Supplemental Material

The cell wall integrity pathway contributes to the early stages of *Aspergillus fumigatus* asexual development

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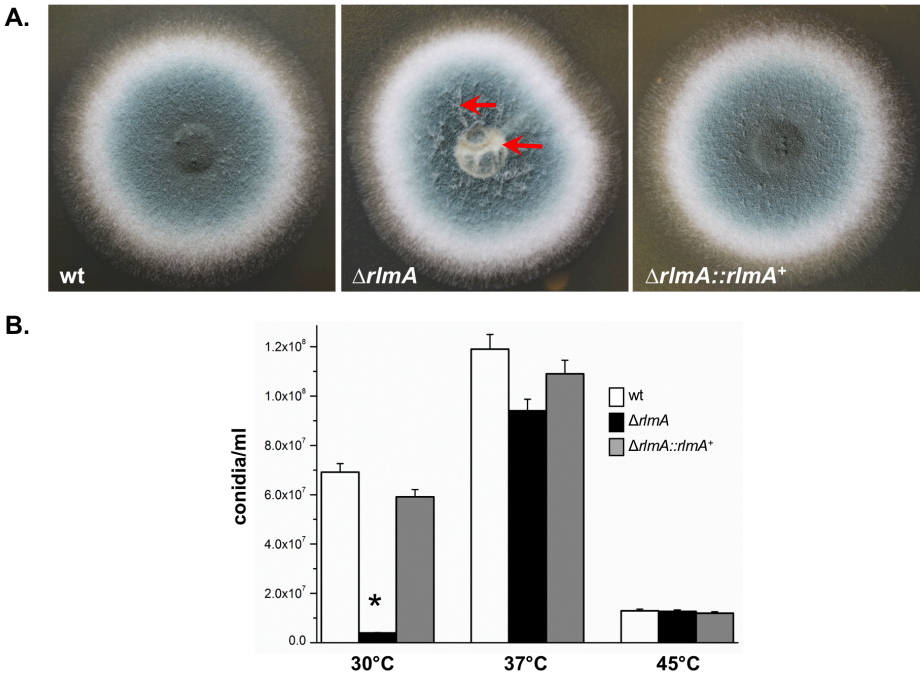


FIG S1 The Δ*rlmA* mutant presents lower number of conidia and a fluffy-like phenotype. (A) Radial growth of the wild-type, Δ*rlmA* and Δ*rlmA::rlmA*$^+$ strains on complete solid medium at 30°C. 1×10^5 conidia from each strain was inoculated onto the center of a solid YG plate and incubated for five days. Arrows indicate undifferentiated aerial hyphae (fluffy phenotype). (B) The conidia were collected by sampling four 0.5 cm^2 agar in 0.01% Tween 20 and counted in a Neubauer chamber. The results are the average ± SD (n = 4; *p ≤ 0.001).

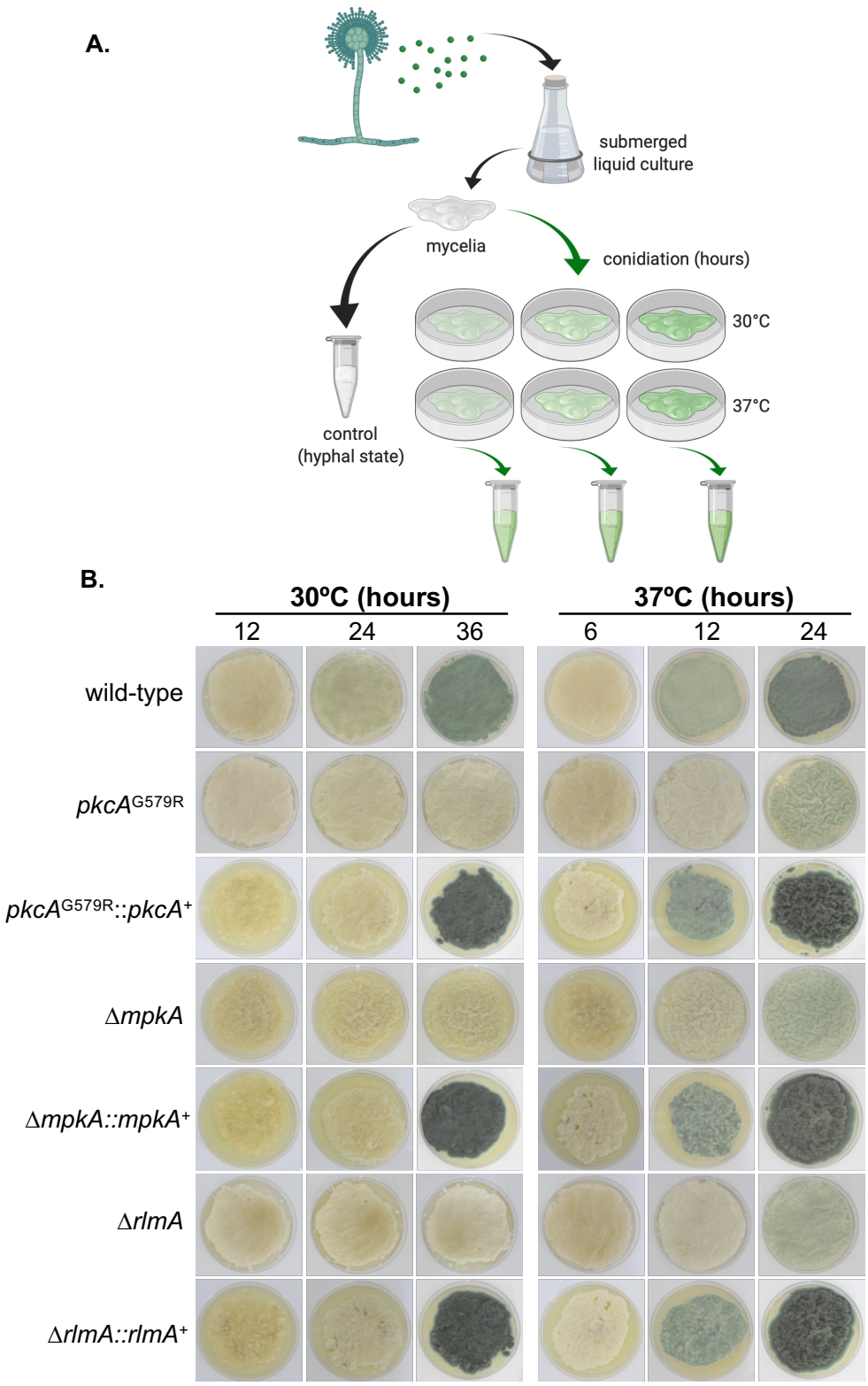


FIG S2 The Δ*rlmA* mutant presents defective conidiation during synchronized asexual differentiation. (A) Scheme of the conditions used to induce synchronized asexual differentiation. Vegetative mycelia grown for 18 hours in liquid MM supplemented with 0.1% yeast extract (37°C) were spread onto solid media and incubated under air-exposed conditions for the required number of hours in each experiment at 30°C or 37°C (green

arrows). The whole cells undergoing asexual differentiation were harvested from the plates at designated time points after transfer and used in the experiments. Vegetative mycelia (hyphal state) served as control. Created with BioRender.com. (B) Delayed conidiation of the CWI pathway mutants. The time (hours) indicates the growth at each temperature after the mycelium was transferred from the liquid-submerged synchronized culture to the air interface onto solid medium.

