

ChIP-seq reveals a role for CrzA in the *Aspergillus fumigatus* high-osmolarity glycerol response (HOG) signalling pathway

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Summary

Aspergillus fumigatus is an opportunistic pathogen and allergen of mammals. Calcium signalling is essential for *A. fumigatus* pathogenicity and is regulated by the CrzA transcription factor. We used ChIP-seq (Chromatin Immunoprecipitation DNA sequencing) to explore CrzA gene targets in *A. fumigatus*. In total, 165 potential binding peaks including 102 directly regulated genes were identified, resulting in the prediction of the A[GT][CG]CA[AC][AG] CrzA-binding motif. The 102 CrzA putatively regulated genes exhibited a diverse array of functions. The *phkB* (Afu3g12530) histidine kinase and the *sskB* (Afu1g10940) MAP kinase kinase kinase of the HOG (high-osmolarity glycerol response) pathway were regulated by CrzA. Several members of the two-component system (TCS) and the HOG pathway were more sensitive to calcium. CrzA::GFP was translocated to the nucleus upon osmotic stress. CrzA is important for the phosphorylation of the SakA MAPK in response to osmotic shock. The Δ *sskB* was more sensitive to CaCl₂, NaCl, and

paraquat stress, while being avirulent in a murine model of invasive pulmonary aspergillosis. The presence of CaCl₂ and osmotic stresses resulted in synergistic inhibition of Δ *crzA* and Δ *sskB* growth. These results suggest there is a genetic interaction between the *A. fumigatus* calcineurin-CrzA and HOG pathway that is essential for full virulence.

Introduction

The calcineurin phosphatase is central to calcium signalling and plays an important role in determining fungal cell morphology and virulence (Fox and Heitman, 2002; Cyert, 2003; Steinbach *et al.*, 2007; Stie and Fox, 2008). Calcineurin mediated Ca²⁺ signalling has been reported to be required for growth in environmental stresses such as osmotic, extreme pH, high temperature, ER stress and prolonged incubation with mating pheromone alpha-factor in *Saccharomyces cerevisiae* (Carafoli, 2002; Miyakawa and Mizunuma, 2007). The reduction in calcineurin activity caused by the immunosuppressant drug, cyclosporine A, leads to the alteration in hyphal morphology and a growth reduction in multiple filamentous fungi including *Aspergillus nidulans* (Rasmussen *et al.*, 1994), *Aspergillus oryzae* (Juvvadi *et al.*, 2003), *Magnaporthe oryzae* (Viaud *et al.*, 2002) and *Neurospora crassa* (Prokisch *et al.*, 1997; Kothe and Free, 1998). The calcineurin heterodimer consists of catalytic (CNA) and regulatory (CNB) subunits (Steinbach *et al.*, 2007). In *Aspergillus fumigatus*, the *cnaA* deletion dramatically affected conidiation, morphology, invasive growth and pathogenicity (Steinbach *et al.*, 2006; da Silva Ferreira *et al.*, 2007). A *MCNA* knocked down mutant, obtained by RNA silencing, had similar effects on *M. oryzae*, including a reduction in hyphal growth, conidiation, and appressorium formation (Choi *et al.*, 2009). Calcineurin functions through the activation of the Crz1 (Calcineurin Responsive zinc Finger 1) transcription factor. Calcineurin dephosphorylates Crz1 upon an increase in cytosolic calcium, allowing its nuclear translocation (Stathopoulos-Gerontides *et al.*, 1999; Thewes, 2014). Crz1 contains a C₂H₂ zinc finger motifs that binds to a CDRE (calcineurin-dependent response element) sequence in the promoter regions of the Crz1-regulated genes (Stathopoulos and Cyert, 1997; Thewes, 2014). Calcineurin and Crz1 deletion strains exhibit both similar and contrasting phenotypes in

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multiple fungi, suggesting the conservation of mode of action and function (Stathopoulos and Cyert, 1997; Karababa *et al.*, 2006; Schumacher *et al.*, 2008; Soriani *et al.*, 2008; Choi *et al.*, 2009).

Multiple transcriptomic projects have investigated the genes regulated by calcineurin/Crz1. A microarray analysis of *S. cerevisiae* identified 163 genes regulated in the calcineurin/Crz1 pathway in response to Na⁺ or Ca²⁺ stimulation (Yoshimoto *et al.*, 2002). Alternatively, Viladevall *et al.* (2004) identified 264 genes from *S. cerevisiae* whose transcription was dependent on CRZ1. In both *S. cerevisiae* data sets, functions of the Crz1-dependent genes were associated with a wide range of biological processes, such as ion/small molecule transport, cell wall synthesis, signalling pathways, and vesicular trafficking (Yoshimoto *et al.*, 2002; Viladevall *et al.*, 2004). Similarly, in *Candida albicans*, analyses of the *cna1* and *crz1* deletion strains identified 60 genes that were transcriptionally regulated by calcineurin/Crz1 in response to Ca²⁺ treatment, which were also involved in a broad range of functions including cellular organization, cell wall organization, cell metabolism, cellular transport, homeostasis and protein fate (Karababa *et al.*, 2006). In the *A. fumigatus* *crzA* deletion strain, 3,622 genes had different expression compared to the wild-type strain. Functional annotation revealed a variety of cellular processes but no enrichment for a specific GO term (Soriani *et al.*, 2008). This diversity of gene functions might be expected due to the involvement of calcium signalling in numerous biological processes influencing the transcription of up to one-third of all genes encoded by the *A. fumigatus* genome.

The limitation of transcriptomic analyses, such as microarray and RNA-seq, is that they are unable to distinguish between genes that are directly or indirectly regulated by Crz1/CrzA. However, chromatin immunoprecipitation (ChIP) used in combination with microarray (ChIP-chip) or next-generation sequencing (ChIP-seq) enables the identification of genes directly regulated by a specific transcription factor. Kim *et al.* (2010) utilized ChIP-chip and microarray approaches to evaluate MoCRZ1 in *M. oryzae*, identifying 346 genes with MoCrz1 binding regions in their promoters. The genes directly regulated by CrzA showed functions involved in cell signalling and transcription, as well as those involved in host association, virulence and secretion. MoCrz1 was also found to regulate its own transcription. This study successfully demonstrated the power of ChIP-chip in the identification of genes regulated by fungal transcription factors such as MoCRZ1.

Aspergillus fumigatus is a major opportunistic pathogen and allergen of mammals (for reviews, see Greenberger, 2002; Dagenais and Keller, 2009). The calcium/calcineurin signalling pathway is essential for *A. fumigatus* pathogenicity and is regulated by the CrzA transcription factor (Steinbach *et al.*, 2007). The present study utilized ChIP-

seq to investigate the targets of CrzA in *A. fumigatus*. Previous fungal ChIP-seq projects served as roadmaps for this study of CrzA (Park, 2009; Kim *et al.*, 2010; Haynes *et al.*, 2011; Nasmith *et al.*, 2011). In total, 165 potential binding peaks including 102 directly regulated genes and a CrzA-binding motif were identified. One of the *A. fumigatus* genes identified, *sskB*, encodes the MAP (mitogen-activated protein) kinase kinase kinase of the HOG pathway. Investigation of the *sskB* null mutant revealed it to be more sensitive to calcium, osmotic and oxidative stresses, while being avirulent in a murine model of invasive pulmonary aspergillosis. CrzA::GFP was translocated to the nucleus upon osmotic stress and this translocation was dependent on calcineurin. Moreover, CrzA was important for the phosphorylation of the SakA MAPK in response to osmotic shock. These findings suggest the existence of a genetic interaction between the *A. fumigatus* calcineurin-CrzA and the HOG pathways.

Results

ChIP sequencing and reads alignment

ChIP-seq was performed to identify the binding sites of the core calcium responsive transcription factor, CrzA. The *A. fumigatus* strain harbouring a functional CrzA::GFP fusion protein (Soriani *et al.*, 2008) was grown in liquid complete media for 16 h and then treated with calcium to promote CrzA entry into nucleus and DNA binding. The protein-DNA complexes were cross-linked and then sonicated into short fragments, after which the CrzA-bound DNA fragments were immunoprecipitated using the GFP antibody. Enriched ChIP DNA fragments were selected by size (about 300 bp) and were sequenced on an Illumina sequencer (Lim *et al.*, 2012). Several negative controls were designed to confirm the accuracy of the technique. These included, mycelia treated with calcium but not immunoprecipitated (TM3), mycelia treated with calcium and precipitated with the human antibody IgG (TM4), mycelia treated with calcium and immunoprecipitated with the anti-GFP antibody (TM5), mycelia treated with both calcium and FK506 (a calcineurin inhibitor that prevents dephosphorylation of CrzA and translocation into nucleus), without immunoprecipitation (TM6), mycelia treated with both calcium and FK506, which were then immunoprecipitated with IgG (TM7) or the anti-GFP antibody (TM8) respectively (Supplementary Table S1). In total, 62 million paired-end and adapter-trimmed short reads with a length of 75 bp were sequenced from all of the six libraries (Supplementary Table S2). High-quality short reads were separately aligned to the *A. fumigatus* Af293 genome using the SOA-Paliner (v2.21) alignment tool developed by BGI (Li *et al.*, 2010) with a maximum of 2 mismatches allowed to accommodate polymorphisms between short reads and the

genome sequence. Approximately, 35–57% of the short reads, in each condition, mapped uniquely to the genome in the form of paired or single reads.

Peak calling, distribution and regulated genes identification

'Peaks' are defined as genomic regions where a significant number of short reads were mapped. These peaks represented candidate CrzA binding sites. Peaks were identified using the Cisgenome software (Ji *et al.*, 2011) utilizing the mapping files for both paired and single-end reads from experimental and control libraries. The six sequenced libraries were divided into two groups and processed by Cisgenome respectively. Group 1 included TM3, TM4, and TM5 without FK506. Group 2 included TM6, TM7, and TM8 with FK506. In group 1, TM3 served as experimental library, while TM4 and TM5 served as negative controls to remove background noise. The genome was scanned in a sliding window fashion. Genomic regions significantly enriched with reads compared to the control libraries were identified using the binomial model (Ji *et al.*, 2011). The ratio between the number of peaks in the control library and the experiment library was used as a cut-off for peak calling. As a result, 165 peaks ranging from 100 to 500 bp, and with an average length of 187.9 bp, were reported with the minimum False Discovery Rate (FDR) = 0 (22 peaks) and maximum FDR = 0.168.

Peak location was compared with *A. fumigatus* Af293 genome and the neighbouring genes were identified as candidates for CrzA regulation. To include all possibly regulated genes, 5 kb upstream of a transcription starting site was defined as the promoter region. Peak distribution in group 1 identified 83 (50%) peaks within gene promoter regions, 46 (28%) peaks were located in coding regions, 18 (11%) peaks were in introns, and the remaining 18 (11%) peaks located to intergenic regions. As showed in Supplementary Table S3, 83 peaks were identified to locate at upstream 3 kb regions of 105 genes, which were identified as CrzA regulated candidate genes. Forty-three peaks located with –1 and –1000 bp upstream of the starting codon of 50 genes, among which seven peaks were in the intergenic region between two genes on opposite strands. Thirty peaks located between –1001 and –2000 bp upstream of 30 genes (Supplementary Table S3). Interestingly, *crzA* (Afu1g06900) was identified as a gene directly regulated by CrzA. Peaks within the coding regions of 32 genes were excluded from further analyses. In the negative control (group 2), 17 peaks were identified within coding regions, while 15 peaks located within the 3 kb upstream regions of 12 genes. The comparison between the peaks found in groups 1 and 2, revealed 3 genes (Afu4g14880, Afu2g18100, and Afu3g15395) were constantly identified in all groups. Subsequently, these genes

were removed from the 105 genes identified in group 1. Figure 1 shows selected examples of peaks that were identified as binding sites in the upstream regions of CrzA target genes.

