1 **Cas9/CRISPR genome editing to demonstrate the contribution of Cyp51A Gly138Ser to**
2 **azole resistance in *Aspergillus fumigatus***
3
4
5 **Supplementary data**
6
7 ***A. fumigatus* media.** *A. fumigatus* cultures were routinely grown in *Aspergillus* minimal
8 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
9 $Mg(NO_3)_2 \cdot 6H_2O$, 1 mL trace elements (1) in 1 L distilled water), Czapek-Dox medium (CD,
10 BD Difco Laboratories Inc., Franklin Lakes, NJ), YG medium (1), or potato dextrose agar
11 medium (PDA, BD Difco). For solid medium, 1.5% agar was added. *A. fumigatus* conidia
12 were obtained from mycelia cultured on AMM or PDA at 30˚C for 3–7 days, harvested with
13 PBS containing 0.05% (v/v) Tween 20 and 20% (v/v) glycerol, and filtered through a 40-µm
14 nylon cell strainer (Greiner Bio-One, Germany).
15
16 **DNA extraction, PCR, and sequencing.** Genomic DNA extractions and purifications were
17 performed using a DNeasy Plant Mini Kit (QIAGEN, Germany). Primers for the
18 amplification and sequencing of *cyp51A* are listed in **Table S1**. Identification was confirmed
19 by sequencing of the internal transcribed spacer (ITS) and D1/D2 regions and the β-tubulin
20 gene. PCR amplification of *cyp51A* was performed using NIID0345 genomic DNA as a
21 template and primers Discheck5 and Discheck3 using Q5 Hot Start High-Fidelity 2× Master
22 Mix (New England Biolabs, Ipswich, MA).
23
24 **sgRNA *in vitro* synthesis.** We manually searched for target sequences consisting of
25 G(N)15(A/T)(N)3NGG near the N-terminus (for sgRNA1) and C-terminus (for sgRNA2) as
26 sgRNA target sequences and synthesized two oligonucleotides (T7-sgRNA1 and T7-
27 sgRNA2, **Table S1**) consisting of the T7 promoter, sgRNA target sequence, and overlap
28 sequence with Cas9 scaffold. These oligonucleotides were used for sgRNA synthesis via the

29 EnGen® sgRNA Synthesis Kit, *S. pyogenes* (New England Biolabs). The synthesized
30 sgRNAs were purified using an RNA clean & concentrator-25 (Zymo Research, Irvine, CA),
31 quantified using a QuantiFluor RNA system (Promega, Madison, WI) and Quantus
32 Fluorometer (Promega), and used for ribonucleoprotein formation with Cas9.
33
34 **Repair templates.** A pHph plasmid harboring a hygromycin B resistance cassette (*hph*) was
35 generated by deletion of two *loxP* sequences and HSV1 thymidine kinase sequences from
36 pSK397 (2). Primers for the repair template construction are listed in **Table S1**. A region
37 from 825-bp upstream to 1503-bp downstream of the *cyp51A* coding region was used for
38 repair templates. The mutations and *hph* marker were introduced via PCR sewing or overlap
39 extension PCR. The *hph* marker for selection of transformants was inserted between
40 nucleotides 500 and 501 downstream of the *cyp51A* stop codon. Q5 Hot Start High-Fidelity
41 2× Master Mix (New England Biolabs) was used for PCR amplification. Primer
42 combinations for overlap extension PCR are listed in **Table S2**. Briefly, NIID0345 or AfS35
43 genomic DNA was used as a template to generate overlapping PCR products with the
44 corresponding site-specific mutations or junctions between *cyp51A* and the *hph* marker. The
45 overlapping PCR products were mixed together and used as a template in the PCR-sewing
46 step using the primers LFH1 and LFH4. Overlapping PCR product combinations are listed in
47 **Table S3**. The fused PCR products were purified using a NucleoSpin® Gel and PCR Clean-
48 up kit (Takarabio, Japan) and used for *A. fumigatus* protoplast transformation.
49
50 ***A. fumigatus* transformation.** *A. fumigatus* protoplasts were generated and fungal
51 transformation was performed as previously described (1), with slight modifications. Briefly,
52 conidia were incubated in YG medium for 6 h at 37°C. Following incubation, the cell walls
53 of germlings were digested with 0.2 g/mL VinoTaste Pro (Novozymes, Denmark) for 1 h at

