SUPPLEMENTAL MATERIAL

Materials and Methods

Strains and media. Six strains of *Aspergillus flavus* were used for susceptibility tests: NRRL3518 (obtained from USDA Agricultural Research Service Culture Collection, Peoria, IL, United States), SS1 and SS2 (both isolated from stored wheat grains), SS3 (isolated from silage), SS4 (isolated from groundnuts), and SS5 (isolated from chickpeas). After determining that VRC resistance was consistent across a range of isolates (0.125 µg/ml for all tested strains), we chose the highly aflatoxigenic strain of *A. flavus* (SS1) to generate all strains used in this study (Table 1). Strains were refreshed from -80 °C by subculturing on potato dextrose agar (PDA; 0.4% potato starch, 2% dextrose, and 2% agar) or broth (PDB) and maintained on PDA plates at 28 °C. Conidia were collected in sterile saline and spore concentrations were determined using a hemocytometer. The viability of inoculum was verified by determining colony forming units (CFUs) on PDA. Stock solutions of VRC (Sigma-Aldrich, St. Louis, MO, USA) were prepared in DMSO at a concentration of 20 mg/ml and kept at −20 °C. Antifungal microdilution susceptibility testing was performed on RPMI 1640 medium (Sigma-Aldrich) buffered with 0.165 M morpholinepropanesulfonic acid (MOPS) pH 7.

Susceptibility testing. The susceptibility of *A. flavus* isolates to VRC was determined using Etest assays and the CLSI method. For Etests, 200 µl of *A. flavus* conidial suspension (containing 10^6 spores/ml) was spread on PDA. After inoculation, a VRC Etest strip (bioMérieux, Marcy l'Etoile, France) was placed in the center of the plate and the plate was incubated at 30 °C for 72 h. The CLSI M38-A2 broth microdilution method was performed according to standard guidelines (25).

Screening for resistance. Conidial suspensions (~1 x 10^3 spores/ml) were plated onto PDA plates amended with various concentrations of VRC (0, 0.125, 0.25, 0.5, 1, and 2 µg/ml). Growth was recorded after 96 h incubation at 28 °C. Isolates were considered resistant to VRC based on their ability to grow on plates containing VRC. Resistant subpopulations were exposed to stepwise increases in VRC concentrations on PDA media.

Highly resistant clones (2 and 4 µg/ml VRC) were used to study the stability of their resistance phenotype. Conidial suspensions of four isolates resistant to high concentrations of VRC were transferred onto drug-free PDA plates and incubated at 28 °C; such transfers were carried out every 96 h. After serial transfers on drug-free media the isolates were tested for their susceptibility to VRC.

Whole-genome sequencing. To obtain tissue for DNA extractions, *A. flavus* SS1 wild-type strain and VRC resistant clones were grown in PDB amended with VRC concentrations corresponding to their resistance (0 – 4 µg/ml) at 28 °C for 4 days. Fungal mycelia were collected, lyophilized, and genomic DNA was isolated using a cetyltrimethylammonium bromide (CTAB) extraction method (Sadhasivam et al., 2017). Paired-end libraries (150 bp) were prepared using TruSeq DNA Nano Library Prep Kit and sequenced using an Illumina NovaSeq 6000 platform. Following quality control and adapter trimming, paired-end reads of the 10 samples were mapped to the *Aspergillus flavus* NRRL3357 reference genome (assembly ASM1411746v1 from the

NCBI database) using the BWA-MEM algorithm (Li, 2013). The resulting mapping files were processed using Picard Tools v1.95 to add read group information, sort, mark duplicates and index. Then, we performed a local re-alignment of reads to minimize mismatched bases using the RealignerTargetCreator of the Genome Analysis Toolkit (GATK) v4.1.9.0. Finally, we called single nucleotide polymorphism (SNPs) and insertions and deletions (indels) using HaplotypeCaller of the GATK toolkit (DePristo et al., 2011). The predicted effect of variants was determined using SnpEff v5.0d (Cingolani et al., 2012) with publicly available gene models (genome annotation GCF_014117465.1 from the NCBI database). Copy number variation (CNV) was identified using CNVkit v0.9.7 (Talevich et al., 2016) in whole-genome sequencing (-wgs) mode. Coverage bigwig files were generated with the bamCoverage tool and ratios were calculated using bigwigCompare (deepTools v3.5.1) (Ramírez et al., 2016).