Binding motif analysis

To predict the CrzA-binding motifs from the 83 peaks located in promoter regions, the nucleotide sequences from the peak regions were used for *de novo* motif identification using the MEME predictor (Bailey *et al.*, 2006). The motif A[GT][CG]CA[AC][AG] (Reverse Complementary: [TC][TG]TG[GC][CA]T) (Fig. 2A) was identified as the top candidate with an E-value of 2.7×10^{-21} . This motif exists in 60 out of the 83 peaks. The distance between motif centre and peak summit was calculated to determine the specific position of motif within the peak. Over half of the motifs located close to the centre of peak summit (Fig. 2B). The identified CrzA-binding motif was compared to the *S. cerevisiae* motif database using motif comparison tool Tomtom (Gupta *et al.*, 2007). The binding motif of the *S. cerevisiae* transcription factor Crz1 has 6 bases overlapping (*P*-value 0.005) with the identified CrzA-binding motif (Fig. 2A). Two additional known motifs for Phd1 (a transcription factor with putative functions in pseudohyphal growth) (Gimeno and Fink, 1994) and Sok2 (which plays a regulation role in the cAMP-dependent protein kinase stimulated signalling) (Ward *et al.*, 1995) were also shown to have a highly similar binding motif.

Functional annotation of CrzA regulated genes

To investigate the expression profile of CrzA target genes, data from a transcriptomic study that compared the wild-type and $\Delta crzA$ strains after a short pulse of calcium was utilized (Soriani *et al.*, 2010). This comparison identified 3622 genes in the $\Delta crzA$ strain as being differentially expressed when compared to the wild-type strain. The 3622 genes were divided into two groups, those which were expressed at either a higher or a lower level in the $\Delta crzA$ strain. Approximately half of 102 CrzA-binding genes identified via ChIP-seq demonstrated differential expression depending on the presence of CrzA (Supplementary Fig. S1). These results suggest that half of the observed direct targets of CrzA had their expression directly modulated at transcriptional level by CrzA.

The functions of the 102 CrzA genes identified via ChIP-seq to be regulated by CrzA were determined. A broad range of biological processes was identified, yet no specific gene ontology (GO) was overrepresented, possibly due to the small sample size. Nonetheless, the most highly represented GO terms included; transport (14.3%), regulation of biological processes (11.4%), response to stress (10.5%), response to chemical (9.5%), and developmental

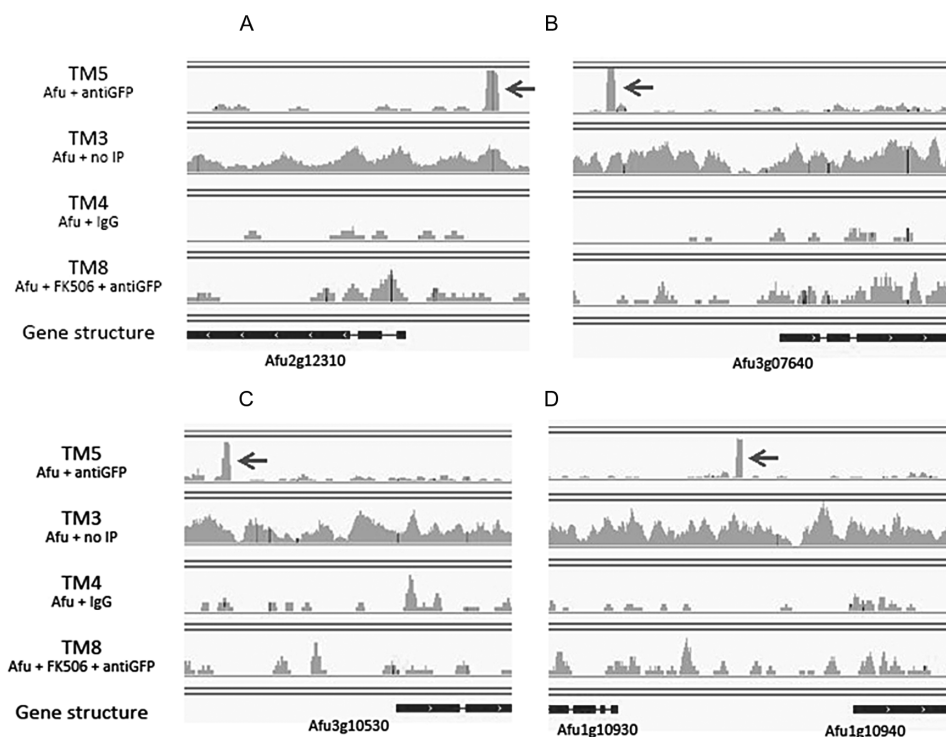


Fig. 1. Selected example of peaks identified in the promoter regions of annotated ORFs. Partial gene structures of (A) *Afu2g12310*, (B) *Afu3g07640*, (C) *Afu3g10530*, and (D) *Afu1g10930* and *Afu1g10940* are shown in black boxes with arrows indicating the 5' to 3' direction. Identified peaks were pointed by a grey arrow. TM5 represents the ChIP library, while TM3 and TM4 served as technical negative control and TM8 serves as biological negative control.

processes (7.6%) (Supplementary Tables S4 and S5). Within these functional categories, a high representation of genes involved in zinc and iron binding were identified, such as the *A. fumigatus* homologues of the *S. cerevisiae* FAD transmembrane transporter *FLC2* (*Afu4g13340*, *flcA*), and the siderophore *SIT1*. In addition, multiple genes involved in the ER-Golgi secretion system were found including several SNARE or SNARE-interacting proteins. Interestingly, the transcription of *crzA* was self-regulated by CrzA binding, while the azole and calcium induced transcription factor, *zfpA*, also demonstrated CrzA binding.

The relation of the CrzA target genes with fungal virulence was assessed by a comparison with the Pathogen–Host Interaction database (PHI-base) version 3.1 (Baldwin *et al.*, 2006; Winnenburg *et al.*, 2006; 2008). Fifteen genes were identified to have more than one PHI-base hit and an E-value cut-off of $10e-10$. Genetic disruption of two homologues of the *A. fumigatus* CrzA-regulated genes, *Afu5g03950* and *Afu3g10530*, proved lethal in the fungal pathogen *Gibberella zeae*. Homologues of the remaining 13 *A. fumigatus* CrzA-regulated genes demonstrated a reduction, or loss, of virulence in the respective host species. This analysis highlighted the importance of CrzA signalling during virulence to calcium homeostasis (*crzA* and *Afu3g07640*), the *sskB* (MAPKK, *Afu1g10940*) of

the osmotic stress pathway, autophagy (*Afu6g12350*, *Afu2g02610* and *Afu1g06400*, *schA*) and iron uptake (*sit1*), plus others (Supplementary Table S6). Subsequently, due to two members of the osmotic stress pathway demonstrating CrzA regulation, the interaction between the calcium and osmotic stress signalling pathways, and its relevance to virulence in *A. fumigatus*, was investigated further.

Validation of the ChIP-seq results

Fourteen genes identified by ChIP-seq as being regulated by CrzA were randomly selected for validation by comparing their expression in the wild-type and $\Delta crzA$ strains in response to a short pulse (10 or 30 min) of calcium (200 mM CaCl_2) via RT-qPCR (Fig. 3). Although the magnitude of gene induction post calcium treatment was different between genes, in the wild-type strain all 14 genes showed increased mRNA accumulation following calcium exposure (Fig. 3A–N). This accumulation was completely abolished in the $\Delta crzA$ mutant strain, except for *Afu1g03210* (*afflbD*; Fig. 3A). To better understand the impact of the CrzA regulatory network, seven of the identified target genes were deleted and the phenotypes of the deletion strains compared to the *crzA*-deletion phenotypes. Four of these genes were transcriptionally down-

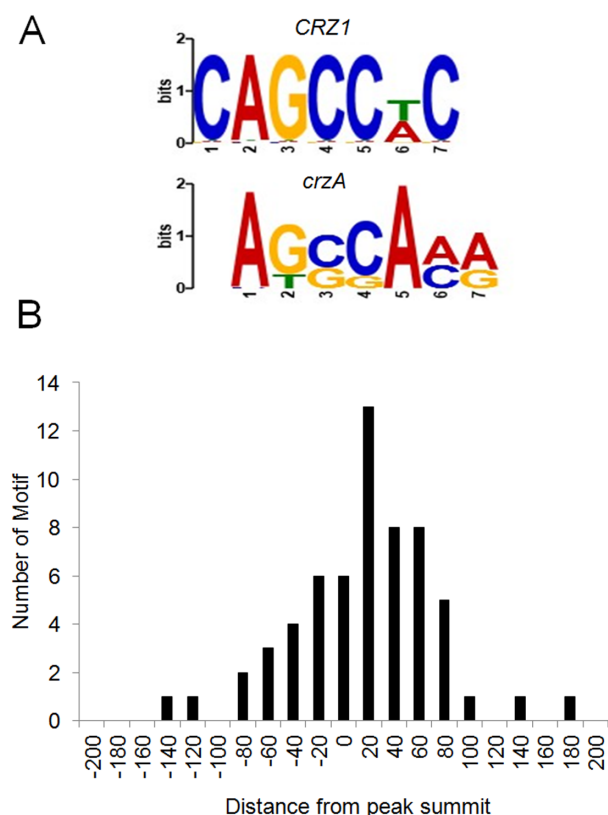


Fig. 2. The predicted CrzA-binding motif.

A. High similarity showed between the *A. fumigatus* CrzA (bottom) and yeast Crz1p (top) binding motifs.
B. Distance between the centre of predicted motifs and the summit of the binding peaks. Majority of motifs located within the summit region of peaks.

regulated in $\Delta crzA$ upon $CaCl_2$ stimulus (see Fig. 3, Supplementary Table S4 and Figs S2 and S3; Afu1g10940, *sskB*; Afu1g06400, *schA*; Afu4g13340, *flcA*; Afu3g06880, *snxA*, which represents *S. cerevisiae* *SNX3* homologue, a sorting nexin for late-Golgi enzymes; Afu1g09950, *cbkA*, which represents *S. cerevisiae* *CKB2* homologue, a regulatory subunit of casein kinase 2; Afu6g12350, *atgl*, a *S. cerevisiae* orthologue of *ATG9* that has a role in CVT pathway and autophagic vacuole assembly, and Afu7g06050, *ddiA*, a *S. cerevisiae* orthologue of *DDI1* that plays a role vesicle-mediated transport and localizes to plasma membrane). The seven mutants plus the corresponding wild-type and $\Delta crzA$ strains were grown in minimal (MM) medium supplemented with agents that affect $\Delta crzA$ growth, such as $CaCl_2$ and caspofungin (Soriani *et al.*, 2008; Fig. 4, Table 1 and Table S1). The null mutants, $\Delta sskB$ (Afu1g10940) and $\Delta schA$ (Afu1g06400), showed sensitivity to $CaCl_2$ comparable to the $\Delta crzA$ mutant (Fig. 4). All the other null mutants, except $\Delta snxA$ (Afu3g06880), were more sensitive to $CaCl_2$ than the wild-type strain (Fig. 4). The wild-type and the $\Delta crzA$ mutant

showed a minimal inhibitory concentration (MIC) 0.50 and <0.094 to caspofungin respectively (Table 1). The $\Delta sskB$ and $\Delta schA$ showed MICs comparable to the $\Delta crzA$ mutant (Table 1); the $\Delta cbkA$, $\Delta flcA$, and $\Delta snxA$ mutants showed MICs lower than the wild-type strain, of 0.19, 0.25, and 0.19 respectively (Table 1). The $\Delta atgl$ and $\Delta ddiA$ showed MICs equal or higher than the wild-type strain (0.50, 1.0, and 0.50 respectively; Table 1). Taken together, these results strongly suggest that these null mutants have phenotypes related to calcium-calcineurin-CrzA.

