Supplemental Materials

Supplemental methods

HPLC and mass spectrometry analysis

Metabolite's extraction: The samples (10mg) were taken respectively and homogenized with 200 μL of methanol (50%) which contained internal standard and centrifuged at 12,000 rpm for 30 min, after leaving it on ice for 30 min. Finally, the supernatant was injected into the LC-MS/MS system for analysis. LC-MS method: The chromatographic mobile phase was: A mobile phase: aqueous solution containing 2.5 mmoL/L ammonium formate, and D mobile phase: acetonitrile. The gradient elution procedure for determination of samples was: 0-1.0 min, 70 -40% A; 1.0-2.0min, 40%-5% A; 2.0-4.0 min, 5% A; 4-4.1 min, 70% A; 4.1-7.0min, 70% A: 5μL per injection, flow rate 0.3 mL/min. Chromatographic column: Thermo Hypersil Gold C18 3μm, 2.1×100 mm, chromatographic column temperature: 30℃. Data were collected in electrospray ionization (ESI) positive, Spray voltage: 3500V; evaporation temperature: 350℃; sheath gas: 40Arb; auxiliary gas: 10Arb; capillary temperature: 320℃; S-lens RF: 50, NCE: 30. Standard solution preparation and standard curve: The standard solution with a concentration of 1, 10, 50, 100, 500, 1000, 5000, and 10000 ng/ml was prepared in acetonitrile. Adding 200 μL (methanol: acetonitrile = 1: 1) solutions into 100 μL standard working fluids, vortexing for 60 s, and centrifuging at 13,000 rpm for 10 min. Taken 100 μL for analysis by LC-MS. Taking the concentration of each compound as the X-axis, the ratio of peak area between each compound, and the internal standard as the Y-axis. The regression operation is carried out by the weighted least square method and the linear regression equation is obtained.

