lentulus* due to increased
276 expression of efflux pumps such as ABC transporters may play a role. However, our
277 present results show that the role of AlCyp51A in azole resistance is the most important.

In the future, it will be necessary to analyze which part of Cyp51A in *A. lentulus* is involved in drug resistance.

Conflicts of interest

The authors declare that there is no conflict of interest.

Acknowledgments

This work was supported by JSPS KAKENHI Grant Numbers JP20K08834. This study was partly supported by the Joint Usage/Research Program of Medical Mycology Research Center, Chiba University (20-5), and by AMED under Grant Numbers JP21fk0108135 and JP21fk0108094.

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Legends to figures

Fig. 1 Strain construction using the CRISPR/Cas9 technique. (A) The first PCR was performed using the genomic DNA (gDNA) of the parental strains Afs35 and NIID0096 as a template. The obtained PCR product was used as a template for the second PCR, and two types of the target repair template were obtained. NIID0096 was used as the host for transformation. (B) RNPs including Cas9 Nuclease and sgRNAs were added to the protoplast solution to induce DSB and homologous recombination with the repair template. Arrows indicate the schematic positions of the primers, AlCyp51A-discheck5 and AlCyp51A-discheck3.

Fig. 2. Colony growth of inoculated AMM and PDA media at 30°C and 37°C for 48 h. The schematic diagram on the left shows the type and position of the strains in each Petri dish. The parent strains were inoculated in the upper part of the Petri dish, and the three clones of recombinant strains NIID0096AF and NIID0096AL were inoculated in the middle and bottom parts. Closed circles, Afs35; open circles, NIID0096; gray circles, NIID0096AF; striped circles, NIID0096AL.

Table 1. *Aspergillus fumigatus* and *Aspergillus lentulus* strains used in this study

Strain(s)	Parent	Genotype*	Source
Aspergillus fumigatus			
Afs35	D141	*akuA*Δ*loxP*	Fungal Genetics Stock Center
Aspergillus lentulus			
NIID0096	clinical isolate		Yoshida et al[3].
NIID0096AF	NIID0096	*Alcyp51A::Afcyp51A*mut1/mut2-hph	Current study
NIID0096AL	NIID0096	*Alcyp51A::Alcyp51A*mut1/mut2-hph	Current study