Electrophoretic mobility shift assays (EMSA) were used to determine if CrzA directly controlled the transcription of two of the ChIP-seq identified genes that were previously validated by RT-qPCR. The promoter regions (~ 1000 bp upstream of the start codon) of Afu3g06880 and Afu1g09950, were scanned for putative CDRE motifs. At 651 and 916 bp upstream of their respective start codons the CDRE-consensus sequences, 5'-AGGTTG-3' (Afu3g06880) and 5'-CACAGCCC-3' (Afu1g09950) were identified (Supplementary Text S1). The recombinant GST::CrzA was produced in *Escherichia coli* and purified. DNA fragments of approximately 200 bp encompassing the CDRE-consensus sequence from both promoters were used as probes (Supplementary Text S1). DNA-protein complexes with reduced mobility were observed for both DNA fragments (Fig. 5). However, the affinities of the two DNA fragments to form complexes with GST::CrzA were different, as 7 and 1 μ g of GST::CrzA were required for binding to Afu3g06880 and Afu1g09950 probes respectively (Fig. 5, lanes 3 and 10 of left and right panels respectively).

The specificity of the DNA-CrzA complexes was confirmed by the addition of unlabelled probes as competitors (Specific Competitors, SC in Fig. 5). The addition of a 30-fold molar excess of unlabelled probes of the motif from the respective genes partially inhibited the complexes formed with Afu3g06880 and Afu1g09950 DNA fragments (Fig. 5, lanes 4 and 11 of left and right panels respectively). The specificity of the DNA-CrzA protein complex was also confirmed by the site-directed mutagenesis of the core sequences. The complex was not observed with the mutated Afu1g09950 probe, while there was not a complete loss, but a substantial reduction, in the formation of a complex with the mutated Afu3g06880 probe (Fig. 5, lanes 7 and 14 of left and right panels respectively).

The *A. fumigatus* CrzA has previously been shown to directly control the mRNA accumulation of the calcium transporters *pmcA*–*C* by binding to their promoters (Dinamarco *et al.*, 2012). CrzA-binding experiments suggested that the 5'-CACAGCCAC-3' and 5'-CCCTGC CCC-3' sequences upstream of *pmcA* and *pmcC* genes, respectively, are CDRE-like consensus motifs. Addition of a 15-fold molar excess of either *pmcA* or *pmcC* unlabelled probes considerably inhibited complex formation with

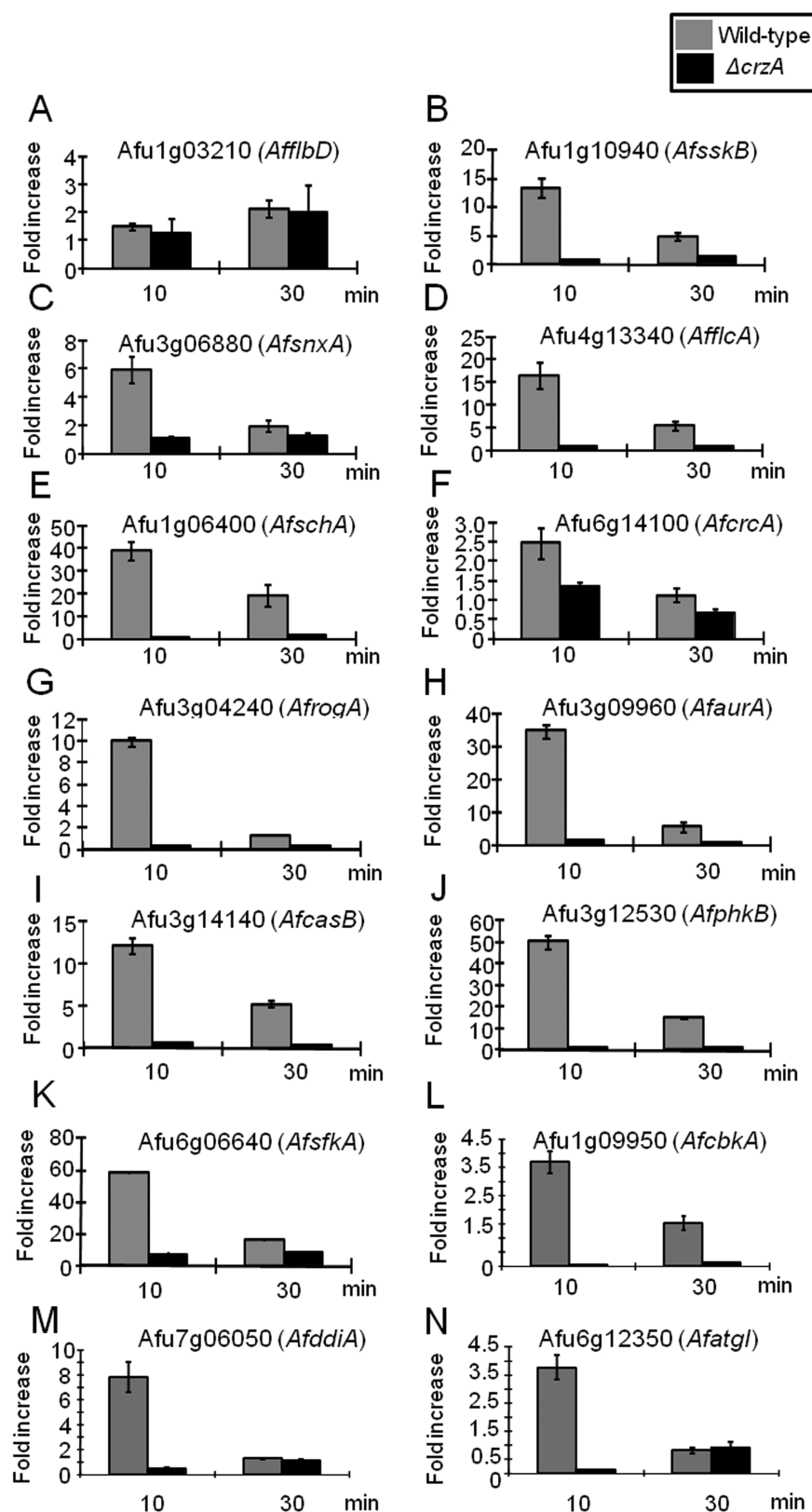


Fig. 3. RT-qPCR was used to validate the ChIP-seq results. The wild-type and the $\Delta crzA$ strains were grown in YG medium for 16 h at 37°C and then transferred to 200 mM CaCl_2 for 10 and 30 min. The absolute quantification of the selected genes (A to N) and *gpdA* (*Afu5g01970*, encoding the glyceraldehyde-3-phosphate dehydrogenase) was determined by a standard curve (i.e. C_T – values plotted against a logarithm of the DNA copy number). Fold increase represents the treatment versus untreated control. The results are the means ($\pm$ standard deviation) of four biological replicates.

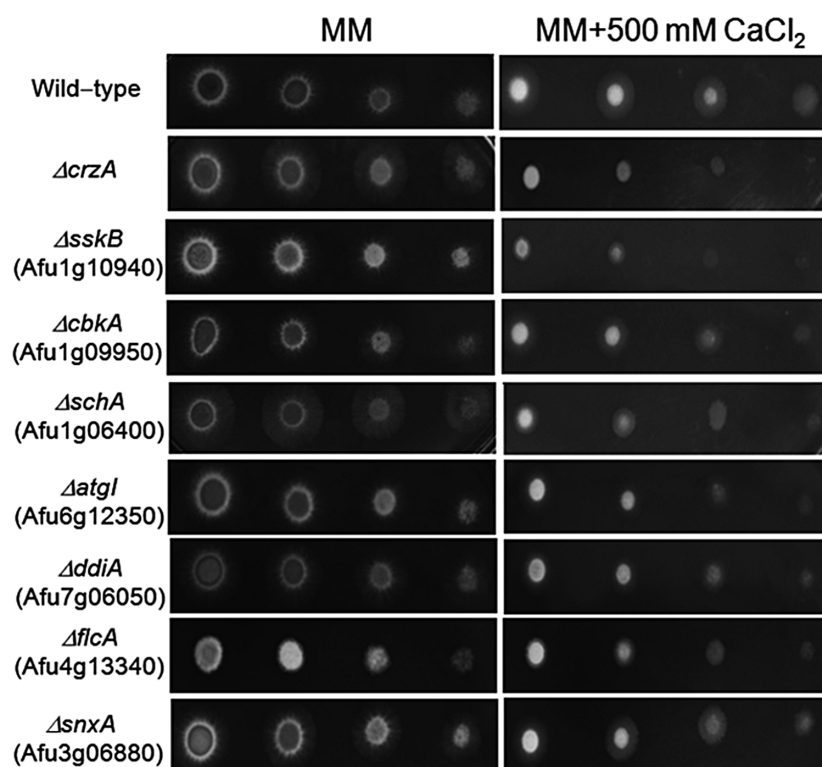


Fig. 4. Growth phenotypes of null mutants of a select subset of genes identified in the ChIP-seq analysis. The *A. fumigatus* wild-type, Afu1g06900, $\Delta crzA$, Afu1g10940, $\Delta sskB$, Afu1g06400, $\Delta schA$, Afu4g13340, $\Delta flcA$, Afu3g06880, $\Delta snxA$, Afu1g09950, $\Delta cbkA$, Afu6g12350, $\Delta atgl$, and Afu7g06050, $\Delta ddiA$ mutants strains were tested for sensitivity to $CaCl_2$. A 10-fold dilution of conidia (10^5 to 10^2) was spotted onto MM plates with or without the indicated $CaCl_2$ concentration. Plates were incubated for 72 h at 37°C.

Afu3g06880 and Afu1g09950 (Fig. 5, lanes 5 and 6 and 12 and 13 of left and right panels respectively). Collectively, these results suggest that the modulation of mRNA accumulation of Afu3g06880 and Afu1g09950, in response to calcium, is directly regulated by CrzA.

Functional characterization of the *A. fumigatus* *SskB*, the MAP kinase kinase kinase of the HOG pathway

The fungal osmotic stress, HOG, pathway is composed of two signalling modules: (i) the MAP kinase module

comprising of a MAP kinase (MAPK), a MAP kinase kinase (MAPKK) and a MAP kinase kinase kinase (MAPKKK), and (ii) the two-component system (TCS)-like phosphorelay module composed of hybrid sensor kinases, a histidine-containing phosphotransfer (HPT) protein and response regulators (RR). The TCS senses and relays environmental signals that subsequently activate the Hog1 MAPK pathway (for a review, see Bahn, 2008). The histidine kinase (HK), *phkB* (Afu3g12530) acting upstream the TCS-Hog1 MAPK pathway and the MAPKKK *sskB* (Afu1g10940) of the HOG pathway were regulated by CrzA (Fig. 3, Supplementary Table S4). In addition, $\Delta sskB$ is more sensitive to $CaCl_2$ (Figs 4 and 6). The function of PhkB has previously been characterized by Hagiwara *et al.* (2013). Subsequently, the role of *sskB* in response to calcium and osmotic stresses was investigated further.