Supplemental figure legends

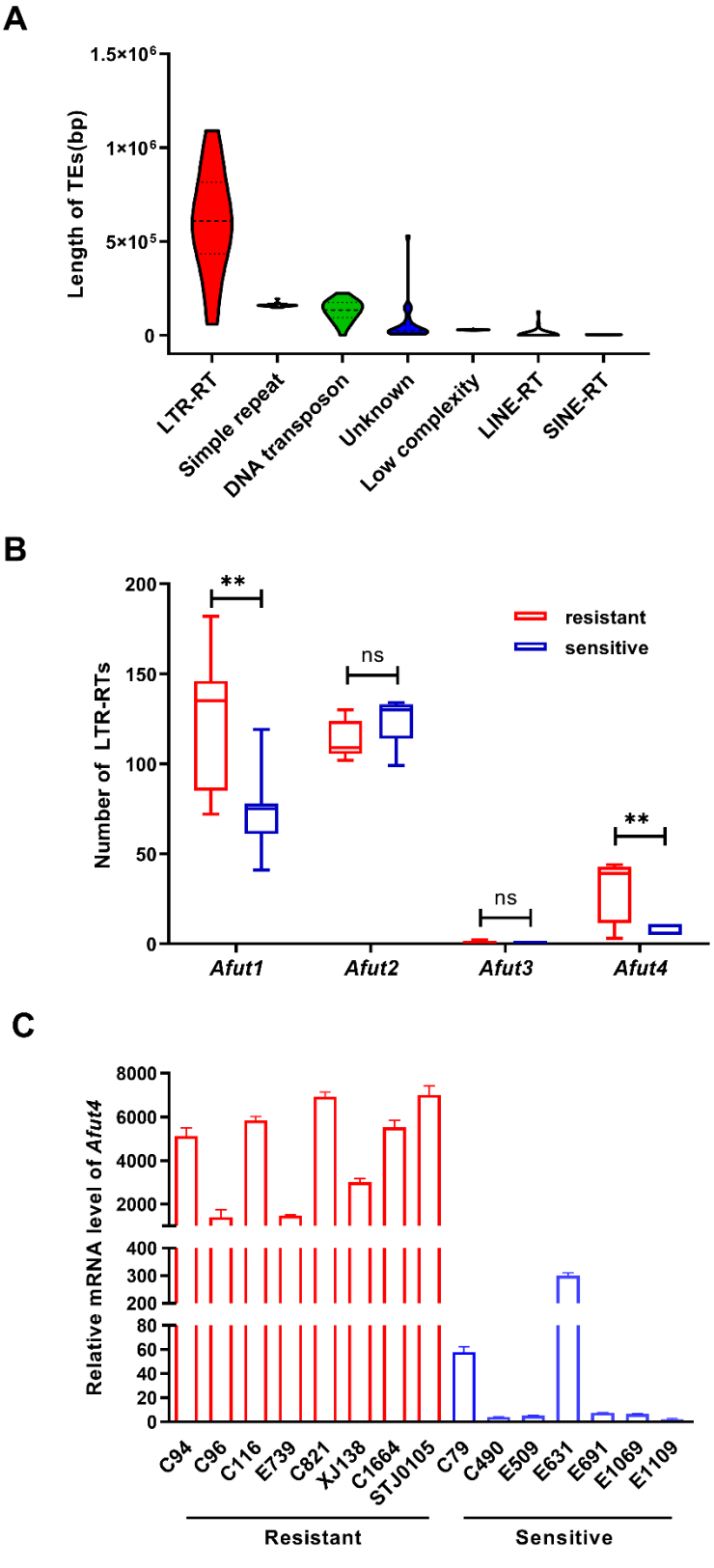

Fig. S1 (A) The total length of different TEs from genomic sequences of 15 *A. fumigatus* strains. TEs families were identified by RepeatModeler-1.0.11 based on the repeat databases (Repbase Update) copyrighted by the Genetic Information Research Institute (G.I.R.I.). **(B)** The number of four LTR-RTs in azole-resistant and azole-sensitive strains. *P*-values were calculated using unpaired Student's t-tests: $*P < 0.05$; ns, $P > 0.05$. **(C)** The specific expression level of *Afut4* in all 15 strains. Total RNA was prepared from the culture of each strain. Levels of the indicated mRNAs were determined by qPCR.