54 30°C; 20 pmol Cas9-NLS protein (New England Biolabs) and 10 pmol each *in vitro*-
55 synthesized sgRNA1 and sgRNA2 were mixed and incubated for 25 min, generating
56 ribonucleoproteins (RNPs). Protoplasts were transformed with 2–3 μg of repair templates and
57 RNPs and plated onto CD supplemented with 1 M sucrose. Using NIID0345 clinical isolate
58 as a host, repair templates 0345-mut1-S138-K248-mut2-hph, 0345-mut1-G138-K248-mut2-
59 hph, 0345-mut1-S138-N248-mut2-hph, or 0345-mut1-G138-N248-mut2-hph were used to
60 generate strains NIID0345-mut1-2, NIID0345-S138G, NIID0345-K248N, or NIID0345-S138G-
61 K248N, respectively. Using AfS35 strain as a host, repair templates 35-mut1-G138-mut2-hph
62 or 35-mut1-S138-mut2-hph were used to generate strains AfS35-mut1-2 or AfS35-G138S,
63 respectively. Following a 15-h incubation at 37°C, plates were overlaid with CD top agar
64 containing 400 μg/mL hygromycin. Positive colonies were confirmed by colony PCR using
65 KOD FX Neo DNA polymerase (TOYOBO, Japan) with the primers Discheck5 and
66 Discheck3 (which were designed at the region outside the repair template sequence),
67 followed by nucleotide sequencing of *cyp51A*, including the promoter region.
68

69 **Antifungal susceptibility testing.** Susceptibility to VRC, ITC, and POS were evaluated with
70 Etest strips according to the manufacturer's instruction (Biomerieux, France). Strains were
71 grown at 37°C, and growth inhibition was visually evaluated after 48 h. Strains with an MIC
72 >0.25 μg/mL for POS were considered resistant according to the epidemiological cutoff
73 values (ECVs) for Etest proposed by Espinel-Ingroff et al. (3). Because ECVs for VRC and
74 ITC have not been established for Etest, strains with an MIC >1 μg/mL for VRC and ITC
75 were considered resistant according to CLSI ECVs proposed by Espinel-Ingroff et al. (4).
76 Susceptibility tests were performed in three independent Cyp51A-sequence-confirmed
77 transformants for each strain.