The $\Delta sskB$ strain was grown in minimal (MM) or complete (YAG) media supplemented with agents that affect calcineurin-CrzA signalling, including $CaCl_2$ and $MnCl_2$, (Soriani *et al.*, 2008), and oxidative or osmotic stresses induced by paraquat, menadione and NaCl (Fig. 6). The $\Delta sskB$ strain showed reduced radial growth in MM compared to the wild-type strain (Fig. 6A and B); however, it showed a wild-type growth rate over 96 h in both MM and YG liquid media (data not shown). The $\Delta sskB$ was more susceptible to NaCl, $CaCl_2$ and paraquat in both media (Fig. 6A and B). The $\Delta sskB$ strain was more resistant to the azole and echinocandin fungicides, itraconazole and

Table 1. Caspofungin E-test for the wild-type, $\Delta crzA$, and deletion strains of selected genes identified in the ChIP-seq as transcriptionally modulated by CrzA.

Strains	Minimal inhibitory concentration (MIC) μg per ml of caspofungin
Wild-type	0.50
$\Delta crzA$	< 0.094
$\Delta sskB$ (Afu1g10940)	0.094
$\Delta cbkA$ (Afu1g09950)	0.19
$\Delta schA$ (Afu1g06400)	0.064
$\Delta atgl$ (Afu6g12350)	1.0
$\Delta ddiA$ (Afu7g06050)	0.50
$\Delta flcA$ (Afu4g13340)	0.25
$\Delta snxA$ (Afu3g06880)	0.19

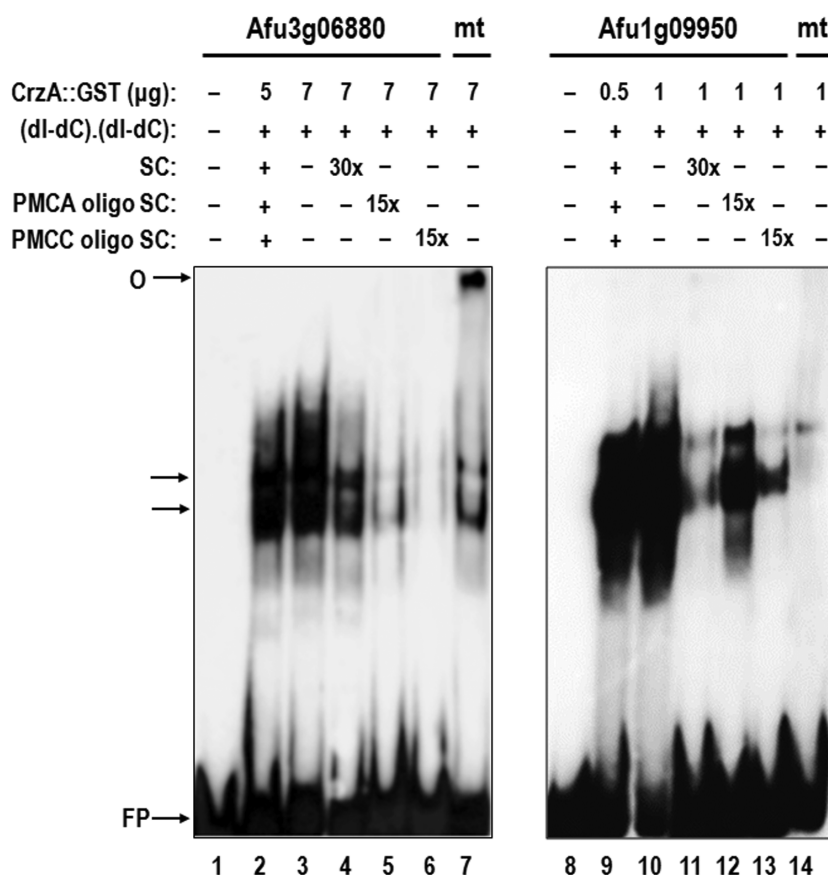


Fig. 5. Binding of GST::CrzA recombinant protein to Afu3g06880 and Afu1g09950 promoters. Gel shift analysis was performed using two DNA fragments of Afu3g06880 and Afu1g09950 promoters as probes and 0.5 to 7.0 μg of the CrzA::GST recombinant protein. Lanes 1 to 6, Afu3g06880 probe; lane 1, no protein added. Lane 7, mutated Afu3g06880 probe. Lanes 8 to 13, Afu1g09950 probe; lane 8, no protein added. Lane 14, mutated Afu1g09950 probe. O, gel origin; SC, specific competitor; FP, free probe; PMCA and PMCC oligos SC, the oligonucleotides that represent the CDRE motif in the calcium transporters *pmcA* and *pmcC* promoter regions and were used as specific competitors. Middle arrows indicate the CrzA::GST-DNA complexes.

caspofungin (Supplementary Fig. S4). The complemented $\Delta sskB::sskB^+$ strain showed the same phenotypes as the wild-type strain, strongly indicating that the observed null phenotypes were due to the loss of gene function (Fig. 6). Interestingly, when the $\Delta crzA$ strain was grown on MM, in the presence of either CaCl_2 (50 mM) plus NaCl (0.5 M), or CaCl_2 (100 mM) plus sorbitol (1 M), there was a synergistic inhibition of growth compared to the wild-type strain (Fig. 7). In the $\Delta sskB$ mutant, this synergistic inhibition is seen only in the presence CaCl_2 plus sorbitol (Fig. 7). As previously reported by Hagiwara *et al.* (2013), in *A. fumigatus* HK Nika, upstream of the Hog1 pathway, is involved in fungicide resistance and osmotic stress adaptation. Subsequently, the impact of a phenylpyrrole and a dicarboximide fungicide, fludioxonil and iprodione respectively, on the radial growth of the *A. fumigatus* wild-type, $\Delta sskB$ and $\Delta crzA$ strains was evaluated (Fig. 8). The $\Delta sskB$ mutant was more resistant to both fungicides, while the $\Delta crzA$ mutant was as sensitive as the wild-type strain (Fig. 8). These results suggest that Calcineurin-CrzA collaborate with the Hog1 pathway upon osmotic stress adaptation but not on fungicide action, and that there is a genetic interaction between the *A. fumigatus* CrzA and the SskB MAPKKK of HOG pathway.

CrzA::GFP translocates to the nucleus upon osmotic stress

The identification of SskB as a possible CrzA target suggests that osmotic stress would induce CrzA translocation to the nucleus. The CrzA::GFP fusion protein predominantly localized within the cytosol during the absence of osmotic stress, but was translocated to 25% of nuclei between 5 and 60 min osmotic stress, while being translocated to 50% of nuclei after 5 h exposure to NaCl or sorbitol (about 100 germlings were observed for each time point; Fig. 9A). The observed nuclear translocation of CrzA::GFP was blocked when germlings were pre-incubated for 30 min with 250 μM of the calcineurin inhibitor cyclosporine A (Fig. 9B). These results strongly indicate that calcineurin and CrzA are involved in the response to osmotic stress.

To determine whether CrzA was involved in the activation of the Saka MAPK cascade in response to osmotic stress, the phosphorylation level of the Saka protein was monitored by immunoblot analysis with the commercial antibodies, anti-phospho-p38 MAPK (Thr180/Tyr182) and anti-Hog1 (y-215). As expected Saka phosphorylation was absent in the $\Delta sskB$ mutant strain (Fig. 10A). In the

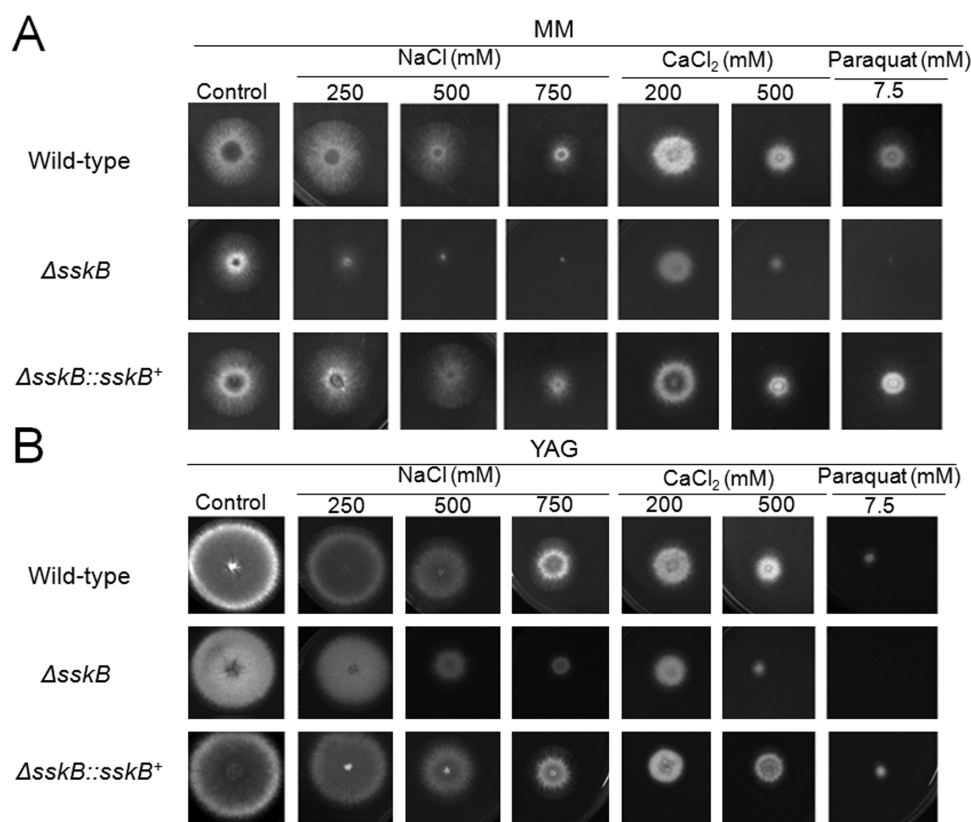


Fig. 6. Growth phenotypes of the $\Delta sskB$ mutant strain. The wild-type, $\Delta sskB$, and $\Delta sskB::sskB^+$ were grown in MM (A) and YAG (B) medium with different concentrations of NaCl, $CaCl_2$, and paraquat for 48 h at 37°C.

wild-type strain, upon transfer to Sorbitol 1 M for 10 and 20 min, the phosphorylation of SakaA increased about 2.8- and 4.4-fold respectively (Fig. 10A). In contrast, in the $\Delta crzA$ mutant upon transfer to Sorbitol 1 M for 10 and 20 min, SakaA phosphorylation showed a decrease of 58% and 72% respectively (Fig. 10A). Interestingly, SakaA phosphorylation has a moderate decrease during calcium stress, which appears to be only partially dependent on CrzA (Supplementary Fig. S5). This indicates that CrzA is important for the phosphorylation of the SakaA MAPK. Taken together these results suggest that calcineurin/CrzA pathway plays a major role in SakaA MAPK cascade activation in response to osmotic shock.

A TCS and a Hog1-type mitogen-activated protein kinase (MAPK) cascade are more sensitive to calcium

The sensitivity of different components of the TCS-HOG cascade to calcium was investigated (Fig. 11A). The TCS-HOG cascade mutants $\Delta nikA$, $\Delta phkB$, $\Delta sskA$, and $\Delta saka$ were described in the study by Hagiwara *et al.* (2013) while the strains $\Delta skn7$, $\Delta pbsB$, $\Delta mpkC$, and $\Delta saka \Delta mpkC$ were constructed in this study (Supplementary Fig. S6). The HKs $\Delta nikA$ and $\Delta phkB$, upstream of the Hog1 pathway,

demonstrated contrasting phenotypes, with only $\Delta nikA$ being more sensitive to calcium (Fig. 11B). Similarly, of the two RRs $\Delta sskA$ and $\Delta skn7$, only $\Delta sskA$ was more sensitive to calcium (Fig. 11B). The MAPKK $\Delta pbsB$ was more sensitive to calcium, but the two MAPKs ($\Delta saka$ and $\Delta mpkC$), were not. However, the double $\Delta saka \Delta mpkC$ mutant was very sensitive to calcium (Fig. 11B). Collectively, these results suggest that NikA and SskA are important for calcium signalling upon activation of MAP kinases during osmotic stress and that SakaA and MpkC play redundant roles during this process.