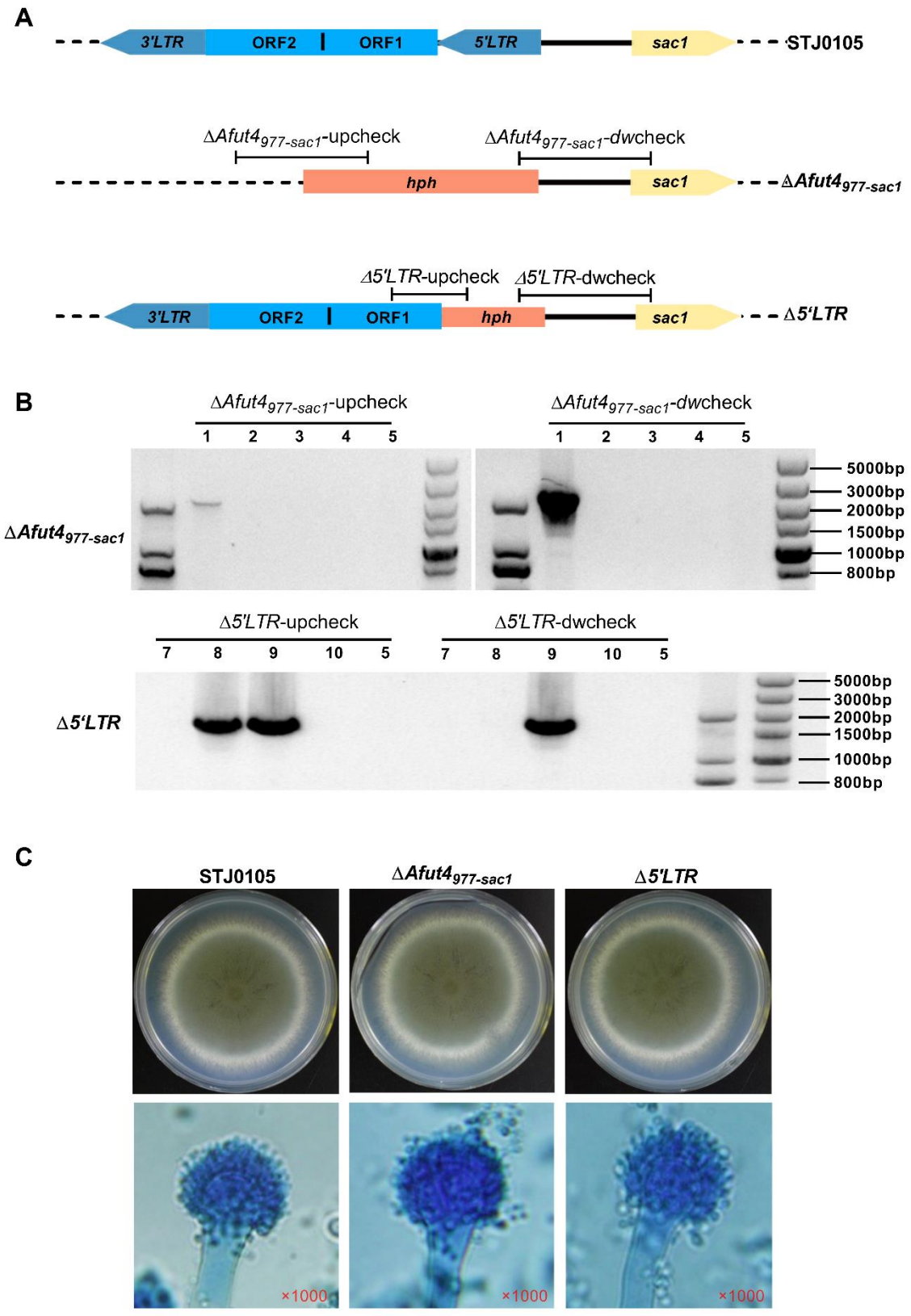

Fig. S2 Schematic depiction and verification of Δ*Afut4*$_{977\text{-}sac1}$ and Δ*5'LTR*

(A) The strategy of verification of Δ*Afut4*$_{977\text{-}sac1}$ and Δ*5'LTR*. **(B)** The primer pairs Δ*Afut4*$_{977\text{-}sac1}$-upcheck-F/R and Δ*Afut4*$_{977\text{-}sac1}$-dwcheck-F/R were used to validate the deletion of full-length *Afut4*$_{977\text{-}sac1}$ (Table S1). The successful verification of both Δ*Afut4*$_{977\text{-}sac1}$-upcheck-F/R and Δ*Afut4*$_{977\text{-}sac1}$-dwcheck-F/R was Δ*Afut4*$_{977\text{-}sac1}$ strain. The primer paris Δ*5'LTR*-upcheck-F/R and Δ*5'LTR*-dwcheck-F/R were used to validate the deletion of *5'LTR* of *Afut4*$_{977\text{-}sac1}$ (Table S1). The successful verification of both Δ*5'LTR*-upcheck and Δ*5'LTR*-dwcheck was Δ*5'LTR* strain. The samples 1~4 were the Δ*Afut4*$_{977\text{-}sac1}$ transformants and the samples 7~9 were the Δ*5'LTR* transformants. Sample 5 was STJ0105. **(C)** Colony and conidiophore morphology of STJ0105, Δ*Afut4*$_{977\text{-}sac1}$, and Δ*5'LTR*.