78
79

80 **Table S1. Oligonucleotide primers used in this study.**

Primer	Sequence (5′-3′) [a]	Orientation	Use
Discheck5	ATGCAGTGAAAAATTCCTAGCAG	Sense	cyp51A amplification and verification of transformants
Discheck3	ATGGTGGTGGTCAAGGTTCAGCAG	Antisense	cyp51A amplification and verification of transformants
T7-sgRNA1	TTCTAATACGACTCACTATAGCTATGGCTTACGGCCTACAGTTTTAGAGCTAGA	Sense	in vitro sgRNA synthesis
T7-sgRNA2	TTCTAATACGACTCACTATAGGGATGAATAGTCAGTTTCAGTTTTAGAGCTAGA	Antisense	in vitro sgRNA synthesis
LFH1	GCGAGCCATGCTGGGAGGAATCTC	Sense	Repair template construction and amplification
Mut1-3	CGGCCATATACGCGGTAAGCCATAGCATCGGCAC	Antisense	Repair template construction
Mut1-5	GGCTTACCGCGTATATGGCCGTTGCGGTGCTGAC	Sense	Repair template construction
G138-3	TGAGTCAAGCCGTACTTGATGAACTTTTTCTGCTC	Antisense	Repair template construction
G138-5	CATCAAGTACGGCTTGACTCAGTCTGCGTTAGAG	Sense	Repair template construction
S138-3	TGAGTCAAGCTGTACTTGATGAACTTTTTCTGCTC	Antisense	Repair template construction
S138-5	CATCAAGTACAGCTTGACTCAGTCTGCGTTAGAG	Sense	Repair template construction
N248-3	GACGGCGCTGATTGATGATGTCAACGTAGATTGAC	Antisense	Repair template construction
N248-5	ACATCATCAATCAGCGCCGTCTTGACGGTGACAAG	Sense	Repair template construction
Mut2-3	AGTCAGTCTCTGGCACTCCTTTCTTTCCATCCAC	Antisense	Repair template construction
Mut2-5	AAGGAGTGCCAGAGACTGACTATTCATCCCTC	Sense	Repair template construction
LFH6	GATATCGGCCTGAGTGGCCTCCAGGTTTTCGCACGAGCTTCTCC	Antisense	Repair template construction
LFH3	GTTGTCGACGGCCATCTAGGCCAGTTTTTGATAGTCTTCAAAAGTCAG	Sense	Repair template construction
LFH4	CGTATTGGTGAGCTGATGATCATC	Antisense	Repair template construction and amplification
Hph5	GAGGCCACTCAGGCCGATATCACC	Sense	hph cassette amplification
Hph3	CTGGCCTAGATGGCCGTCGACAAC	Antisense	hph cassette amplification
proseq3	TTAGTAATTAGGCAACTTTCATTC	Antisense	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

81 [a] Letters in bold indicate the mutated nucleotide. Underlining indicates an additional

82 sequence for in vitro gRNA synthesis.

83 **Table S2. Combination of primers for overlapping PCR used in this study.**

Name of PCR product	Primers	Template DNA
0345-A	LFH1/Mut1-3	NIID0345 genomic DNA
0345-mut1-G138	Mut1-5/G138-3	NIID0345 genomic DNA
0345-mut1-N248	Mut1-5/N248-3	NIID0345 genomic DNA
0345-mut1-mut2	Mut1-5/Mut2-3	NIID0345 genomic DNA
0345-G138-mut2	G138-5/Mut2-3	NIID0345 genomic DNA
0345-G138-N248	G138-5/N248-3	NIID0345 genomic DNA
0345-N248-mut2	N248-3/Mut2-3	NIID0345 genomic DNA
0345-C	Mut2-5/LFH6	NIID0345 genomic DNA
0345-B	LFH3/LFH4	NIID0345 genomic DNA
35-A	LFH1/Mut1-3	AfS35 genomic DNA
35-mut1-mut2	Mut1-5/Mut2-3	AfS35 genomic DNA
35-mut1-S138	Mut1-5/S138-3	AfS35 genomic DNA
35-S138-mut2	S138-5/Mut2-3	AfS35 genomic DNA
35-C	Mut2-5/LFH6	AfS35 genomic DNA
35-B	LFH3/LFH4	AfS35 genomic DNA
Hph	Hph5/Hph3	pHph plasmid DNA

84

85 **Table S3. Combination of PCR products for repair template amplification used in this**

86 **study.**

Name of repair template	PCR products
0345-mut1-S138-K248-mut2-hph	0345-A, 0345-mut1-mut2, 0345-C, Hph, 0345-B
0345-mut1-G138-K248-mut2-hph	0345-A, 0345-mut1-G138, 0345-G138-mut2, 0345-C, Hph, 0345-B
0345-mut1-S138-N248-mut2-hph	0345-A, 0345-mut1-N248, 0345-N248-mut2, 0345-C, Hph, 0345-B
0345-mut1-G138-N248-mut2-hph	0345-A, 0345-mut1-G138, 0345-G138-N248, 0345-N248-mut2, 0345-C, Hph, 0345-B
35-mut1-G138-mut2-hph	35-A, 35-mut1-mut2, 35-C, Hph, 35-B
35-mut1-S138-mut2-hph	35-A, 35-mut1-S138, 35-S138-mut2, 35-C, Hph, 35-B

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