**mut1* and *mut2* are silent mutations for Cas9-nuclease resistance.

Table 2. Oligonucleotide primers used in this study

Primer	Sequence(5'-3')[a]	Orientation	Use
AlCyp51A-discheck5	AGGCGGCTATAGCGGGAATAGACG	Sense	*cyp51A* amplification and verification of transformants
AlCyp51A-discheck3	GGAGAAATAGTAGATAGAACCTTC	Antisense	*cyp51A* amplification and verification of transformants
T7-sgRNA1	TTCTAATACGACTCACTATAGCTATGGCTTACGGCCTAC AGTTTTAGAGCTAGA	Sense	in vitro sgRNA synthesis
T7-sgRNA2	TTCTAATACGACTCACTATAGGGATGAATAGTCAGTTTC AGTTTTAGAGCTAGA	Antisense	in vitro sgRNA synthesis
T7-AlCyp51A-gRNA1	TTCTAATACGACTCACTATAGCTATTGCTCACGGCCTAC AGTTTTAGAGCTAGA	Sense	in vitro sgRNA synthesis
T7-AlCyp51A-gRNA2	TTCTAATACGACTCACTATAGCGGATGATGCTTGGCTTC AGTTTTAGAGCTAGA	Antisense	in vitro sgRNA synthesis
Al-LFH1	CTTTTACACCAGAAGCAGTAGCGCAC	Sense	Repair template construction and amplification
AfCyp51A-mut1-5	ATGGTGCCGATGCTATGGCTTAC**T**GC**A**TA**T**ATGGCCGTT GCGGTGCTGACG	Sense	Repair template construction
Al-AfCyp51A-mut1-3	AGCCATAGCATCGGCACCATTTCGAGGGCACACAGGGA GGGTCAG	Antisense	Repair template construction
AlAf-mut2-5	CATCCAAGTGAGACTGTTGTA	Sense	Repair template construction
Al-AfCyp51A-mut2-3	TACAACAGTCTCACTTGGATGTGTTTTTCGACCGCTTCTC CCAGCCGATGAT**A**CT**A**GG**T**TTCATGGGGCCCGAAAAG	Antisense	Repair template construction
Al-LFH6	GGTGATATCGGCCTGAGTGGCCTCCGAGCTTCTCCCCGT CATCTAGAC	Antisense	Repair template construction
Al-LFH3	TCGACGGCCATCTAGGCCAGTGCAAAAACCTGCTTTGAT AGTCC	Sense	Repair template construction
Al-LFH4	GAATCTACTTGCCTCTTCAGAAGAG	Antisense	Repair template construction and amplification
AlCyp51A-mut1-5	ATGGTATCGATGCTATTGCTCAC**T**GC**A**TA**T**ATGGCCGTT GCTATGCTGACGGTG	Sense	Repair template construction
Al-AlCyp51A-mut1-3	AGCAATAGCATCGATACCATTTCGAGGGCACACAGGGA GGGTCAG	Antisense	Repair template construction
Al-AlCyp51A-mut2-3	TACAACAGTCTCACTTGGATGAGTCTTTAGAACGCTTCT CCCAGCGGATGAT**A**CT**A**GG**T**TTCATGGGCCCTG	Antisense	Repair template construction
397-5	GAGGCCACTCAGGCCGATATCACC	Sense	*hph* cassette amplification
397-3	CTGGCCTAGATGGCCGTCGACAAC	Antisense	*hph* cassette amplification
AlCyp51A-seq1	AGATAACATGACTTTCATATC	Sense	*cyp51A* sequencing
AlCyp51A-seq1r	GCGGTAATGCTGCAGTTATTC	Antisense	*cyp51A* sequencing
AlCyp51A-seq2	CTTAGCTCATACTACGGTAGG	Sense	*cyp51A* sequencing
AlCyp51A-seq2r	GTCCGATTCCAAAGCCGGTAG	Antisense	*cyp51A* sequencing
AlCyp51A-seq3	CAGGGGAACGAGTTCATTCTC	Sense	*cyp51A* sequencing
AlCyp51A-seq3r	TGCTCCTGATAAAGCTCTTCC	Antisense	*cyp51A* sequencing
AlCyp51A-seq4	CAGCAGGTGCCTGATAAAGAG	Sense	*cyp51A* sequencing
AlCyp51A-seq4r	CACTGTTATGTGGCTCTATCG	Antisense	*cyp51A* sequencing
AlCyp51A-seq5	ACGACTTTTCAACGTGGATGG	Sense	*cyp51A* sequencing
seq1	CCAATGGTCTTTCATTGGGTC	Sense	*cyp51A* sequencing
seq1r	TCCCGTAACTGATGGTACTAC	Antisense	*cyp51A* sequencing
seq2	TTTACCGCTGCTCGAGCCCTC	Sense	*cyp51A* sequencing
seq2r	GGAACGAACTTCCTGGCCTTG	Antisense	*cyp51A* sequencing
seq3	AACTTCCCTTCCATCAACATG	Sense	*cyp51A* sequencing
seq3r	CGAATAACATGTTGATGGAAG	Antisense	*cyp51A* sequencing

[a]Letters in bold indicate the mutated nucleotides. Underlining indicates an additional sequence for *in vitro* gRNA synthesis.