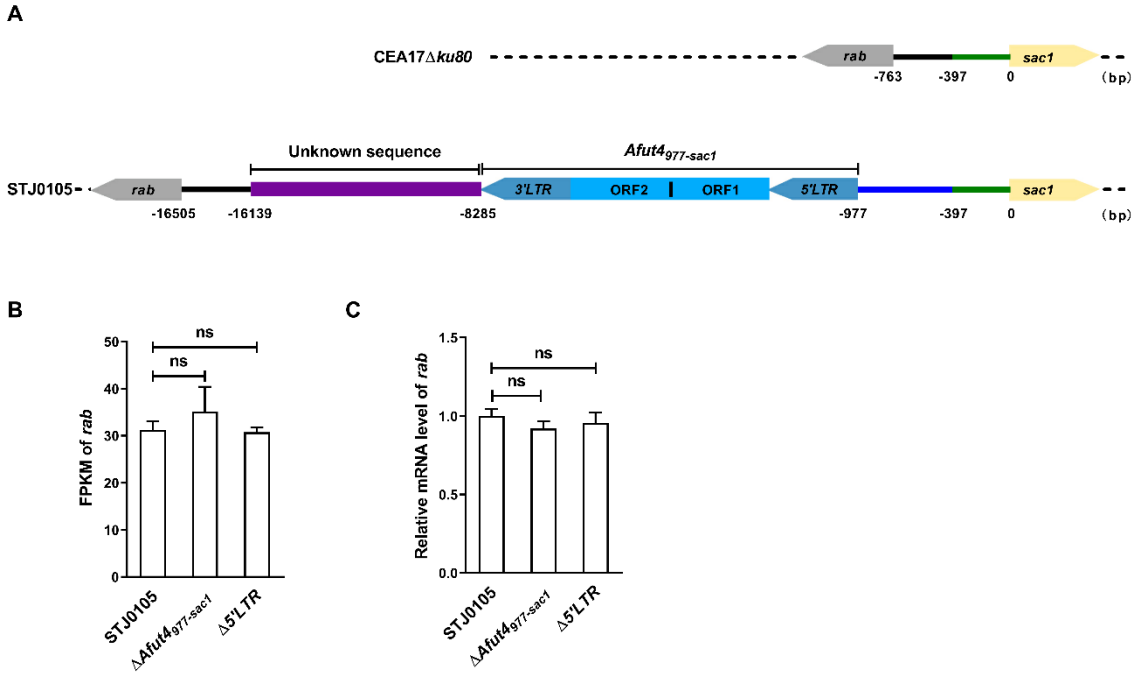

Fig. S3 (A) Schematic depiction and verification of the location of *sac1* (AFUA_4G08050) and *rab* (AFUA_4G08040) in CEA17Δ*ku80* and STJ0105. The yellow tapered rectangle is

sac1 (AFUA_4G08050) and the gray tapered rectangle is *rab* (AFUA_4G08040). The 762 bp sequence between *rab* and *sac1* in CEA17Δ*ku80* consists of both the green and black sequences. The green line is a 396 bp sequence and the black line is a 366 bp sequence. The insertion of $Afut4_{977\text{-}sac1}$ separates the black and green sequence with a more than 15,000bp sequence and the 579 bp blue sequence. **(B)** FPKM measurements of *rab* of the parental STJ0105, Δ$Afut4_{977\text{-}sac1}$, and Δ*5'LTR*. FPKM is fragments per kilobase per million mapped reads. *P*-values were calculated using unpaired Student's t-tests: ns, $P > 0.05$. **(C)** The mRNA level of *rab* of the parental STJ0105, Δ$Afut4_{977\text{-}sac1}$, and Δ*5'LTR* by RT-qPCR. *P*-values were calculated using unpaired Student's t-tests: ns, $P > 0.05$.