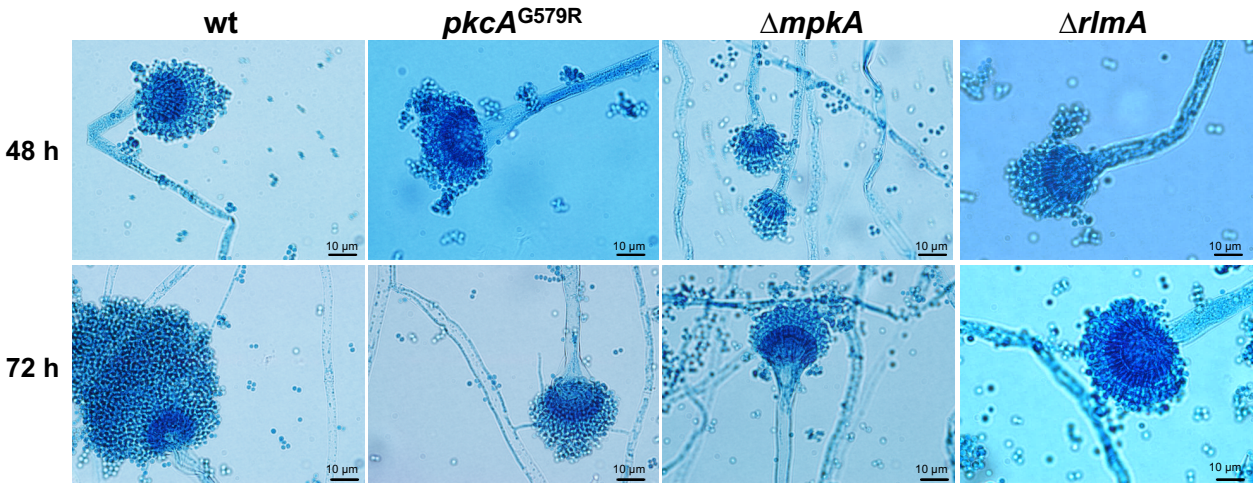


FIG S3 Conidiophore morphology of the wild-type, *pkcA*^{G579R}, Δ*rlmA* and Δ*mpkA* mutant strains was analyzed after 48 and 72 hours of cultivation on solid YG medium. Coverslips containing the adhered conidiophores were stained with lactophenol cotton blue and inspected under bright field microscope (100× magnification).

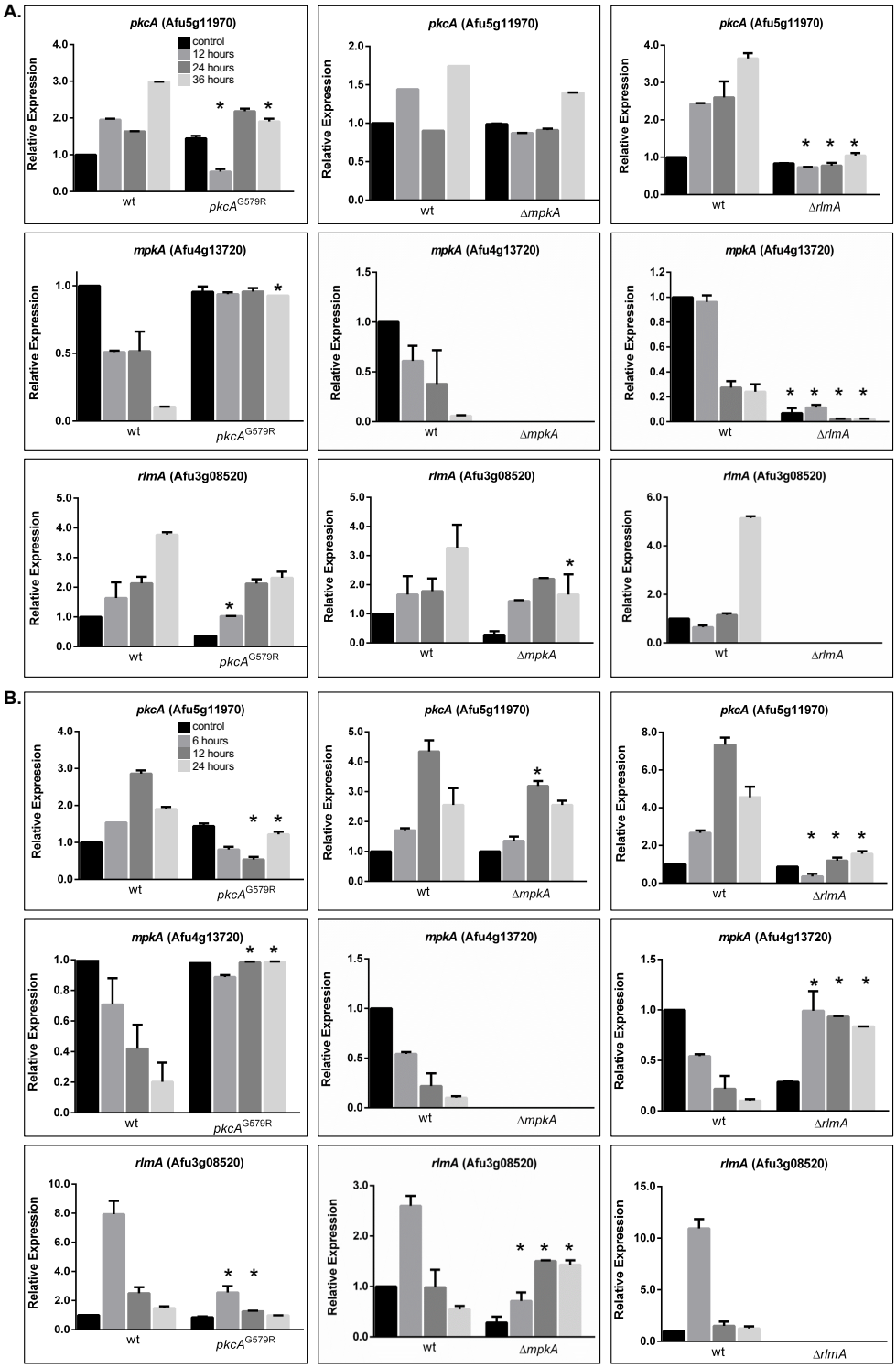


FIG S4 Asexual differentiation is accompanied by up-regulation of the CWIP genes. Total RNA was isolated from the strains subjected to synchronized asexual differentiation at 30°C (A) and 37°C (B). cDNA was obtained and used in RT-qPCR. The fold increase in each strain represents the normalized mRNA abundance relative to the wild-type strain. The data represent the average value of at least three independent experiments (with 2 technical repetitions each). *$p \leq 0.05$ (One-Way ANOVA).