Table 3. Combination of primers for overlapping PCR used in this study

Name of PCR product	Primers	Template DNA
AlAf-LFHA	Al-LFH1/Al-AfCyp51A-mut1-3	NIID0096 genomic DNA
AlAl-LFHA	Al-LFH1/Al-AlCyp51A-mut1-3	NIID0096 genomic DNA
AlCyp51A	AlCyp51A-mut1-5/Al-AlCyp51A-mut2-3	NIID0096 genomic DNA
Al-LFHB	Al-LFH3/Al-LFH4	NIID0096 genomic DNA
Al-LFHC	AlAf-mut2-5/Al-LFH6	NIID0096 genomic DNA
AfCyp51A	AfCyp51A-mut1-5/Al-AfCyp51A-mut2-3	AfS35 genomic DNA
Hph	397-5/397-3	pHph plasmid DNA

Table 4. Combination of PCR products for repair template amplification used in this study

Name of repair template	PCR products
Al-AfCyp51A	AlAf-LFHA, AfCyp51A, Al-LFHC, Hph, Al-LFHB
Al-AlCyp51A(control)	AlAl-LFHA, AlCyp51A, Al-LFHC, Hph, Al-LFHB

Table 5. Amino acid differences among Cyp51A in *A. fumigatus* and *A. lentulus*

Amino acid	Accession No. (strain)			
	A. fumigatus		*A. lentulus*	
	LC649066 (Afs35)	LC649065 (NIID0096)	ADI80344 (CM-1290)[13]	KAF4159341 (CNM-CM6069）[28,29]
Pro[3]	P3 (CCG)	P3S (TCG)	*	*
Trp[6]	W6 (TGG)	W6L (TTG)	*	*
Met[11]	M11 (ATG)	M11 (ATG)†	M11T (ACG)	M11T (ACG)
Val[15]	V15 (GTG)	V15M (ATG)	*	*
Ala[18]	A18 (GCA)	A18V (GTG)	*	*
Phe[29]	F29 (TTT)	F29Y (TAC)	F29 (TTC)†	F29 (TTC)†
Ser[49]	S49 (AGT)	S49N (AAT)	*	*
Lys[67]	K67 (AAG)	K67R (AGG)	*	*
Asp[161]	D161 (GAT)	D161N (AAC)	*	*
Arg[171]	R171 (CGG)	R171Q (CAG)	*	*
Met[172]	M172 (ATG)	M172V (GTG)	*	*
Asp[255]	D255 (GAC)	D255G (GGA)	*	*
Cys[270]	C270 (TGC)	C270S (AGC)	*	*
Lys[314]	K314 (AAA)	K314Q (CAG)	*	*
Ala[330]	A330 (GCC)	A330I (ATT)	*	*
Ser[335]	S335 (AGT)	S335N (AAT)	*	*
His[352]	H352 (CAT)	H352 (CAT)†	H352Q (CAG)	*†
Ile[354]	I354 (ATT)	I354V (GTT)	*	*
Ile[360]	I360 (ATT)	I360L (CTT)	*	*
Ile[367]	I367 (ATC)	I367L (CTC)	*	*
Met[383]	M383 (ATG)	M383V (GTG)	*	M383 (ATG)†
Thr[420]	T420 (ACT)	T420A (GCC)	*	*
Leu[464]	L464 (CTT)	L464I (ATT)	*	*
Val[466]	V466 (GTG)	V466 (GTA)†	*†	V466I (ATA)
Glu[488]	E488 (GAA)	E488D (GAT)	*	*
Gly[505]	G505 (GGC)	G505R (CGC)	*	*
Asn[512]	N512 (AAC)	N512D (GAC)	*	*
Thr[513]	T513 (ACA)	T513S (TCA)	T513 (ACA)†	*

* same as NIID0096 † same as Afs35 amino acid

Table 6. MICs or MECs or IC_{50}s of different antifungals against *A. fumigatus* and *A. lentulus* isolates and their derived mutant strains