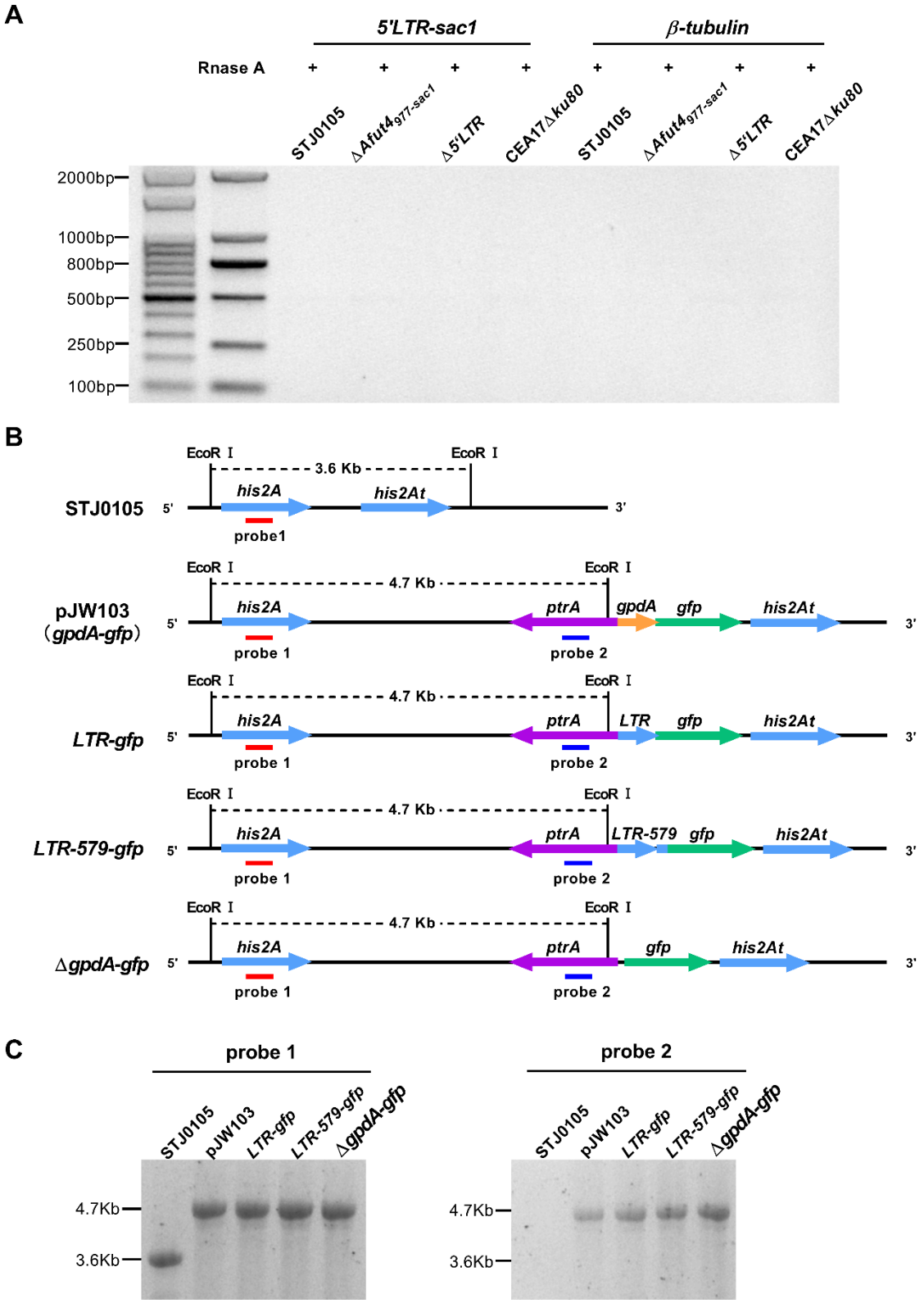

Fig. S4 (A) The negative control of reverse transcription PCR detection of the *5'LTR-sac1*

transcript. The negative control: cDNA of STJ0105, Δ*Afut4*$_{977\text{-}sac1}$, Δ*5'LTR*, and CEA17Δ*ku80* were reverse transcribed from the RNA with RNase A added. Then the *5'LTR-sac1* transcript and *β-tubulin* were detected again. **(B) and (C)** Schematic depiction and verification of single integration of pJW103 or other derived plasmids into STJ0105. The pJW103 or pJW103-derived plasmids are designed to specifically integrate into the histone 2A locus of the *A. fumigatus* genome via a single crossing over because of the presence of *his2A* 3' flanking region, called *his2At*. The genomic DNA of STJ0105 carrying pJW103 or pJW103-derived plasmids were extracted and digested with the enzyme EcoRI. Probe 1 was designed to verify the correct insertion of pJW103 or pJW103-derived plasmids into the genome and was obtained by PCR using the primer pairs His-F/R (Table S1). The expected signal is 3.6 Kb for STJ0105. Following single homologous integration, the size expected would be 4.7 Kb. Probe 2 was designed to verify the single insertion of pJW103 or pJW103-derived plasmids in the genome and constructed using the primer pairs ptrA-F/R (Table S1). No signal is expected for STJ0105 while for plasmid-carried STJ0105 the expected signal is 4.7 Kb.