The A. fumigatus ΔsskB strain is avirulent in low-dose murine infection

The importance of SskB to *A. fumigatus* pathogenicity was evaluated in a neutropenic murine model of invasive pulmonary aspergillosis (Fig. 12A). Wild-type infection resulted in a 100% mortality after 13 days post infection, while $\Delta sskB$ infection resulted in a significantly reduced mortality rate, at approximately 35% after 15 days post infection ($P < 0.005$). Full virulence was restored in an independent strain that resulted from a single ectopic reintegration of the wild-type *sskB* locus (Supplementary

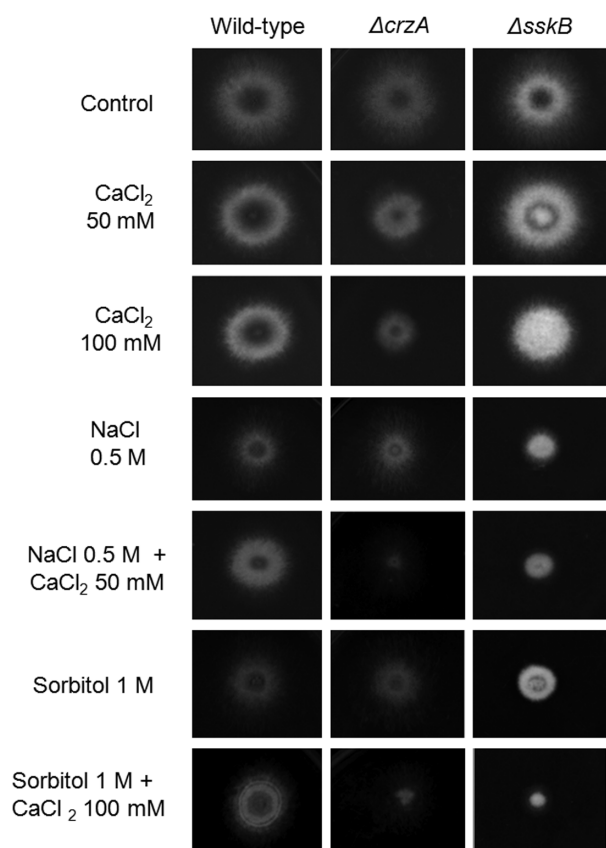


Fig. 7. There is a synergistic growth inhibition of $\Delta crzA$ and $\Delta sskB$ when these strains are grown in the presence of calcium and osmotic stresses. The wild-type, $\Delta crzA$, and $\Delta sskB$ were grown for 48 h at 37°C at different growth conditions.

Fig. S2) and the $\Delta sskB::sskB^+$ complemented strain (Fig. 12A), directly linking the attenuation of $\Delta sskB$ virulence to SskB function.

Histopathological examination revealed that at 72 h post infection the lungs of mice infected with the wild-type strain contained multiple foci of invasive hyphal growth, manifesting as both penetration of the pulmonary epithelium in major airways and pockets of branched invading hypha originating from the alveoli (Fig. 12B). In contrast, $\Delta sskB$ infections were typified by inflammatory infiltrates in bronchioles, with some containing poorly germinated or ungerminated conidia (Fig. 12B). Fungal burden was measured by real-time PCR, showing that the $\Delta sskB$ strain did not grow within the lungs as well as the wild-type and the complemented $\Delta sskB$ strains (Fig. 11C, $P < 0.001$ for the comparison between the wild-type and the deletion mutant, $P < 0.01$ between the deletion mutant and the complemented strain, and $P < 0.05$ between the wild-type and the complemented strain). Taken together, this strongly indicates that SskB plays a role in *A. fumigatus* virulence.

Discussion

The calcium/calcineurin signalling pathway plays an important role in fungal morphogenesis, virulence and ion tolerance. The presented study of *A. fumigatus* set out with the goal of identifying the gene targets directly regulated by the CrzA transcription factor of the calcium/calcineurin pathway. *A. fumigatus* *crzA*-deficient mutants show decreased virulence and differential expression of

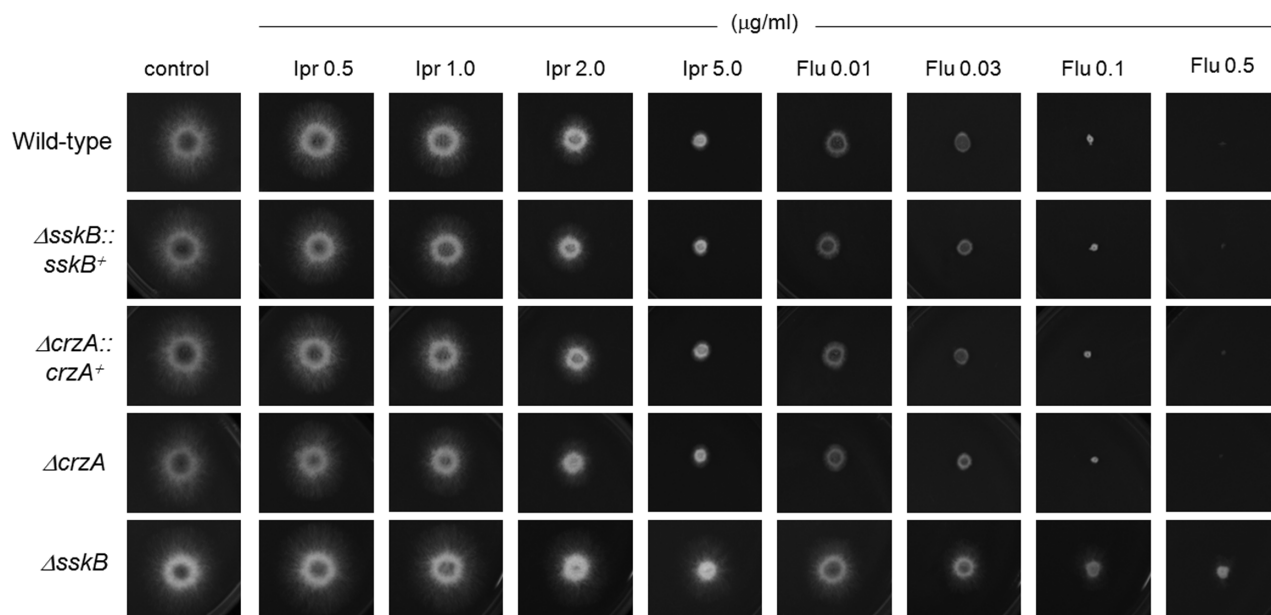


Fig. 8. The $\Delta sskB$ mutant is more resistant to fungicides fludioxonil and iprodione. The wild-type, $\Delta sskB$, $\Delta sskB::sskB^+$, $\Delta crzA$, and $\Delta crzA::crzA^+$ were grown in MM with different concentrations of fludioxonil and iprodione for 48 h at 37°C.

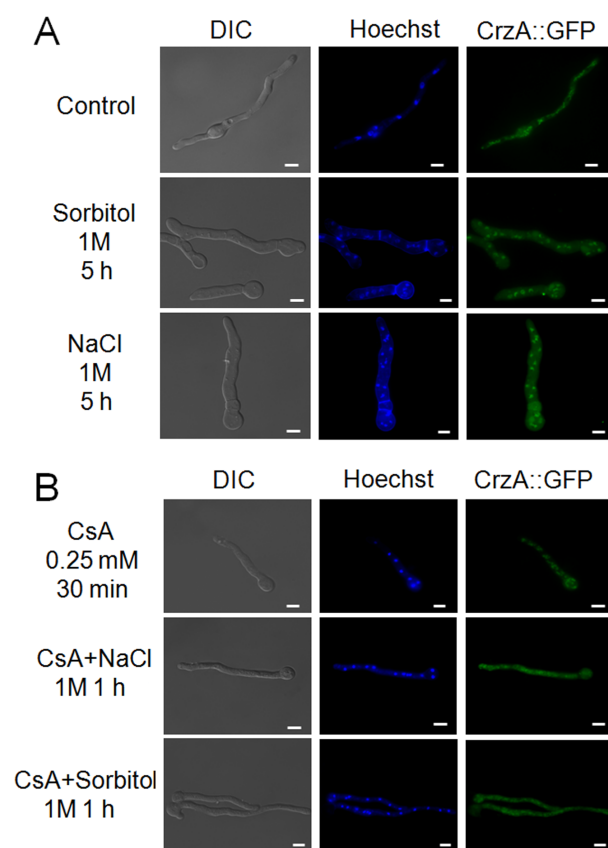


Fig. 9. CrzA::GFP translocates to the nucleus upon osmotic stress.

A. CrzA::GFP strain was grown for 16 h at 30°C and then transferred to either Sorbitol 1 M or NaCl 1 M for 5 h (DIC, differential interference contrast).
 B. CrzA::GFP strain was grown for 16 h at 30°C, 0.25 mM cyclosporine was added for 30 min, and the germlings were transferred to either Sorbitol 1 M or NaCl 1 M for 1 h. Bar, 5 µm.

over 3000 genes in response to calcium when compared to wild-type strain (Soriani *et al.*, 2008; 2010). Genes encoding calcium transporters, transcription factors and enzymes involved in metabolism were influenced by CrzA. However, the transcriptomic analysis failed to differentiate between the direct and indirect impacts of CrzA, while little is known about CrzA-binding targets of CrzA (Dinamarco *et al.*, 2012). Subsequently, ChIP-seq was utilized to identify the direct targets of CrzA, revealing 102 genes as being regulated by CrzA in the calcium/calcineurin signalling pathway.

A CDRE-binding motif within the promoters of genes regulated by calcium/calcineurin has been predicted in the study of the *S. cerevisiae* homologue Crz1p (Yoshimoto *et al.*, 2002). This study identified 5'-GNGGC[G/T]-3' as the CDRE sequence, while the 5'-GAGGCTG-3' sequence was identified as a common motif in the 500 bp upstream region of 40 genes whose expression level was regulated by Crz1p. In *C. albicans* the 5'-G[C/T]GGT-3' motif was identified in the upstream regions of 60 calcineurin/Crz1p-dependent genes (Karababa *et al.*, 2006). Similarly, Hagiwara *et al.* (2008) reported a 5'-G[T/G]GGC[T/A]G[T/G]G-3' consensus motif for genes involved in calcium/calcineurin signalling in *A. nidulans* by performing an *in silico* analysis of the promoters of 25 CrzA-dependent genes using the predictor MEME. Finally, in *M. oryzae* two candidate binding motifs in the promoters of 83 MoCrz1 target genes 5'-GGC[A/T]GTG-3' and 5'-TTGNTTG-3' were identified and confirmed by EMSA (Kim *et al.*, 2010). Compared to the reported putative Crz1/CrzA-binding motifs, the *A. fumigatus* motif predicted in the presented CrzA study demonstrated the highest level of similarity to the *A. nidulans* motif. Despite possessing differences, a similar core (5'-[G/T]T[G/C][G/C]CT-3') was identified. These results indicated that in different fungi the Crz1/CrzA

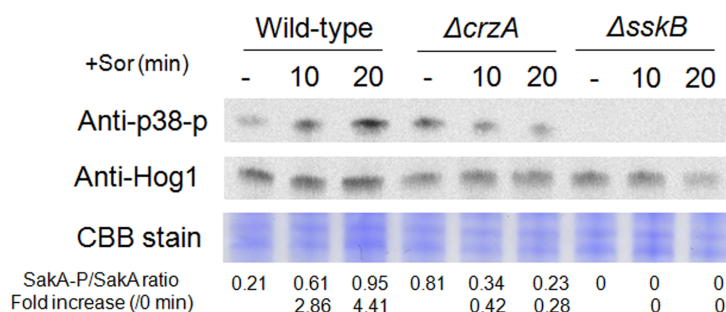


Fig. 10. The lack of CrzA affects SakA phosphorylation upon stress. Immunoblot analysis for phosphorylation of the SakA MAPK in response to osmotic stress. The wild-type, $\Delta crzA$, and $\Delta sskB$ mutant strains were grown for 18 h at 37°C. Then, sorbitol (Sor, 1 M final concentration) was added for 0 (control), 10 and 20 min. The mycelium was harvested at the indicated times, and total proteins were extracted. Anti-phospho-p38 was used to detect the phosphorylation of SakA, and anti-Hog1p was used to detect the total protein of SakA. A Coomassie Brilliant Blue (CBB)-stained gel is shown as a loading control. Signal intensities were quantified using the Image J software by dividing the intensity of SakA-P/SakA ratio and expressed as fold increase from the control (0 min).