Isolate	Clone number	Origin and/or background	Species	*cyp51A* gene copy origin	MIC or MEC* or IC_{50}** (μg/mL)							
					MCFG*	CPFG*	AMPH-B	5-FC**	FLCZ**	ITCZ	VRCZ	MCZ
Afs35		reference	*A. fumigatus*	*A. fumigatus*	≦0.015	0.25	1	>64	>64	1	0.5	>16
NIID0096		clinical isolate	*A. lentulus*	*A. lentulus*	0.03	0.25	4	>64	>64	>8	2	>16
NIID0096AF	1	Mutant; NIID0096	*A. lentulus*	*A. fumigatus*	0.03	0.5	4	>64	>64	0.5	0.5	8
NIID0096AF	2	Mutant; NIID0096	*A. lentulus*	*A. fumigatus*	≦0.015	0.5	4	>64	>64	1	1	>16
NIID0096AF	3	Mutant; NIID0096	*A. lentulus*	*A. fumigatus*	≦0.015	0.5	4	>64	>64	1	1	>16
NIID0096AL	1	Mutant; NIID0096	*A. lentulus*	*A. lentulus*	≦0.015	0.12	4	>64	>64	>8	4	>16
NIID0096AL	2	Mutant; NIID0096	*A. lentulus*	*A. lentulus*	≦0.015	0.5	8	>64	>64	>8	4	>16
NIID0096AL	3	Mutant; NIID0096	*A. lentulus*	*A. lentulus*	≦0.015	0.5	4	>64	>64	>8	4	>16