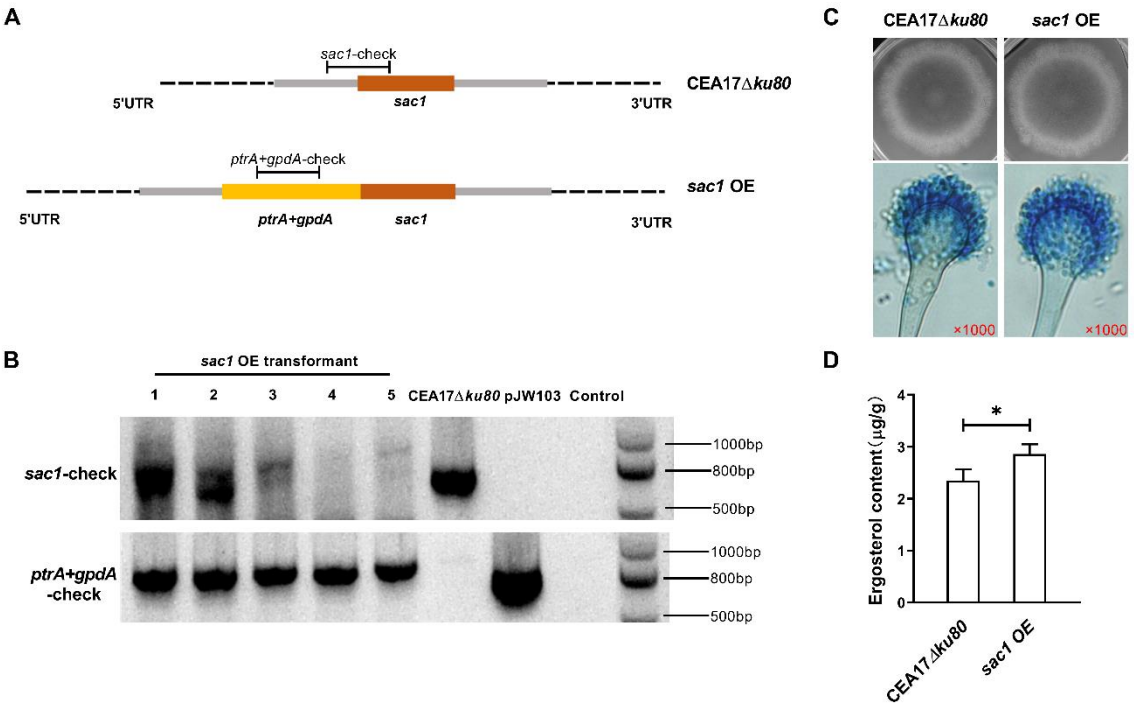

Fig. S5 Schematic depiction and verification of *sac1* OE

(A) The strategy of verification of *sac1* OE. **(B)** The primer Δ*sac1*-check and Δ*ptrA*+*grdA*-check were used to validate the construction of *sac1* OE (Table S1). The successful verification of *sac1* OE was transformant 4. **(C)** Colony and conidiophore morphology of CEA17Δ*ku80* and *sac1* OE. (D) Ergosterol content of the parental CEA17Δ*ku80* and *sac1* OE was analyzed by LC/MS assays. *P*-values were calculated using unpaired Student's t-tests: $*P < 0.05$.