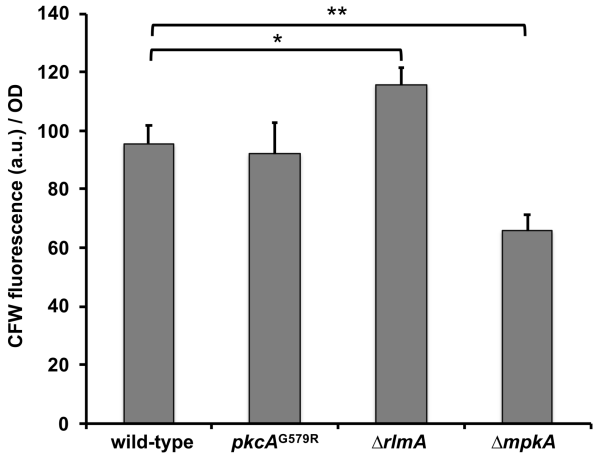


FIG S5 Chitin detection during growth on solid media. Strains were grown on solid media for 18-30 hours at 37ºC and CFW was used to quantify chitin by fluorescence detection. The data shown are the slope of the plot of CFW fluorescence against biomass (measured as absorbance at 600 nm). The results are the average ± SE (n = 4, *$p \leq 0.02$ or **$p \leq 0.002$ *t*-test).

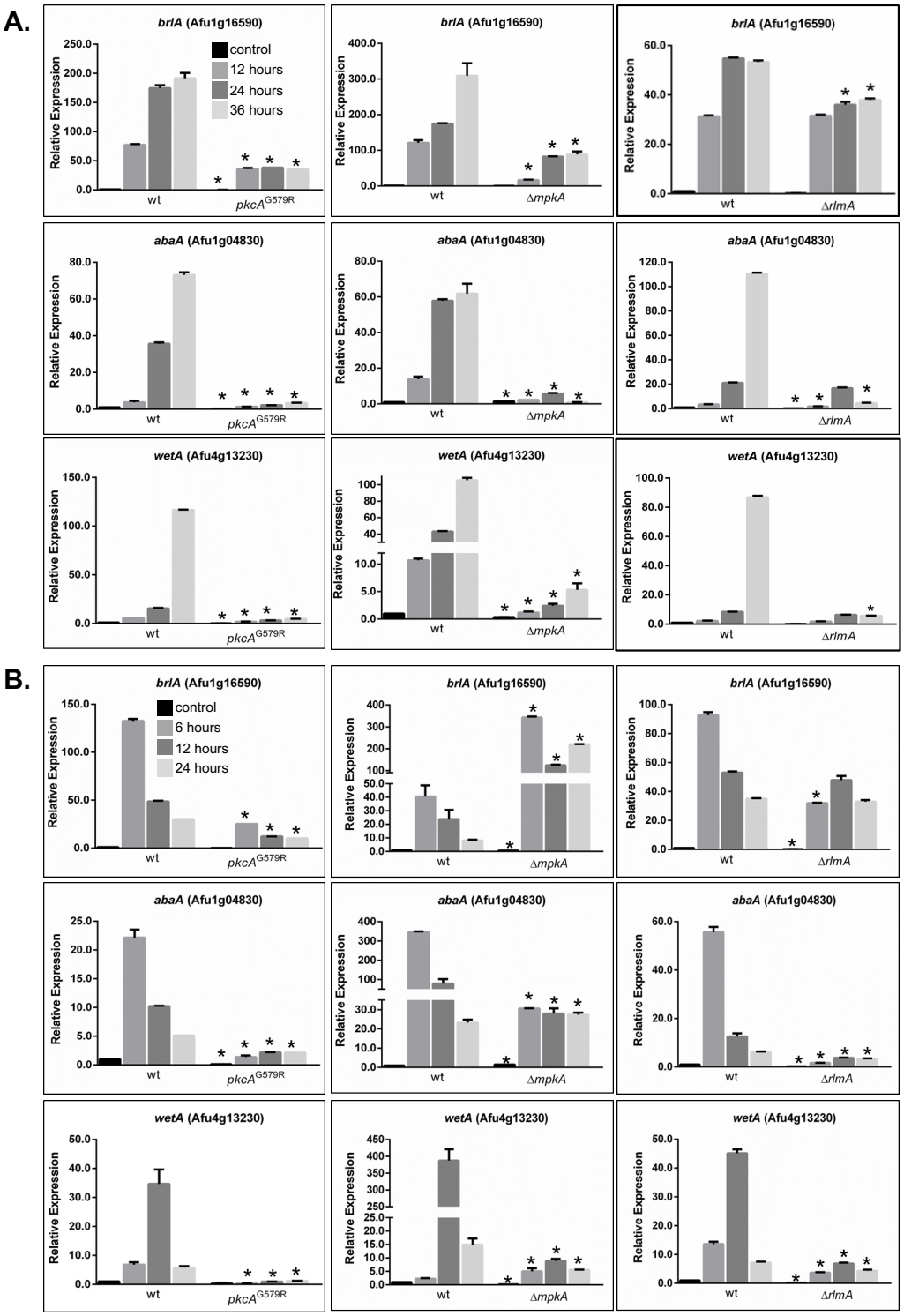


FIG S6. mRNA abundance of genes encoding the central regulators of asexual development is lower in the CWIP mutants. Total RNA was isolated from the strains subjected to synchronized asexual differentiation at 30°C (A) or 37°C (B). cDNA was obtained and used in RT-qPCR. The fold increase in each strain represents the normalized mRNA abundance relative to the wild-type strain. The data represent the average value of at least three independent experiments (with 2 technical repetitions each). *$p \leq 0.05$ (One-Way ANOVA).