Quantitative real-time PCR. We performed qPCR assays to quantify the copy number of genes located on specific chromosomes in both wild-type and VRC-resistant strains. CNVs identified using whole-genome sequence data were confirmed by qPCR using the same sample of genomic DNA. Experiments were run in triplicate using Fast SYBR Green Master Mix in a StepOnePlus Real-Time PCR System (Applied Biosystems). The copy number of genes across seven chromosomes were standardized using the gene G4B84_009880 on Chr6 as endogenous control (Table S2). The relative copy number of each gene ($2^{(-\Delta\Delta Ct)}$) was obtained in comparison to the endogenous control. Results were analyzed with StepOne software. The sequences of the primers used for the qPCR are listed in Table S2.

TABLE S1. VRC MIC values of *Aspergillus flavus* wild type strains. The name, source, and VRC MIC (determined using Etest strips and CLSI method) is summarized for each of the six WT strains.

#	Strain name	Source	VRC MIC (µg/ml)
1	NRRL3518	ARS culture collection	0.125
2	SS1	Wheat	0.125
3	SS2	Wheat	0.125
4	SS3	Silage	0.125
5	SS4	Peanut	0.125
6	SS5	Chickpea	0.125

TABLE S2. Primers used for qPCR assays

Target	Chromosome location	Locus tag	Gene name	Primer sequence	Product size	Amplification efficiency	Source
chr1A	Chr1 (1.71/6.39 Mb)	G4B84_000547	*mdr1*	CCTCCCACAAGAGTGATAGA CGCATCGAGCTGTTTCTT	107 bp	101.1%	Paul et al., 2018
chr2A	Chr2 (5.44/6.25 Mb)	G4B84_003868	*mdr2*	GATATCTCCAACTAAGGGTACAAAGG TGAAGATGGGAACAGCAGGTC	104 bp	100.9%	Paul et al., 2018
chr3A	Chr3 (3.73/5.10 Mb)	G4B84_005397	*creA*	TGCCGGACAAGAGAATGCA AGCGGGTCATAGGCTTTGTG	80 bp	100.7%	This study
chr3B	Chr3 (4.12/5.10 Mb)	G4B84_005516	*chsE*	ATCTTCATCTTCCGCCGCAAGTG GTCATCCATGTGCCAGAACGAGTAG	114 bp	100.6%	Li et al., 2021
chr4A	Chr4 (1.90/4.66 Mb)	G4B84_006515	*mdr4*	GGTCAGCGTCAACGACTTTC ATCGTGTTGAGCCGGTGAG	176 bp	103.5%	Paul et al., 2018
chr5A	Chr5 (2.23/4.45 Mb)	G4B84_008177	*β-actin*	ACGGTGTCGTCACAAACTGG CGGTTGGACTTAGGGTTGATAG	130 bp	102.0%	Lan et al., 2019
chr6A	Chr6 (2.84/3.94 Mb)	G4B84_009880	*bgt1*	GTACTGCCGATCCCATCATCCAAG CCTTAGCAAGCCTCGTGTCATTCC	126 bp	95.8%	Li et al., 2021
chr7A	Chr7 (1.04/3.03 Mb)	G4B84_010605	*cdr1B*	GAAGTATCTGCCACCGAGAA GCCATCGACATGATCGAGAA	150 bp	103.5%	Paul et al., 2018
chr8A	Chr8 (0.28/3.18 Mb)	G4B84_011340	*mfs1*	ACAACGCCCCGCATAGTC TTGCTGTTACGCAGTTCATCG	103 bp	98.1%	Paul et al., 2018
chr8B	Chr8 (0.38/3.18 Mb)	G4B84_011371	*nrps-mrp*	CATCGGAGTGCCACAAGATG ACCATGTGACCCATTAGCCA	148 bp	104.7%	This study
chr8C	Chr8 (3.05/3.18 Mb)	G4B84_012248	*pks8.12*	ACCCGTCGATGGGAACAAT AGCAATTATGCGGTGATCGTT	70 bp	97.8%	This study
chr8D	Chr8 (2.08/3.18 Mb)	G4B84_011919	*nrps8.6*	TTGTGGCGGATGTATTTGGA AATGAGGACCCGCCCATTAC	70 bp	100.0%	This study