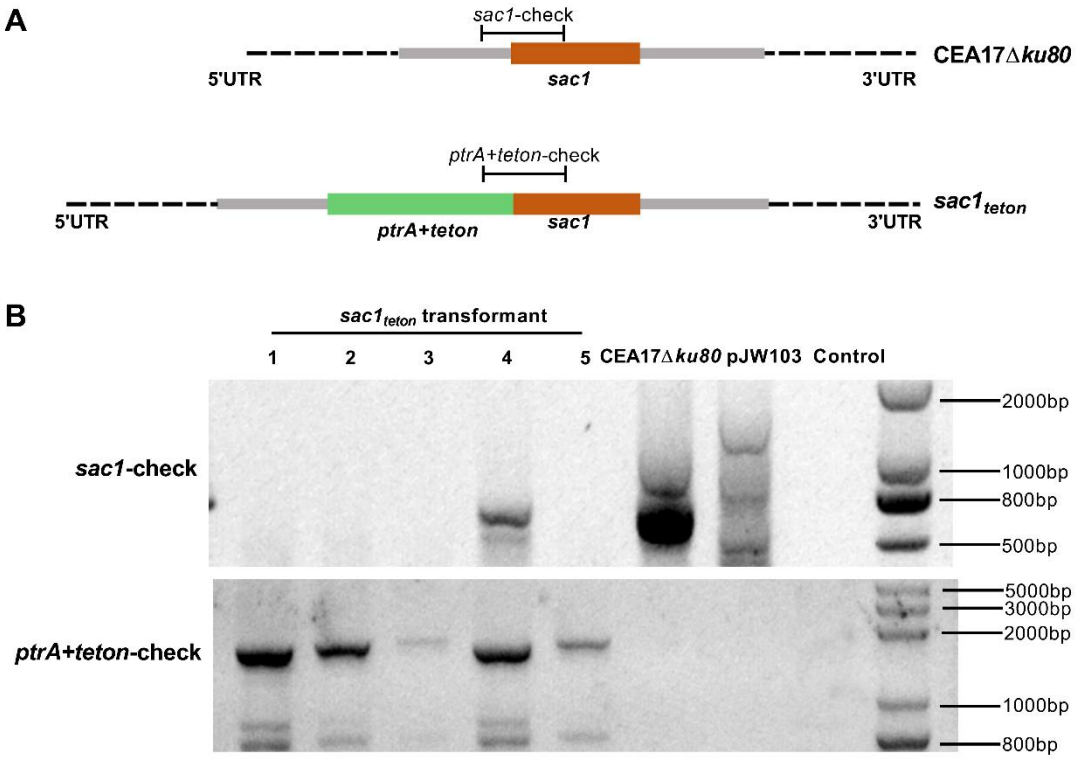

Fig. S6 Schematic depiction and verification of *sac1*$_{\text{teton}}$ (A) The strategy of verification of *sac1*$_{teton}$. **(B)** The primer pair *sac1*-check-F/R and *ptrA+teton*-check-F/R were used to validate the construction of *sac1*$_{\text{teton}}$ (Table S1).

Table S1. Primers used in this study

Primer name	Primer sequence (5' to 3')
Strains construction	
Δ*Afut4*$_{977\text{-}sac1}$-up-F	CTAGTCTGTAGCTGCCCTTAACCC
Δ *Afut4*$_{977\text{-}sac1}$-up-R	GAGCTCCAGCTTTTGTTCCGATTCCAACCTAGTAAAGGCAAATC
Δ *Afut4*$_{977\text{-}sac1}$-hph-F	GATTTGCCTTTACTAGGTTGGAATCGGAACAAAAGCTGGAGCTC
Δ *Afut4*$_{977\text{-}sac1}$-hph-R	GGCCTAGTGAGGTAAAAGATAAAGGAAGTCGACGGTATCGATAAGCTT
Δ *Afut4*$_{977\text{-}sac1}$-dw-F	AAGCTTATCGATACCGTCGACTTCCTTTATCTTTTACCTCACTAGGCC
Δ *Afut4*$_{977\text{-}sac1}$-dw-R	CCAGTTCGGAGCAATTCTTTCA
Δ *Afut4*$_{977\text{-}sac1}$-upcheck-F	CTATTTCCGCCTACTTAGGACATCT
Δ *Afut4*$_{977\text{-}sac1}$-upcheck-R	ACACCCCTGGCTTCACATTCT
Δ *Afut4*$_{977\text{-}sac1}$-dwcheck-F	GACTGAGGAATCCGCTCTTGG
Δ *Afut4*$_{977\text{-}sac1}$-dwcheck-R	CCGCCGCATCGTTGAGTATC
Δ5'*LTR*-up-F	TTGGCTAAGTAAAGCAGACCGC
Δ5'*LTR*-up-R	GAGCTCCAGCTTTTGTTCCGTCCATGTTTGTTCCAGAGGGT
Δ5'*LTR*-hph-F	ACCCTCTGGAACAAACATGGACGGAACAAAAGCTGGAGCTC
Δ5'*LTR*-hph-R	AGCATATCCAGGCTAAAGATGTAGGGTCGACGGTATCGATAAGCTT
Δ5'*LTR*-dw-F	AAGCTTATCGATACCGTCGACCCTACATCTTTAGCCTGGATATGCT
Δ5'*LTR*-dw-R	AACAGTGCCAGGTAGGTGTCTTC
Δ5'*LTR*-upcheck-F	GAACTGCTCTGTTGGGCACTTAT
Δ5'*LTR*-upcheck-R	CTTCACATTCTCCTTCGCTTACTG
Δ5'*LTR*-dwcheck-F	TTTCATTTTCGGGAGACGAGATC
Δ5'*LTR*-dwcheck-R	CTGAGATTGACGCTGGAAGCTGT
*sac1*OE-up-F	CGACGACTGTTTGCCAATT
*sac1*OE-up-R	CAGCGCGAGTGTGCTGAGTAATGTCAATGTCTCCCAGTGTTG
*sac1*OE-ptrA-F	CAACACTGGGAGACATTGACATTACTCAGCACACTCGCGCTG
*sac1*OE-ptrA-R	GAAAGGGCAGCACAGAAGGAGCCATGTGATGTCTGCTCAAGCGG
*sac1*OE-dw-F	CCGCTTGAGCAGACATCACATGGCTCCTTCTGTGCTGCCCTTTC
*sac1*OE-dw-R	TCCGGACGACGCTTGACT
sac1-check-F	ACATTGTCGGTTGGTTTGG
sac1-check-R	CGTGAAGTCCGTCTTGAGG
sac1$_{tet}$-up-F	GAAGTCGTTCCAGATCTATACCCTA
sac1$_{tet}$-up-R	ACCACGAACCATATTAATGATGTCT
sac1$_{tet}$-ptrA-F	CTGTATCGACAACGAGGCTCT
sac1$_{tet}$-ptrA-R	AGTTGAGCTGACCAGGGAAA
sac1$_{tet}$-dw-F	CAGGACTACACGGGCGTC
sac1$_{tet}$-dw-R	TTGAGCAGAGGCATTGGAAG
sac1-check-F	AGAGCGATGAAGGCGGGATC
sac1-check-R	AACGAGCGTCTGAGCATTAGGT
teton-check-F	TACTCCATCCTTCCCATCCCTT
teton-check-R	GAGACAACATCGGCATTATCAGC
ptrA+gpdA-check-F	GCGATGAAGTGGGAAAGCTC