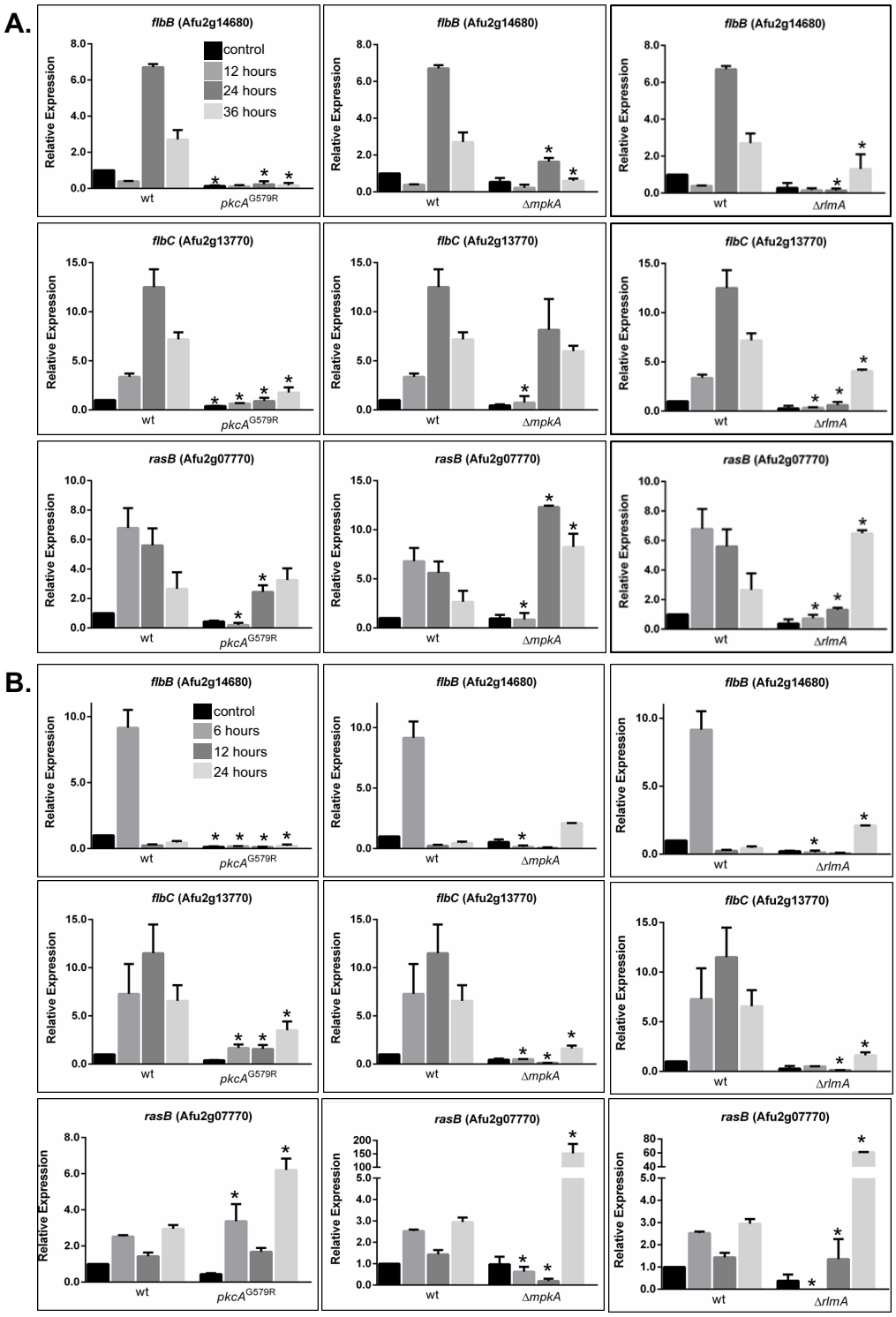


FIG S7 CWIP genes are required for the expression of *flbB* and *flbC.* Total RNA was isolated from the strains subjected to synchronized asexual differentiation at 30°C (A) or 37°C (B). cDNA was obtained and used in RT-qPCR. The fold increase in each strain represents the normalized mRNA abundance relative to the wild-type strain. The data represent the average value of at least three independent experiments (with 2 technical repetitions each). *$p \leq 0.05$ (One-Way ANOVA).

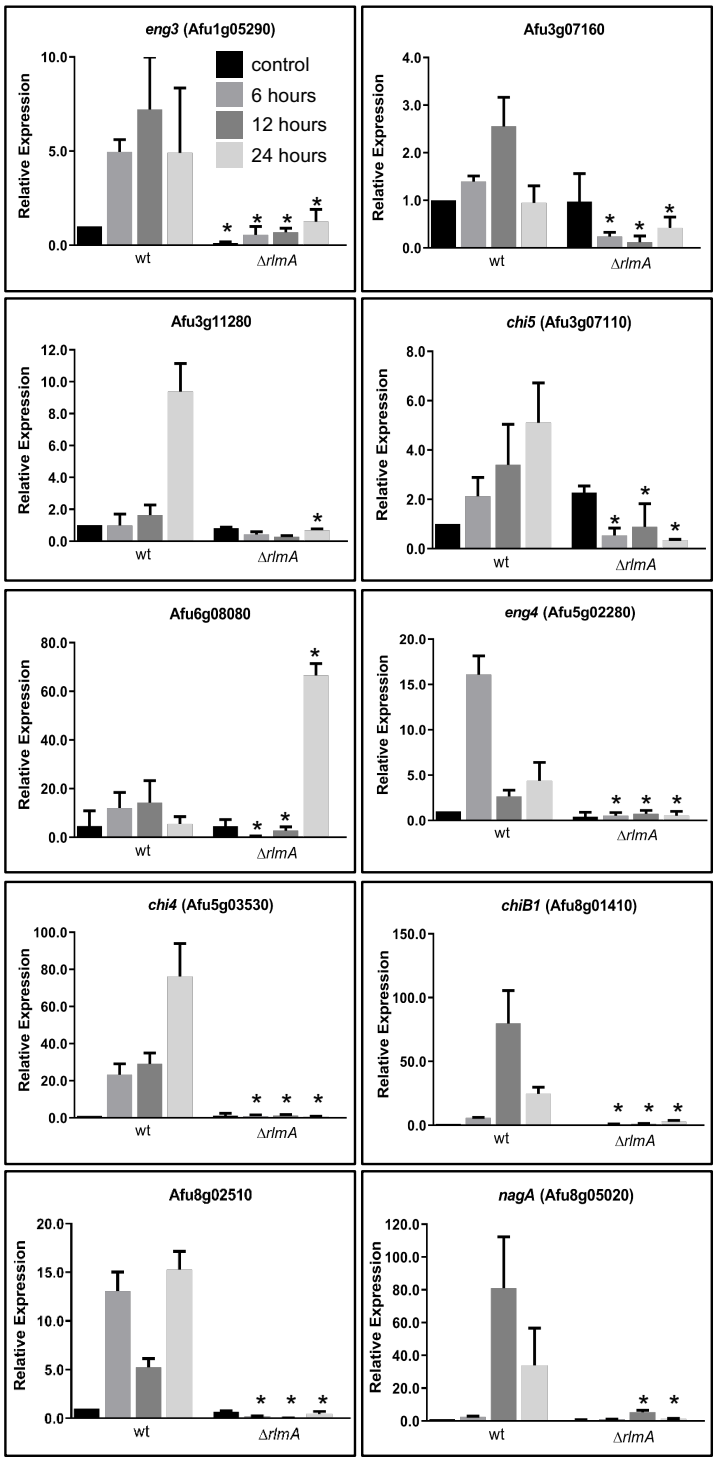
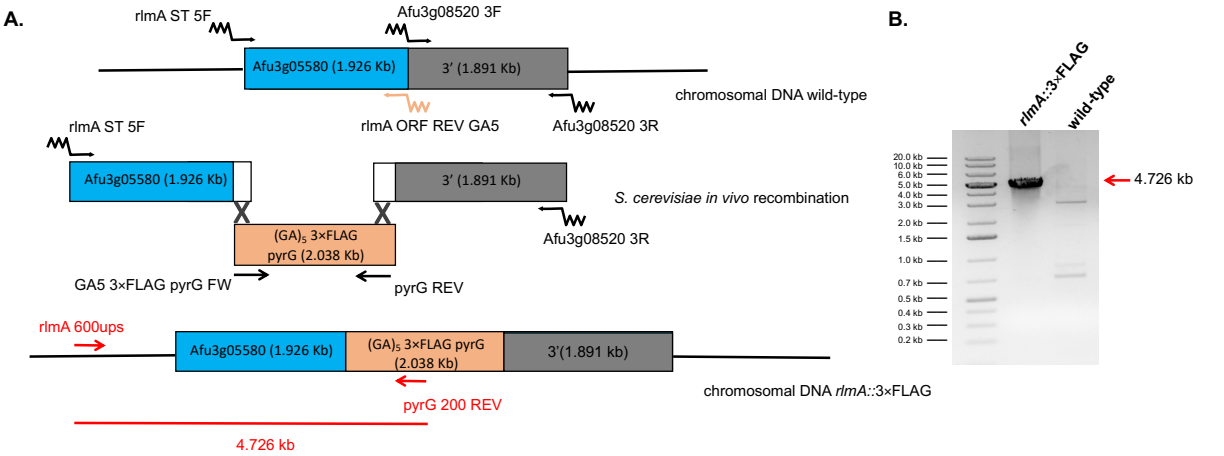