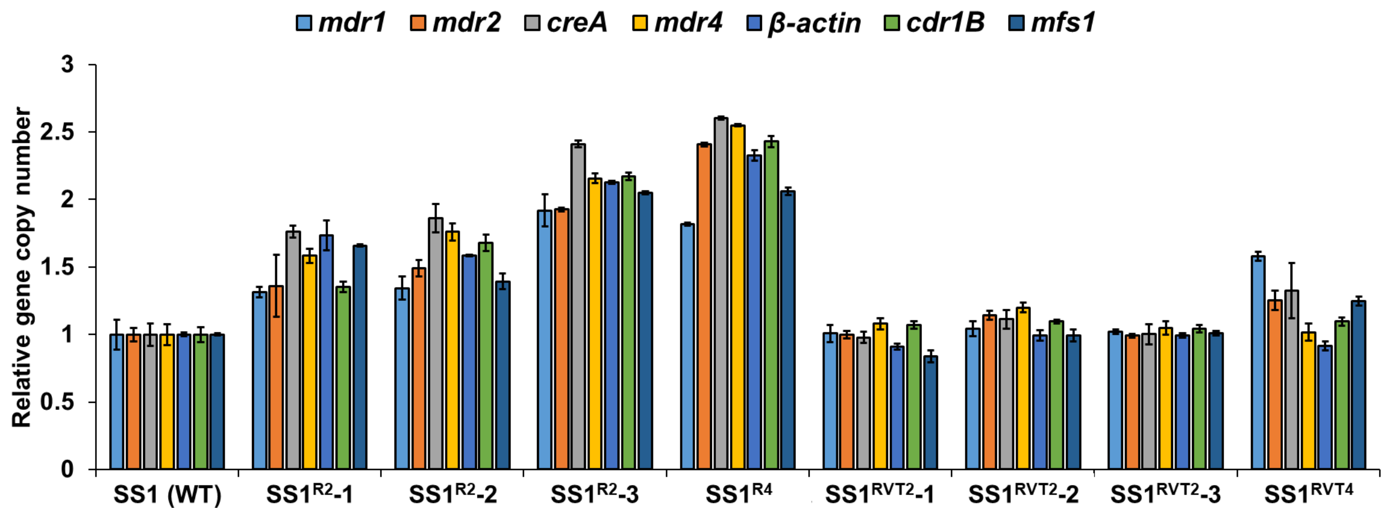

FIG S1. Gene copy number determined by qPCR in VRC resistant and reverted strains. The relative copy number of seven genes (*mdr1*, *mdr2*, *creA*, *mdr4, β-actin, cdr1B, mfs1*) located on Chr1, 2, 3, 4, 5, 7 and 8 was determined relative to the control gene (*bgt1*) on Chr6. Resistant strains are named $SS1^{R2}$-1, $SS1^{R2}$-2, $SS1^{R2}$-3, $SS1^{R4}$ and reverted strains are named $SS1^{RVT2}$-1, $SS1^{RVT2}$-2, $SS1^{RVT2}$-3, $SS1^{RVT4}$.

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