ptrA+gpdA-check-R	TTACATTGTCGGTTGGTTTGG
sac1$_{tet}$-up-F	GAAGTCGTTCCAGATCTATACCCTA
sac1$_{tet}$-up-R	ACCACGAACCATATTAATGATGTCT
sac1$_{tet}$-ptrA-F	CTGTATCGACAACGAGGCTCT
sac1$_{tet}$-ptrA-R	AGTTGAGCTGACCAGGGAAA
sac1$_{tet}$-dw-F	CAGGACTACACGGGCGTC
sac1$_{tet}$-dw-R	TTGAGCAGAGGCATTGGAAG
ptrA+teton-check-F	TACTCCATCCTTCCCATCCCTT
ptrA+teton--check-R	GAGACAACATCGGCATTATCAGC
Plasmid construction	
5'*LTR*-up-F	TCCTCGCCCTTGCTCACCATGTTTAAACTGTCGCATAGCGTACGCT
5'*LTR*-up-R	GGCCTGAGTGGCCATCGAATTCCTGCAGGTGGCAGACTAACATGAT
5'*LTR*-579-up-F	CGCCCTTGCTCACCATGTTTAAACTCCGTGCCATACGCACCC
5'*LTR*-579-up-R	TGAGTGGCCATCGAATTCCTGCAGGTGGCAGACTAACATGATTTGAATGG
Blank-F	TCGCCCTTGCTCACCATGTTTAAACGTTTAAAC
Blank-R	TGAGTGGCCATCGAATTCCTGCAGCTGCAG
pJW103-check-F	AGCTCGATGCGGTTCACCAG
pJW103-check-R	TCAGCGGCACCCGATTCTAT
His-F	GTAACTACGCTCAACGTGTT
His-R	GAAAGCTGTCGGTATCATTC
ptrA-F	CTTCCTGTTGATGGAATGG
ptrA-R	GACGGCGCATGACCATAG
q-PCR or RT-PCR	
qRT-*Afut1*-F	GAAGTCGTTCCAGATCTATACCCTA
qRT-*Afut1*-R	ACCACGAACCATATTAATGATGTCT
qRT-*Afut4*-F	AGATCGCATCTAGGGAGTGAAGT
qRT-*Afut4*-R	AGGTGCGGAATGCTATCTTCT
qRT-*sac1*-F	CGATTTCCACAATGAGACCAA
qRT-*sac1*-R	TTCAACCCCACGGAAGTAGC
qRT-*gfp*-F	TGCCGTTCTTCTGCTTGTCG
qRT-*gfp*-R	GTCCAGGAGCGCACCATCTT
qRT-*tub1*-F	CTGTATCGACAACGAGGCTCT
qRT-*tub1*-R	AGTTGAGCTGACCAGGGAAA
qRT-*rab*-F	TCTTGCTTGGTGAATCTGCTG
qRT-*rab*-F	TTTGACCGTTGTGCTTTCGT
5'LTR-sac1-F	AGATGGGGCTATCTGTCTACCTGT
5'LTR-sac1-R	GACAACGAGCGTCTGAGCATTAG
sphk-692/392-F	TGACTTTGGGTAACGATGCTCTT
sphk-692/392-R	TATCTCGGTGGCGTGTCCTC

The DNA sequences ranging from the gene rab to gene sac1 in STJ0105.

>STJ0105_*rab_Afut4_sac1*

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2 *Aspergillus fumigatus* retrotransposon *Afut4*

3 579bp flanking sequence

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4 AFUA_4G08050 phosphoinositide phosphatase Sac1, putative