FIG S8 RlmA is required for the expression of genes involved in glucan and chitin metabolism. Total RNA was isolated from the wild-type and Δ*rlmA* strains subjected to synchronized asexual differentiation at 37°C. cDNA was obtained and used in RT-qPCR. The fold increase in each strain represents the normalized mRNA abundance relative to the wild-type strain. The data represent the average value of at least three independent experiments (with 2 technical repetitions each). *$p \leq 0.05$ (One-Way ANOVA).


A.
rlmA ST 5F
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Afu3g05580 (1.926 Kb)
3′ (1.891 Kb)
chromosomal DNA wild-type
rlmA ORF REV GA5
Afu3g08520 3R
rlmA ST 5F
Afu3g05580 (1.926 Kb)
3′ (1.891 Kb)
S. cerevisiae in vivo recombination
(GA)5 3×FLAG pyrG (2.038 Kb)
Afu3g08520 3R
GA5 3×FLAG pyrG FW
pyrG REV
rlmA 600ups
Afu3g05580 (1.926 Kb)
(GA)5 3×FLAG pyrG (2.038 Kb)
3′(1.891 kb)
chromosomal DNA rlmA::3×FLAG
pyrG 200 REV
4.726 kb
B.
rlmA::3×FLAG
wild-type
20.0 kb
10.0 kb
6.0 kb
5.0 kb
4.0 kb
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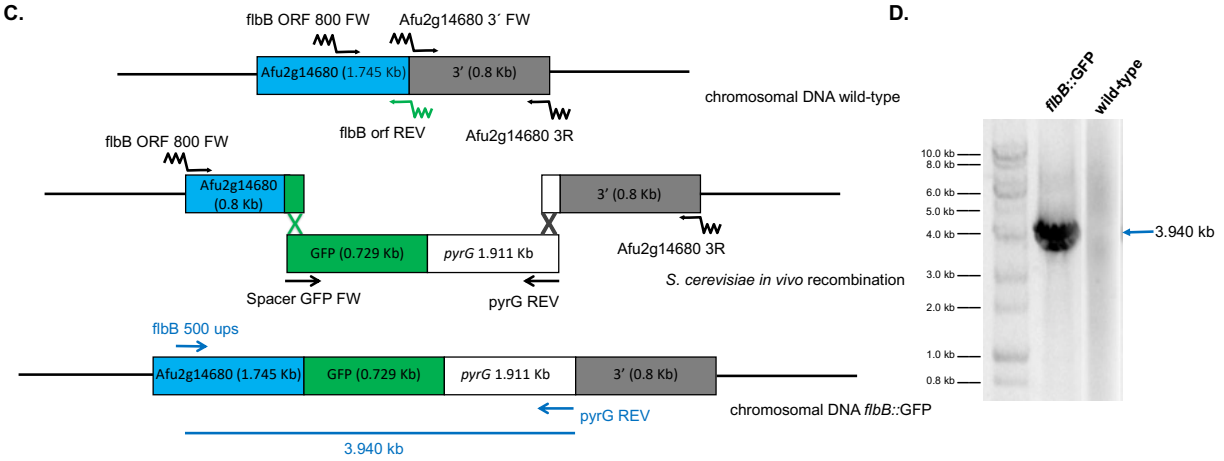
4.726 kb