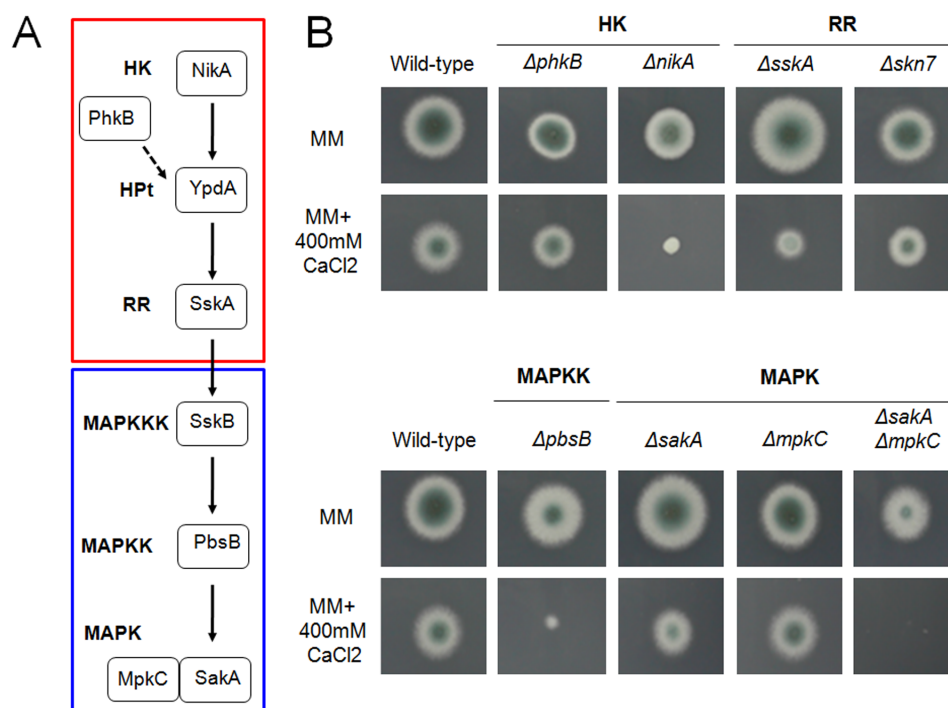


Fig. 11. A two-component system (TCS) and a Hog1-type mitogen-activated protein kinase (MAPK) cascade are more sensitive to calcium. **A.** Schematic representation of the TCS-HOG-MAPK cascade (HK, histidine kinase; HPT, histidine-containing phosphotransfer protein; MPKKK, mitogen-activated protein kinase kinase kinase; MPKK, mitogen-activated protein kinase kinase; MPK, mitogen-activated protein kinase; and RR, response regulator). **B.** The wild-type, $\Delta phkB$, $\Delta nikA$, $\Delta sskA$, $\Delta skn7$, $\Delta pbsB$, $\Delta sakA$, $\Delta mpkC$, and $\Delta sakA \Delta mpkC$ were grown for 48 h at 37°C.

homologues may have species-specific recognition sites. To further confirm the functionality of the motif identified in this project additional EMSAs are required. Recently, CrzA was shown to directly control the mRNA accumulation of the calcium transporters *pmcA–C*, by binding to their promoters (Dinamarco *et al.*, 2012). CrzA-binding experiments indicated that the 5'-CACAGCCAC-3' and 5'-CCCTGCCCC-3' sequences upstream of *pmcA* and *pmcC*, respectively, are possible CDREs-like consensus motifs. These two motifs display between 50% (*pmcA*) and 80% (*pmcC*) identity with the *A. fumigatus* CrzA CDRE motif proposed via ChIP-seq.

Comparative analyses of the *A. fumigatus* CrzA ChIP-seq data and published microarrays of the wild-type and $\Delta crzA$ strains in response to calcium, has provided valuable information. These complementary techniques describe the genes bound and transcriptionally regulated by CrzA. For example, *ZfpA* (Afu8g05010) showed increased expression in response to calcium treatment in the wild-type strain (Malavazi *et al.*, 2009), but this fold change decreased in the $\Delta crzA$ mutant (Soriani *et al.*, 2010), while ChIP-seq predicted a CrzA-binding motif in the *zfpA* promoter. Collectively, these observations clearly show that the transcription of *zfpA* is directly regulated by CrzA, and that it may play an important role in the calcium

signalling. Interestingly, the cAMP-dependent PKA signalling pathway has also been reported to regulate the increased expression of *zfpA* during the adaptation to voriconazole (da Silva Ferreira *et al.*, 2006). Therefore, *ZfpA* possibly forms part of a transcriptional response regulated by calcineurin and CrzA, co-ordinating cellular stress responses.

On the other hand, the large number of genes that showed differential expression in $\Delta crzA$ mutant during calcium treatment, but were not bound by CrzA, could represent downstream events that are subsequently regulated by CrzA in an indirect manner. Some of these genes are involved in calcium transport, such as vacuolar (Afu2g007630) and calcium-translocating P-type ATPase (Afu7g01030), or transcription factors (Afu4g03960 or Afu4g10200), or a phospholipase D (Afu2g16520). The remaining circumstance is the genes that were bound by CrzA but showed no transcriptional modulation in $\Delta crzA$ strain. This would suggest that the CrzA loss of function could be complemented by other proteins and/or pathways. Examples in this category include genes related to transport (Afu8g05090, Afu7g06060, Afu2g12310, and Afu3g12580), and genes predicted as involved in metabolic processes (Afu1g07550, Afu3g04240, Afu6g14400, and Afu8g02350).

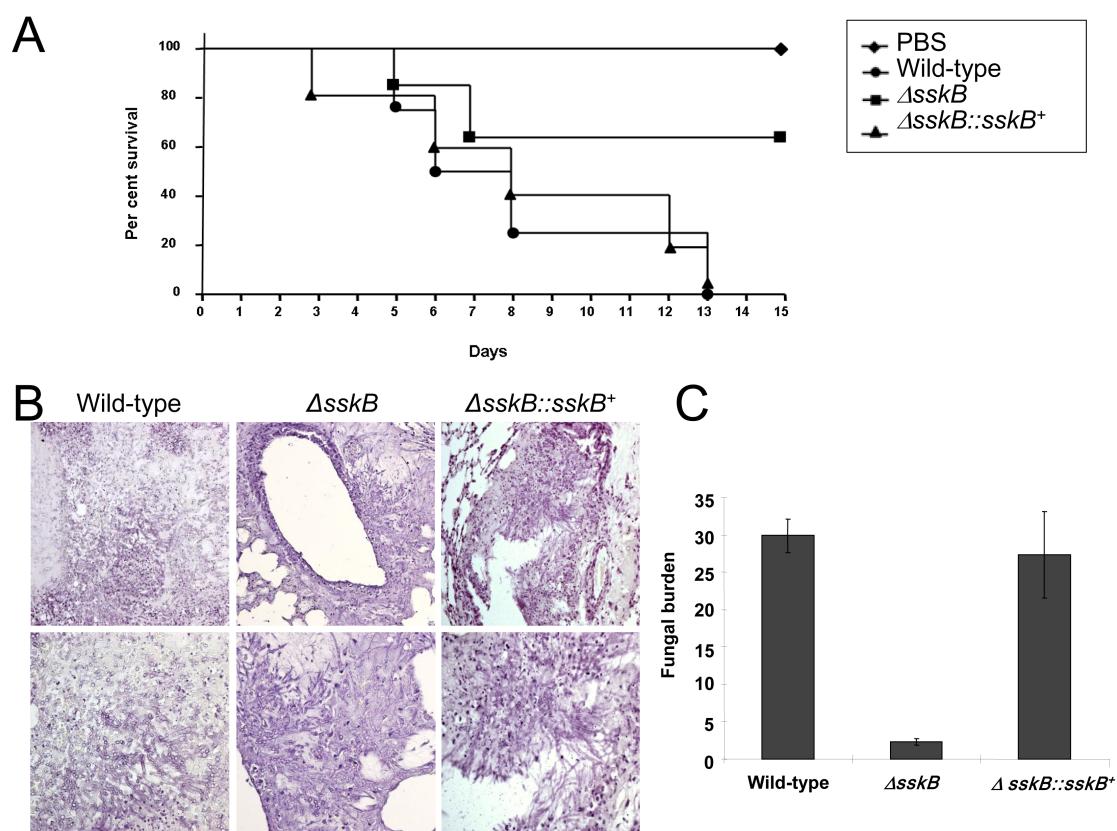


Fig. 12. *A. fumigatus sskB* contributes to virulence in neutropenic mice. Comparative analysis of wild-type and the mutants strains in a neutropenic murine model of pulmonary aspergillosis. Mice in groups of 10 per strain were infected intranasally with a $20 \mu\text{l}$ suspension of conidia at a dose of 10^5 (A). Fungal burden was determined 72 h post infection by qPCR based on 18S rRNA gene of *A. fumigatus* and an intronic region of the mouse GAPDH gene (B). Histological analyses of infection murine lung were performed 72 h after infection (C).