Fig1

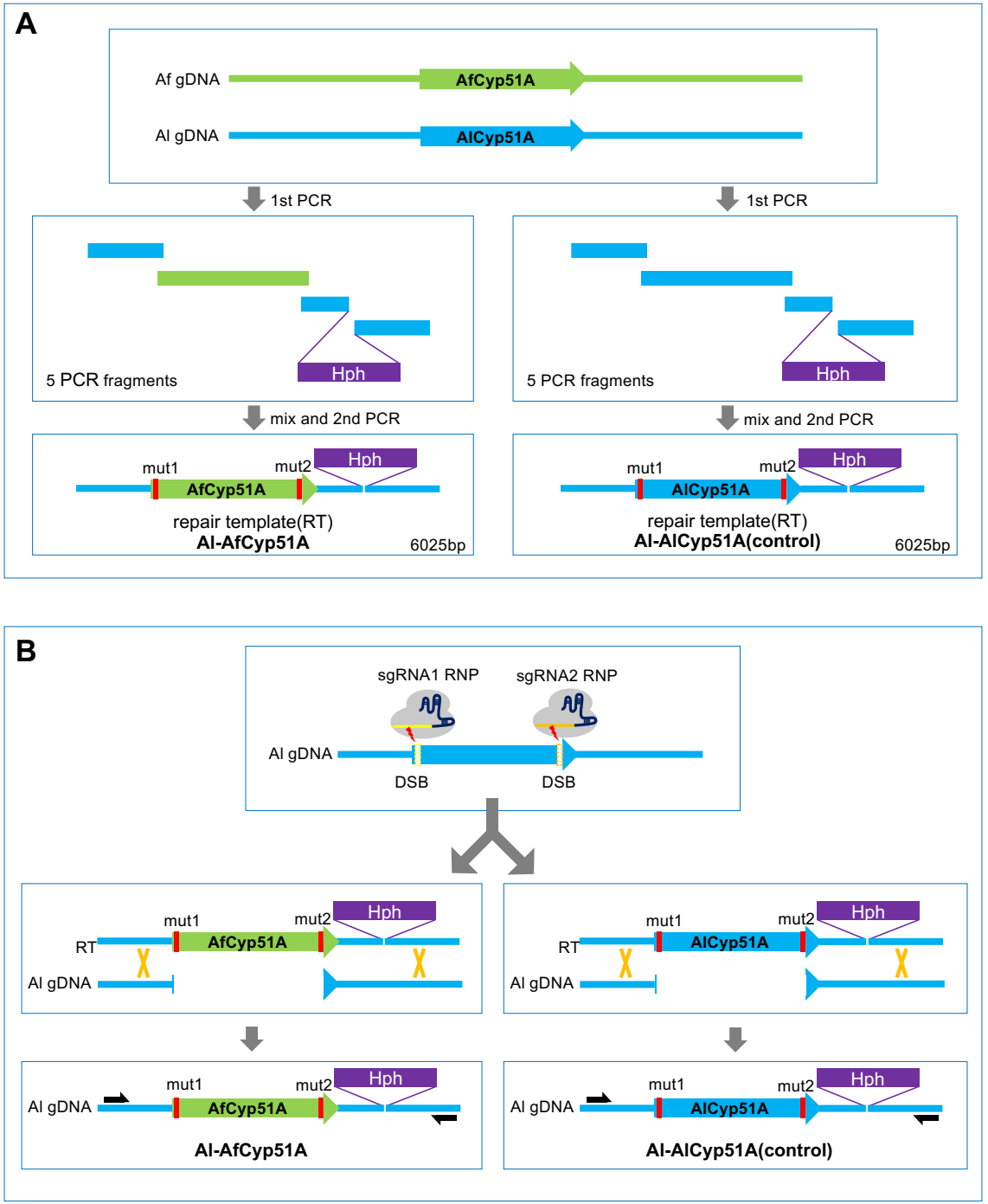
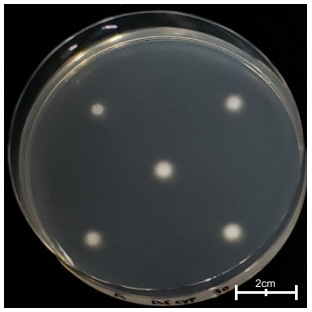
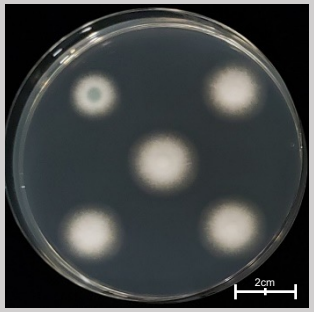
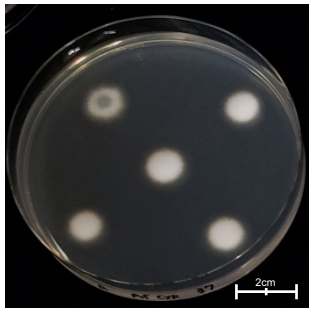
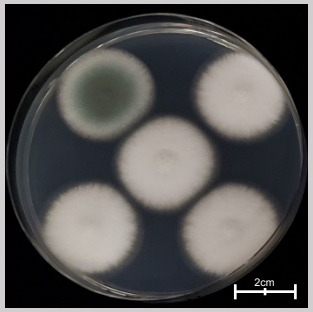

A
Af gDNA
AfCyp51A
Al gDNA
AlCyp51A
1st PCR
1st PCR
5 PCR fragments
Hph
5 PCR fragments
Hph
mix and 2nd PCR
mix and 2nd PCR
mut1
mut2
Hph
AfCyp51A
repair template(RT)
Al-AfCyp51A
6025bp
mut1
mut2
Hph
AlCyp51A
repair template(RT)
Al-AlCyp51A(control)
6025bp
B
sgRNA1 RNP
sgRNA2 RNP
Al gDNA
DSB
DSB
RT
mut1
mut2
Hph
AfCyp51A
Al gDNA
RT
mut1
mut2
Hph
AlCyp51A
Al gDNA
mut1
mut2
Hph
AfCyp51A
Al-AfCyp51A
Al gDNA
mut1
mut2
Hph
AlCyp51A
Al-AlCyp51A(control)

Fig. 2

48 h

Strain \ Temperature / Culture medium	30℃		37℃	
	AMM	PDA	AMM	PDA
AfCyp51A Afs35 NIID 0096 NIID0096AF				
AlCyp51A Afs35 NIID 0096 NIID0096AL