C.
flbB ORF 800 FW
Afu2g14680 3′ FW
Afu2g14680 (1.745 Kb)
3′ (0.8 Kb)
chromosomal DNA wild-type
flbB orf REV
Afu2g14680 3R
flbB ORF 800 FW
Afu2g14680 (0.8 Kb)
3′ (0.8 Kb)
GFP (0.729 Kb)
pyrG 1.911 Kb
Afu2g14680 3R
S. cerevisiae in vivo recombination
Spacer GFP FW
pyrG REV
flbB 500 ups
Afu2g14680 (1.745 Kb)
GFP (0.729 Kb)
pyrG 1.911 Kb
3′ (0.8 Kb)
pyrG REV
chromosomal DNA flbB::GFP
3.940 kb
D.
flbB::GFP
wild-type
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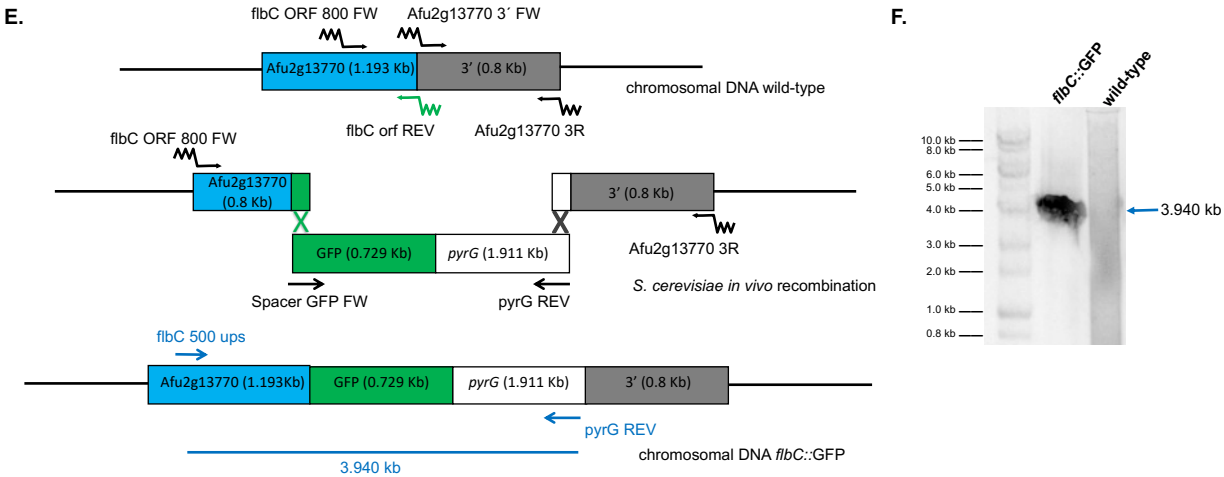
E.
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Afu2g13770 3′ FW
Afu2g13770 (1.193 Kb)
3′ (0.8 Kb)
chromosomal DNA wild-type
flbC orf REV
Afu2g13770 3R
flbC ORF 800 FW
Afu2g13770 (0.8 Kb)
3′ (0.8 Kb)
GFP (0.729 Kb)
pyrG (1.911 Kb)
Afu2g13770 3R
S. cerevisiae in vivo recombination
Spacer GFP FW
pyrG REV
flbC 500 ups
Afu2g13770 (1.193Kb)
GFP (0.729 Kb)
pyrG (1.911 Kb)
3′ (0.8 Kb)
pyrG REV
chromosomal DNA flbC::GFP
3.940 kb
F.
flbC::GFP
wild-type
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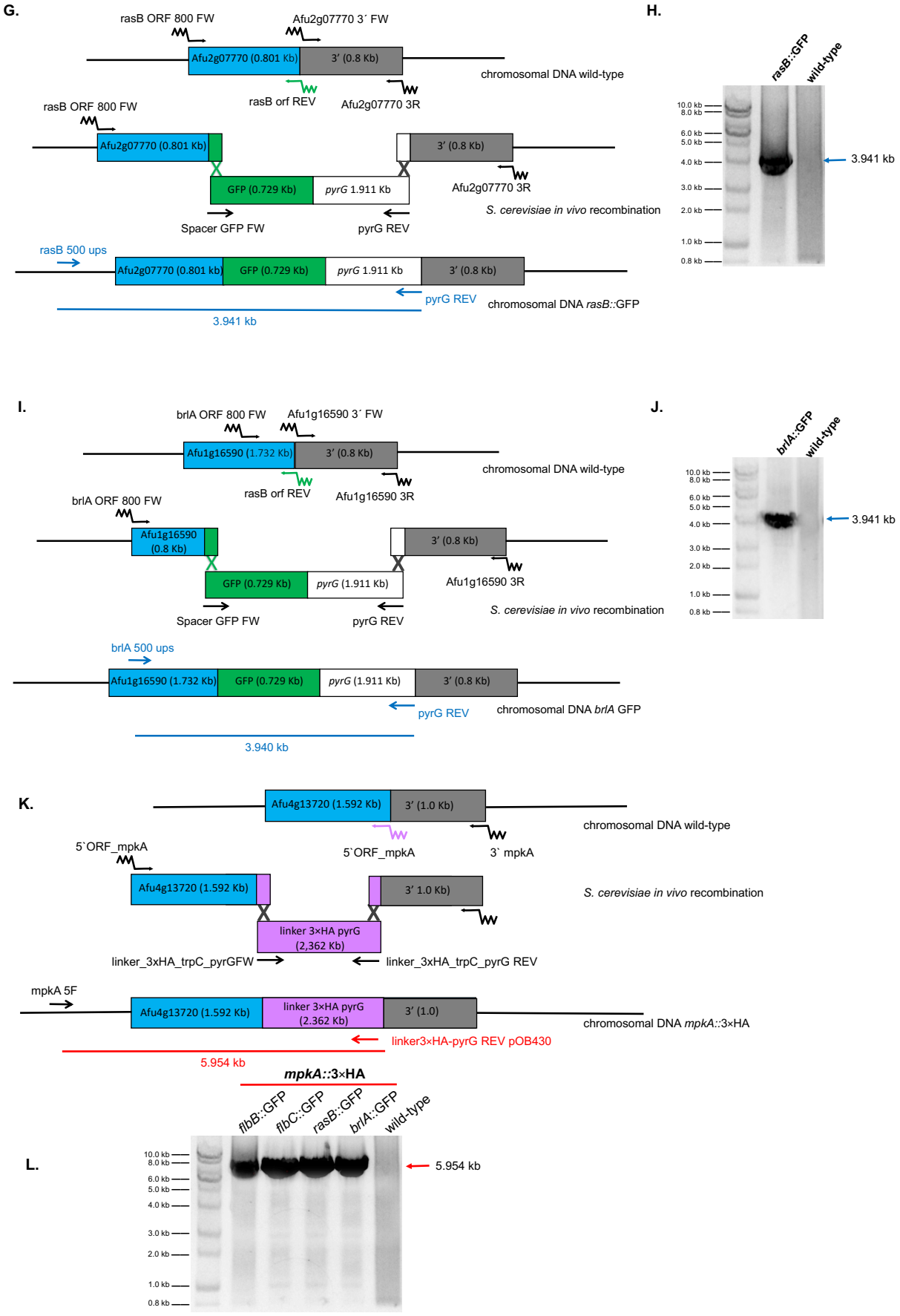

FIG S9 Graphical representation of the substituted loci for the *A. fumigatus* C-terminal GFP, 3×HA and 3×FLAG tagged strains. The genes of interest and their flanking regions are

shown in blue and grey boxes, respectively. (A) Gene replacement strategy for the *rlmA::*3×FLAG strain construction. The *rlmA* genomic sequence without stop codon was cloned in-frame with the 3×FLAG in a C-terminal fusion separated by a $(GA)_5$ linker. The *pyrG* gene was used as a prototrophy marker. The *rlmA*::3×FLAG cassette was transformed into the *A. fumigatus* wild-type strain. (B) Transformants were validated by PCR with primer set rlmA 600 ups and pyrG 200 REV to confirm the *rlmA* locus replacement by the amplification of the 4.726 kb band in the mutant strain. (C, E, G, I) Gene replacement strategy for *flbB*::GFP *mpkA*::3×HA, *flbC*::GFP *mpkA*::3×HA, *rasB*::GFP *mpkA*::3×HA and *brlA*::GFP *mpkA*::3×HA strains construction. Fragments spanning 0.8 kb of 3' region of *flbB*, *flbC*, *rasB* and *brlA* genomic sequences (without stop codon) were individually cloned in-frame with the green fluorescent protein (GFP) gene in a C-terminal fusion separated by a Gly-Thr-Arg-Gly linker. Each cassette was transformed into the *A. fumigatus* wild-type strain. (D, F, H, J). Transformants were validated by PCR with primer set *flbB*, *flbC*, *rasB, brlA*, (located 500 bp upstream from the start of substitution cassette) and pyrG REV to confirm each locus replacement (blue lines). (K) The *mpkA::*3×HA*::ptrA* cassette was amplified form the plasmid pUC19-mpkA::3×HA and used to transform each GFP fused strain described in C-J. (L) The pyrithiamine-resistant transformants were selected and validated by PCR with primers mpkA 5F and prtA 3R to confirm the *mpkA* locus replacement by the amplification of the 5.954 kb band in the double tagged strains.