This study extensively validated the ChIP-seq analysis by RT-qPCR, gene deletion studies and EMSA analyses. All these analyses provided strong evidence of the regulation of approximately 10% of the identified target genes by the calcium-calcineurin-CrzA pathway. The presented ChIP-seq analysis identified the *phkB* histidine kinase and the *sskB* MAPKKK, which are components of the TCS and HOG-MAPK cascade, as being transcriptionally regulated by CrzA. Subsequently, three observations suggested that the calcineurin-CrzA pathway contributes to the activation of the TCS and HOG-MAPK cascade in *A. fumigatus*: (i) osmotic stress induced the translocation of CrzA::GFP to the nucleus and that this translocation can be blocked by cyclosporine, (ii) reduced Saka phosphorylation upon osmotic stress in the $\Delta crzA$ mutant, and (iii) different null mutants of TCS and HOG-MAPK cascade were sensitive to calcium. There are 13 HK in *A. fumigatus* and Hagiwara *et al.* (2013) have demonstrated that Nika plays an important role in modulating the Saka MAPK cascade in response to osmotic stress, while the other HKs apparently do not impact upon the HOG-MAPK pathway. The HOG pathway does not only affect osmolality regulation, but also cell wall integrity and hyphal growth are also affected, as

demonstrated by deletion of *A. nidulans saka* or the *A. fumigatus ssk1* (Du *et al.*, 2006; Hagiwara *et al.*, 2007). The *A. fumigatus* $\Delta saka$ strain has dysregulated conidial germination and deficiencies in sensing environmental nitrogen or mounting a response to hypertonic stress (May *et al.*, 2005; Du *et al.*, 2006). The HOG-MAPK pathway has been reported as being involved in the regulation of morphogenesis and virulence in fungal pathogens of humans, insects and plants (Alonso-Monge *et al.*, 1999; Bahn *et al.*, 2005; Igbaria *et al.*, 2008; Zhang *et al.*, 2009), and in the response to oxidative, osmotic, citric acid, arsenite, heat and ultraviolet light stresses (Blomberg and Adler, 1992; Degols and Russell, 1997; Rep *et al.*, 2000; Winkler *et al.*, 2002; Bilsland *et al.*, 2004; Lawrence *et al.*, 2004; Sotelo and Rodríguez-Gabriel, 2006; Yaakov *et al.*, 2009; Ma and Li, 2013). This study has shown that SskB is involved on *A. fumigatus* virulence in mice since $\Delta sskB$ mutant has attenuated virulence. *C. albicans* MAPKK Pbs2 mutants displayed attenuated virulence in a mouse model of disease (Cheetham *et al.*, 2011). The fungal phytopathogens *Cochliobolus heterostrophus* and *Fusarium graminearum* Hog1 mutants cause decreased disease symptoms (Igbaria *et al.*, 2008; Nguyen *et al.*, 2012). Interestingly,

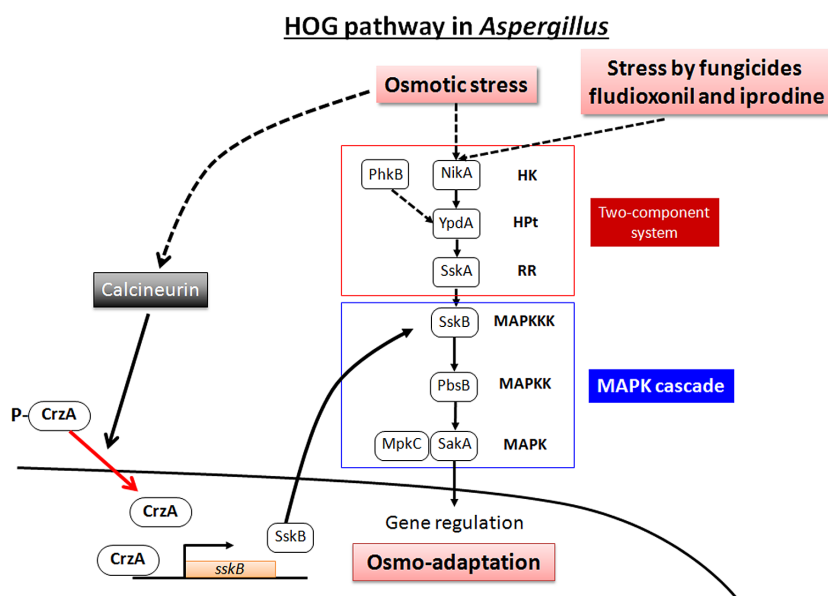


Fig. 13. Proposed model for the interaction between *A. fumigatus* CrzA and TCS-HOG-MAPK cascade.

several HKs and RR from the TCS pathway from human and plant pathogens are involved in virulence and pathogenicity (Nathues *et al.*, 2007; Lin and Chung, 2010; Oide *et al.*, 2010; Risipail and Di Pietro, 2010; Boyce *et al.*, 2011; Luo *et al.*, 2012).

In *S. cerevisiae* several investigations describe a link between calcium metabolism and the HOG pathway (Shitamukai *et al.*, 2004; Takatsume *et al.*, 2010). Shitamukai *et al.* (2004) showed the antagonistic role of the Hog1 and Ca^{2+} signalling pathways in the regulation of *S. cerevisiae* growth, where the calcineurin-Crz1 pathway was required for the downregulation of the Hog1 pathway. In *S. cerevisiae*, glyoxalase 1 (*GLO1*) metabolizes the toxic by-product of glycolysis, methylglyoxal. The expression of *GLO1* is induced by methylglyoxal, osmotic stress in a Hog1-dependent manner and following treatment with Ca^{2+} (Takatsume *et al.*, 2010). The Hog1 and Crz1 regulation of *GLO1* was dependent on a stress response element in the promoter and Crz1 was proposed to destabilize the stress response transcription factors Msn2/Msn4 in response to calcium. The aforementioned studies imply that the calcineurin-Crz1 and Hog1 signalling pathway converge during stress response.

In *Cryptococcus neoformans*, resistance to the fungicide fludioxonil is due to a contribution of both the calcineurin and Hog1 pathways (Kojima *et al.*, 2006). In the mycoparasitic fungus *Trichoderma harzianum* a hog1 mutant was highly resistant to the calcineurin inhibitor cyclosporine A, suggesting interactions between both pathways in filamentous fungi (Delgado-Jarana *et al.*, 2006). The presented study of *A. fumigatus* emphasizes the collaboration between the calcineurin-CrzA, TCS and HOG pathways in the response to osmotic stress. A model for the cross-talk

between the calcineurin-CrzA and the TCS-HOG pathways during osmotic stress is proposed (Fig. 13). During osmotic stress, but probably not on during exposure to the fungicides fludioxonil and iprodione, an increase in the cytosolic calcium may activate calcineurin which dephosphorylates CrzA. The transcription factor CrzA subsequently translocates to the nucleus and induces the *sskB* mRNA accumulation. Upon the activation of the TCS cascade, SskB is activated and phosphorylates the MAPKK PbsB activating MAP kinases SakA and MpkC.

In conclusion, this study revealed a breadth of target genes that are directly regulated at transcriptional level by CrzA, including members of the osmotic stress pathway. In addition, evidence linking the TCS-HOG and the Ca^{2+} -calcineurin pathways was presented. Importantly, the MAPKK of the osmotic stress pathway, SskB, which was transcriptionally regulated by CrzA, was required for full virulence in an animal infection model. Further studies are necessary to fully understand biochemical interaction and how these two pathways cross-talk during a response to different environmental stresses and pathogenicity. In *A. fumigatus*, both the TCS-HOG and calcium-calcineurin pathways are important to stress responses and virulence in a mammalian host. Therefore, the identification of the link between these central pathways will provided new avenues for research into the identification of novel targets for disease intervention.

Experimental procedures

Ethics statement

The principles that guide our studies are based on the Declaration of Animal Rights ratified by the UNESCO in 27 January

1978 in its articles 8th and 14th. All protocols used in this study were approved by the local ethics committee ('Comitê Central de Ética Experimental do Campus') for animal experiments from the Campus of Ribeirão Preto from Universidade de São Paulo (Permit Number: 08.1.1277.53.6; studies on the interaction of *A. fumigatus* with animals). All animals were housed in groups of five within individually ventilated cages and were cared for in strict accordance with the principles outlined by the Brazilian College of Animal Experimentation (Princípios Éticos na Experimentação Animal – Colégio Brasileiro de Experimentação Animal, COBEA) and Guiding Principles for Research Involving Animals and Human Beings, American Physiological Society. All efforts were made to minimize suffering. Animals were clinically monitored at least twice daily and humanely sacrificed if moribund (defined by lethargy, dyspnoea, hypothermia and weight loss).

Strains, media and culture methods

The *A. fumigatus* strains used in this study are CEA17 (pyrG⁻), CEA17-80 (as the wild-type in most of the experiments), Afs35 (*akuA::loxP*) that was a parental strain for the TCS-HOG cascade experiments, $\Delta nikA$, $\Delta phkB$, $\Delta sskA$, $\Delta sakA$ (Hagiwara *et al.*, 2013), $\Delta skn7$, $\Delta pbsB$, $\Delta mpkC$, and $\Delta sakA \Delta mpkC$, $\Delta crzA$, CrzA::GFP (Soriani *et al.*, 2008), $\Delta sskB$, and $\Delta sskB::sskB$; Afu1g06400, $\Delta schA$, Afu4g13340, $\Delta flcA$, Afu3g06880, $\Delta snxA$, Afu1g09950, $\Delta cbkA$, Afu6g12350, $\Delta atgI$, and Afu7g06050, $\Delta ddiA$. The media used were of two basic types, i.e. complete and minimal. The complete media comprised the following three variants: YAG (2% w/v glucose, 0.5% w/v yeast extract, 2% w/v agar, trace elements), YUU [YAG supplemented with 1.2 g l⁻¹ (each) of uracil and uridine], and liquid YG or YG+UU medium with the same composition (but without agar). The minimal medium (MM) consist of 1% glucose, trace elements and salt solution 20× (Kafer, 1977), 2% agar, pH 6.5. Strains were grown at 37°C.

Chromatin immunoprecipitation and ChIP-seq library preparations

The *A. fumigatus* strain used in this study was CEA17 (CrzA::eGFP). Fungal conidia (10⁶ conidia ml⁻¹) were incubated in 50 ml YG media (2% glucose, 0.5% yeast extract, 2% agar, trace elements) (Kafer, 1977) at 37°C, under shaking (200 r.p.m.), for 16 h. The mycelia was harvested, dried with two layers of miracloth and the separated into that which was treated with 200 mM CaCl₂ with or without adding 10 µg ml⁻¹ calcineurin inhibitor FK506, and the two treatments were returned into YG media for 30 min with shaking (200 r.p.m.). Mycelia from the two treatments was collected with 1 miracloth and washed excessively with water. Cross-linking was performed by resuspending the mycelia in 20 ml of Buffer A (0.4 M Sucrose, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM PMSF, 1% formaldehyde) with shaking (~ 100 r.p.m.) for 20 min. Then 1 ml (1/20 volume) of 2 M glycine (final concentration 0.1 M) was added and incubated for an additional 10 min to stop cross-linking. The mycelia was harvested with miracloth and rinsed with water. The excessive water was removed by squeezing. Then the sample was frozen in liquid nitrogen and stored at -80°C. The frozen mycelia were then weighed and

~ 200 mg of mycelia were grinded in a chilled pestle and mortar. Genomic DNA was extracted with Plant Nuclear Isolation Kit (Sigma CELLYT PN1-1; CelLytic™ PN Isolation/Extraction Kit for Plant leaves). Genomic DNA was sonicated with a Biorupter (Cosmo Bio, Tokyo, Japan) using an operation pattern of 30 s ON and 30 s OFF, at power level H, for a total 30 min. Six libraries were designed for identifying CrzA binding site: (i) mycelia+Ca-nolP, (ii) mycelia+Ca+IgG, (iii) mycelia+Ca+anti-GFP, (iv) mycelia+Ca+FK506-nolP, (v) mycelia+Ca+FK506+IgG, and (vi) mycelia+Ca+FK506+anti-GFP. For each library, 30 µl of dynabeads were washed with 500 µl lysis buffer (50 mM HEPES pH7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Deoxycholate (Sigma D6750), 0.1% SDS (10%), 10 mM Na butyrate (Sigma B5887), 1 mM PMSF (100×), 1× plant proteinase inhibitor cocktail) and were kept on a magnetic stand. One hundred microlitres of blocking buffer [BSA (10 mg ml⁻¹) 10 µl, yeast transfer RNA (100 µg µl⁻¹) 2 µl in 1 ml of lysis buffer] with 1 µl of antibody were transferred into dynabeads and incubated overnight at 4°C with rotation. Precipitated sample was then washed and the immunoprecipitated DNA was purified with RNase A. DNA samples were prepared using Illumina multiplex system and sequenced by Illumina Genome Analyser II (Life Technologies).