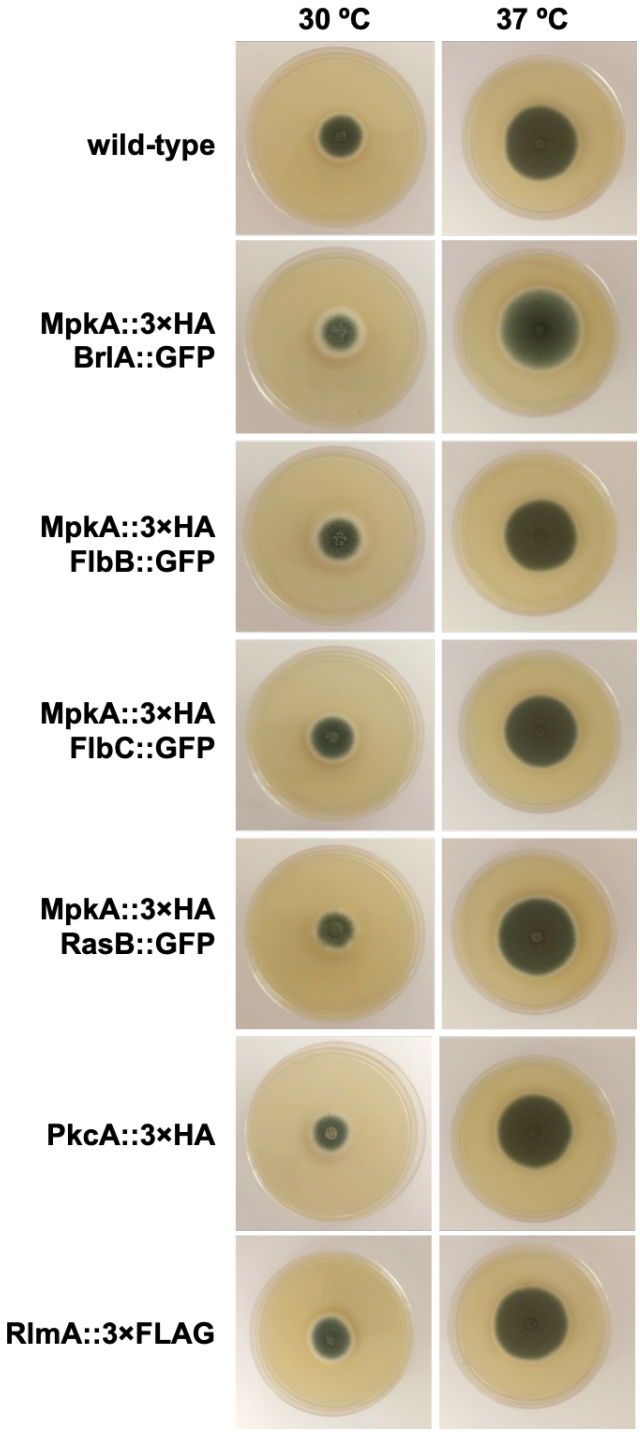

FIG S10 Growth phenotype of the double-tagged strains. 1×10^5 conidia were inoculated on YG medium and incubated at the indicated temperature for three days and photographed.

TABLE S1 Primers used in this study for strains construction

Primer*	Sequence (5'→3')	Systematic name
Afu3g08520 5F	gtaacgccagggttttcccagtcacgacgCTTGCTTGCTTGCTTGCTT'	Afu3g08520
Afu3g08520 5R	GCATCAGTGCCTCCTCTCAGACAGAATTCCGCAGTAAACAAGGAACACCACG'	
pyrG FW	GGAATTCTGTCTGAGAGGAGGC	Afu2g08360
pyrG REV	GATATCGAATTCGCCTCAAAC	
Afu3g08520 3F	AAGAGCATTGTTTGAGGCGAATTCGATATCACTTTCTTGAATATTGCGATGG	Afu3g08520
Afu3g08520 3R	gcggataacaatttcacacaggaaacagcTCAGCGTCCGGTAAGTTG	
rlmA ST 5F	gtaacgccagggttttcccagtcacgacgATGGGTCGAAGAAAGATCGA	Afu3g08520
rlmA ORF REV	AGTTCTTCTCCTTTACTCATTCCCCGTGTTCCCGTCTTGGATTTCTTCGCC	
rlmA 600 ups	GAATGAGAAGAAAGGAGGAATGA	
MpkA_5'_FW	CTCATTCCTTGTTCTGATGCG	Afu4g13720
MpkA_3'_REV	GACTGTCGCAGAAATCCGCTT	
mpkA 600 ups	GAGCCCTGACTTCACTGCA	
flbC 500 ups	TCAACACACTTGAACCGTGG	Afu2g13770
flbC 800 FW	gtaacgccagggttttcccagtCACGACGACTTATGCGCCGATCAGCTA	
flbC ORF REV	AGTTCTTCTCCTTTACTCATTCCCCGTGTTCCCTCCTCCTCGCCACCAGATA	
flbC 3F	AAGAGCATTGTTTGAGGCGAATTCGATATCAAAGCCCGTTCTCTACTCTG	
flbC 3R	gcggataacaatttcacacaGGAAACAGCCAAGATGACTCCAGACATTC	
flbB 500 ups	TGAAAACCCCCACTTCAATG	Afu2g14680
flbB 800 FW	gtaacgccagggttttcccagtCACGACGCAGGCGCCGGCAGCACTAGC	
flbB ORF REV	AGTTCTTCTCCTTTACTCATTCCCCGTGTTCCCGAGTACATCGTATCGTCGC	
flbB 3F	AAGAGCATTGTTTGAGGCGAATTCGATATCTGTGATATCGGATACTTCTT	
flbB 3R	gcggataacaatttcacacaGGAAACAGCGTTTTCTATTAAAGGCTTAA	
rasB 500 ups	ATCGACGAGCTTATACTGCG	Afu2g07760
rasB 800 FW	gtaacgccagggttttcccagtCACGACGATGACGTTGTACAAATTGGT	
rasB ORF REV	AGTTCTTCTCCTTTACTCATTCCCCGTGTTCCCAGGATGACGCACTTGATGC	
rasB 3F	AAGAGCATTGTTTGAGGCGAATTCGATATCGGAACACATTAGCATTCGCA	
rasB 3R	gcggataacaatttcacacaGGAAACAGCAGGACTCGCAAGAACCGGGT	
brlA 500 ups	TGAACTAGAAGATACAGAGG	Afu1g16590
brlA 800 FW	gtaacgccagggttttcccagtCACGACGCTCCGTATTTCCCTGAATCGG	

brlA ORF REV brlA 3F brlA 3R	AGTTCTTCTCCTTTACTCATTCCCCGTGTTCCCTCATCCCATTCCATACTGA AAGAGCATTGTTTGAGGCGAATTCGATATCAAAAAAAAAGACGAAAAAAG gcggataacaatttcacacaGGAAACAGCGCGATTCGTCCGTGTCATTT	
Spacer GFP FW	**GGAACACGGGGA**ATGAGTAAAGGAGAAGAACTTTTCA	-
GFP REV pyrG	GCATCAGTGCCTCCTCTCAGACAGAATTCCTTATTTGTATAGTTCATCCATGCCATG	-
GA5 3×FLAG pyrG FW	GGAGCTGGTGCAGGCGCTGGAGCCGGTGCCGATTACAAGGATGACGACGATAAGGATTACAAGGATGACGACGATAAGGATTACAAGGATGACGACGATAAG**TAA**GGAATTCTGTCTGAGAGGAGGC	-
linker_3×HA_trpC_pyrG FW linker_3×HA_trpC_pyrG REV	GGAGGTGGTAGCGGTGGT CTGTCTGAGAGGAGGCACTGA	-

* For primers location on each construction refer to Fig. S9.
Small letters indicate homology to the plasmid pRS426 flanking sequence for *in vivo* recombination in *S. cerevisiae*.
Underlined letters indicate homology to a fragment in the cassette.
Bold letters indicate the Gly-Thr-Arg-Gly linker separating *flbB*, *flbC*, *brlA* and *rasB* C-terminus and GFP start codon.
Red letters indicate the $(GA)_5$ linker and blue letters indicate the 3×FLAG peptide sequence followed by a stop codon (TAA) in bold.

TABLE S2 RT-qPCR primers used in this study

Primer name	Sequence (5'→3')	Systematic name
pkcA FW *pkcA* REV	CCGAAGTTCTGTTGGCTCTC CAGAGACCGTAATCGGCAAT	Afu5g11970
tubA FW *tubA* REV	TTCCCAACAACATCCAGACC CGACGGAACATAGCAGTGAA	Afu1g10910
rlmA FW *rlmA* REV	GACGCCGATCTCTGCTCTAC GGAGTGGGGAAGGTTAGAGG	Afu3g08520
mpkA FW *mpkA* REV	GGCCATCAAGAAGGTTACCA TGAAATTGTCTGGTCGTGGA'	Afu4g13720
abaA FW *abaA* REV	GCACGACCTGTTGCATCAAA GCGGTGGCGGGTACAA	Afu1g04830
brlA FW *brlA* REV	TGCAAAGAACCTGGCTGCAA AACCCAGCAGACATGAGGCTT	Afu1g16590
wetA FW *wetA* REV	CGAGATTCCCATGAGCGTAAA GGAAGGGCGCATCAAGCT	Afu4g13230
flbB FW *flbB* REV	TCCCCCACGCACGAAAT CGAAGCCGTCATGGCAAT	Afu2g14680
flbC FW *flbC* REV	TGGACTTCTGCACACACGTCTT CCCATGGAAGTTGCGTACACT	Afu2g13770
rasB FW *rasB* REV	TGTCGTTCGCATGCTCCGACA CCAAGCCAGTCGGTCGACG	Afu2g07770
*chiB*1 FW *chiB*1 REV	GAATCGCTGCCTTATCTCTT AGCGTCGCTCCCTGGTCAAG	Afu8g01410
Afu3g07160 FW Afu3g07160 REV	CTTCAAAATATAACTAGCTC AAGACCGACGGCTCTGAGGT	Afu3g07160
Afu3g11280 FW Afu3g11280 REV	CGTCTATCACTGTCCTTGCT AGATAGGATGAAAGAGACT	Afu3g11280
nagA FW *nagA* REV	ATCCGCTGCCTCATCTTCCA GACCCCTTTTCCAACGCTTG	Afu8g05020
chi5 FW *chi5* REV	AAGGGGCCGCTTGTATATTA AGGTGCCAACCTTGGCAAAA	Afu3g07160
chi4 FW *chi4* REV	CGATTCTGGGCGGCTGCACT GGAAAGAATCAATGACAACT	Afu5g03530
chi5 FW *chi5* REV	GGACTCCGTAGGTATCTGGC GAATACAATACCGAACCAGT	Afu3g07110
eng3 FW *eng3* REV	GACCCCTTTTCCAACGCTTG ACAGCGGCTTGGATCAATCA	Afu1g05290
eng4 FW *eng4* REV	TATATGCTGGCTGCTGTGCC ATGCCTCATCAGCGCTTGGG	Afu5g02280
Afu6g08080 FW Afu6g08080 REV	TAGAACCCAGCGAATTTTTC AGAAGTCCAATGAACGCACC	Afu6g08080

TABLE S3 ChIP-qPCR primers used in this study

Primers	Sequence (5'→3')	Systematic name
flbB FW	CTCTTATTCTTTGTGGCCCTCTCT	Afu2g14680
flbB REV	GAGCTCAAGGAACGAGATTATTTTTAA	
flbC FW	CAAGATGAAAGAAGAAAAAGAAGCAGTT	Afu2g13770
flbC REV	CCTTTCGATTACTGTGTGCAAGTT	
abaA FW	GCTGGAACTCGTTATACATGAATGTG	Afu1g04830
abaA REV	GACACCAAGTCGCTCGGCCACCGAAG	
brlA FW	AAATGCTCCGAAGACAAGAAAC	Afu1g16590
brlA REV	CTCTTTTCTTTTTTGATTTAATTCCTT	
rasB FW	CGAGCTTATACTGCGACCGG	Afu2g07770
rasB REV	AGAGACATGACATACCTTTG	
*chiB*1 FW	GAATCGCTGCCTTATCTCTT	Afu8g01410
*chiB*1 REV	AGCGTCGCTCCCTGGTCAAG	
Afu3g07160 FW	CTTCAAAATATAACTAGCTC	Afu3g07160
Afu3g07160 REV	AAGACCGACGGCTCTGAGGT	
Afu3g11280 FW	CGTCTATCACTGTCCTTGCT	Afu3g11280
Afu3g11280 REV	AGATAGGATGAAAGAGACT	
nagA FW	CGATTCTGGGCGGCTGCACT	Afu8g05020
nagA REV	GGAAAGAATCAATGACAACT	
chi5 FW	AAGGGGCCGCTTGTATATTA	Afu3g07160
chi5 REV	AGGTGCCAACCTTGGCAAAA	
chi4 FW	ATTCCGTTACATAAAATATG	Afu5g03530
chi4 REV	AGTGCTCTACATATTGTCAG	
chi5 FW	CCGCGAGTTCGTCAAGCTAC	Afu3g07110
chi5 REV	ATCCGCTGCCTCATCTTCCA	
eng3 FW	GACCCCTTTTCCAACGCTTG	Afu1g05290
eng3 REV	ACAGCGGCTTGGATCAATCA	
eng4 FW	GTATGAAATTCATCCAGTGT	Afu5g02280
eng4 REV	AGCAATGGATGTACTGTGTG	
Afu6g08080 FW	TAGAACCCAGCGAATTTTTC	Afu6g08080
Afu6g08080 REV	AGAAGTCCAATGAACGCACC	
prxB FW	CTCTACTGCCCCTGACTTCG	Afu5g15070
prxB REV	GGGTGGGAGAAGAGGATAGC	