Binding site and downstream gene identification

The 75 bp paired-end Illumina short reads were trimmed and aligned to the *A. fumigatus* Af293 genome (AspGD) with SOAPaligner 2.21 (Conesa *et al.*, 2005). Some of the key parameters used for alignment were: insert size allowed for pair-end: 100–400 bp, maximum two mismatches allowed. Alignment files were then formatted to be uploaded into Cisgenome (v2.0) (Ji *et al.*, 2011) for binding sites identification. The 'two samples peak calling' mode was used, which allowed the user to upload two library files, as one served as negative control. For group 1, which was not treated with FK506, TM5 immunoprecipitated by anti-GFP was used as the ChIPed library, while TM3 (no immunoprecipitation, IP) and TM4 (random human antibody anti-IgG) were used to provide background noise information that was used for normalization. For group 2, which was treated with FK506, TM8 was ChIPed, while TM6 and TM7 served as background libraries. Key parameters used in peaking calling process were: read extension length E = 150bp, Bin size B = 50 bp, half window size W = 2, max gap = 50 bp, min peak length = 100 bp, checked standardize window statistics, win stat cut-off C ≥ 3, check apply local read sampling rate filter and boundary refinement. Peaks with FDR < 0.2 were chosen as candidate binding sites and located back to the reference genome sequence. Downstream genes were identified if peaks located within their promoter regions (about 5 kb). The identified downstream genes were BLAST against NCBI 'NR' database, using BLAST2GO (Conesa *et al.*, 2005), to provide function annotation.

Binding motif analysis

De novo motif finding program MEME (Bailey *et al.*, 2006) was used to predict CrzA-binding motifs from the over-represented motifs in the 'peak sequences'. The sequences

of the 165 identified 'peaks' were uploaded into MEME. The top candidate motif with an E-value of 2.7×10^{-21} was chosen as a predicted CrzA-binding motif, which was then searched against a yeast binding motif databases using the motif comparison tool Tomtom (Gupta *et al.*, 2007) to determine its reliability. Possible functions associated with identified motifs were analysed using GOMO (Gene Ontology for Motif, Buske *et al.*, 2010) against yeast databases.

Construction of the *A. fumigatus* mutants

A gene replacement cassette was constructed by 'in vivo' recombination in *S. cerevisiae* as previously described by Colot *et al.* (2006). Briefly, approximately 2.0 kb either side of the respective open reading frame (ORF) were selected for primer design. For construction, the primers named as 5F and 5R, were used to amplify the 5'-untranslated region (UTR) flanking region, while the primers 3F and 3R were used to amplify the 3'-UTR ORF flanking region. The primers 5F and 3R primers contained a short homologue sequences to the multiple cloning site (MCS) of the plasmid pRS426. Both the fragments, 5- and 3-UTR, were PCR-amplified from *A. fumigatus* genomic DNA (gDNA). The *pyrG* was amplified by external primers containing short homologue sequences with upstream and downstream of the *sskB* gene and it was used in the *A. fumigatus* cassette for generating the mutant strains was used as marker for prototrophy. Deletion cassette generation was achieved by transforming each fragment along with the BamHI/EcoRI cut pRS426 plasmid into the *S. cerevisiae* strain SC9721 via the lithium acetate method (Schiestl and Gietz, 1989). DNA from positive yeast transformants was extracted by the method described by Goldman *et al.* (2003). The cassette was PCR-amplified directly from yeast genomic DNA utilizing TaKaRa Ex Taq™ DNA Polymerase (Clontech Takara Bio) and used for *A. fumigatus* transformation. Southern blot analyses demonstrated that the transformation cassettes had integrated homologously at the targeted *A. fumigatus* locus. The single gene deletion strains were complemented by co-transforming a DNA fragment (approximately 1 kb from each 5' and 3'-flanking regions plus the ORF) together with the pHAT α vector (Herrera-Estrella *et al.*, 1990) and selecting for hygromycin resistance in MM plates with 150 mg ml⁻¹ of hygromycin B.

The strains $\Delta skn7$, $\Delta pbsB$, $\Delta mpkC$, and $\Delta sakA, mpkC$ were constructed as follows. All the deletion mutants were generated using *A. fumigatus* strain Afs35 (*akuA::loxP*) as a parental strain. For construction of the strains, the gene replacement cassettes were generated by GeneArt system (Invitrogen). Briefly, approximately 1.0–1.5 kb either side (up or down) of the targeted ORF was amplified by using appropriate primer sets (U-F/U-R and D-F/D-R). The respective primer contains a homologous sequence (15–20 bp) to the MCS of cloning vector (pUC119) or the end of selection marker (HygBr or ptrA). The fragments of HygBr and ptrA were obtained from plasmids pCB1004 and pPTR I using primer sets, HygBr-FW/HygBr-RV and ptrA-F/ptrA-R respectively. The flanking region set and marker fragment were fused into the pUC119 using GeneArt system. The resulting plasmids, pUC119-skn7::HygBr, pUC-pbsB::ptrA, and pUC-mpkC::ptrA, were used for transformation to construct the $\Delta skn7$, $\Delta pbsB$, $\Delta mpkC$, and $\Delta sakA, mpkC$. Homologous

recombination and gene replacement were confirmed by PCR or Southern blot from the genome DNA, and the absence of the mRNA of the target gene was verified using qPCR analysis. The primers used in this work are described in Supplementary Table S7.

RNA extraction and real-time PCR reactions

Post treatment, mycelia were harvested by filtration, washed twice with H₂O and immediately frozen in liquid nitrogen. For total RNA isolation, mycelia were ground in liquid nitrogen using a pestle and mortar. Total RNA was extracted with Trizol reagent (Invitrogen, USA). The RNA from each treatment was analysed using an Agilent 2100 Bioanalyser system to assess the integrity of the RNA. For real-time PCR experiments RNase free DNase I treatment was carried out as previously described by Semighini *et al.* (2002). Twenty micrograms of total RNA was treated with DNase, purified using a RNeasy kit (Qiagen) and cDNA was generated using the SuperScript III First Strand Synthesis system (Invitrogen) with oligo(dT) primers, according to the manufacturer's protocol.

All the PCR reactions were performed using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, USA) and Taq-Man™ Universal PCR Master Mix kit (Applied Biosystems, USA). The reactions and calculations were performed according to Semighini *et al.* (2002). The primers and Lux™ fluorescent probes (Invitrogen, USA) used in this work are described in Supplementary Table S7.

Cloning the *crzA* gene into the pDEST15 vector, production and purification of GST::CrzA

Gateway Technology (Invitrogen) was used to construct the *E. coli* expression system for the N-terminal GST tagged CrzA. Subsequently, CrzA was expressed as a GST fusion protein from the pDEST15-GST/CrzA vector in *E. coli* Rosetta™ (DE3) pLysS strain (Novagen). All the cloning, production and purification procedures were carried out as previously described by Dinamarco *et al.* (2012).

Electrophoretic mobility shift assay

GST::CrzA recombinant protein was assayed in DNA–protein binding reactions using 200 bp DNA fragments of the Afu1g9950 (CKB2) and Afu3g06880 (SNX3) promoters as probes, containing the putative *cis*-regulatory calcineurin-dependent response elements (CDREs) for the transcription factor CrzA. Binding reactions were carried out in 1× binding buffer (25 mM HEPES-KOH, pH 7.9, 20 mM KCl, 10% w/v glycerol, 1 mM DTT, 0.2 mM EDTA, pH 8.0, 0.5 mM PMSF, 12.5 mM benzamidine, 5 mg ml⁻¹ of each antipain and pepstatin A) containing 2 mg poly(dI-dC). (dI-dC) as non-specific competitor and different amounts of GST::CrzA recombinant protein, at room temperature for 10 min. After that, DNA probes (10⁴ cpm) were added and the binding reactions were incubated at room temperature for 20 min prior to being loaded onto a native 5% polyacrylamide gel in 0.5× TBE buffer. Gels were run at 10 mA, 15°C, dried, and exposed to

X-ray film. For competition assays, an excess of the specific DNA competitors were added prior to incubation with the radiolabelled probe.

DNA probes and specific competitors for EMSA

Putative *cis crzA* motifs were visually identified in the promoter regions of the genes *ckb2* and *snx3* by using the *A. fumigatus* CDRE consensus. To produce the probes, a 200 bp DNA fragment of the genes promoters was amplified from *A. fumigatus* genomic DNA using the primers *ckb2*-5'Fw: 5'-CCTTTCACGATATTGGACAGAA-3', *ckb2*-3'Rv: 5'-TGAA GTGCCATATACAAA TGC-3' and *snx3*-5'Fw: 5'-TGTTTGT TTCTCCCTGTGGA-3', *snx3*-3'Rv: 5'-CTTCTT GAAGCC GGGAAAAA-3' in the presence of [α -³²P]-dATP (3000 Ci mmol⁻¹) and purified on 2% low-melting-point agarose gel. The unlabelled 200 bp *Ckb2* and *Snx3* probes were used as specific DNA competitors and added to the binding reaction in a 30-fold molar excess, 10 min prior to the addition of the respective probes. Mutated probes (*mCkb2* and *mSnx3*) were prepared by changing the core sequence by site-directed mutagenesis in a two-step PCR. In the *mCkb2* probe the sequence 5'-CACAGCCC-3' was changed to 5'-GTGTCTG GG-3' by using the oligonucleotides pairs *mCkb2*-A-Fw: 5'-CTGCTGGGCATATCAACAAG-3' and *mCkb2*-A-Rv: 5'-TGTTGGAAAACCCGACACTTCTTCTCGT-3', and *mCkb2*-B-Fw: 5'-ACGAGAAGAAGTGTGCGGGTTTCCAACA-3' and *mCkb2*-B-Rv: 5'-GTGAGTGTGGTCTCTGATTG-3' in the first reaction to amplify two fragments: A and B. The oligonucleotide pair *ckb2*-5'Fw and *ckb2*-3'Rv was used in a second reaction to amplify the whole DNA fragment containing the mutation. In the *mSnx3* probe the sequence 5'-AGGTTG-3' was changed to 5'-TCCAAC-3' by using the oligonucleotides pairs *mSnx3*-A-Fw: 5'-CAGATGCTCAT CCTGTCCA-3' and *mSnx3*-A-Rv: 5'-AACGTCAATGTGTTGGATGGGGTTCG CC-3', and *mSnx3*-B-Fw: 5'-GGACGACCCCATCCAACACAT TGACGTT-3' and *mSnx3*-B-Rv: 5'-TCCTTCACTTGGCCT TCAGC-3'. The oligonucleotide pair *snx3*-5'Fw and *snx3*-3'Rv was used in a second reaction to amplify the whole DNA fragment containing the mutation. For EMSA, both mutated fragments were used as templates in PCR amplifications in the presence of [α -³²P]-dATP (3000 Ci mmol⁻¹) and purified on 2% low-melting-point agarose gel.

Murine model of pulmonary aspergillosis

Outbred female mice (BALB/c strain; body weight, 20 to 22 g) were housed in vented cages containing five animals. Mice were immunosuppressed with cyclophosphamide (150 mg per kg of body weight), which was administered intraperitoneally on days -4, -1, and 2 prior to and post infection. Hydrocortisonacetate (200 mg kg⁻¹ body weight) was injected subcutaneously on day -3. *A. fumigatus* strains were grown on YAG for 2 days prior to infection. Fresh conidia were harvested in PBS and filtered through a Miracloth (Calbiochem). Conidial suspensions were spun for 5 min at 3000 g, washed three times with PBS, counted using a haemocytometer, and resuspended at a concentration of 5.0×10^6 conidia ml⁻¹. The viability of the administered inoculum was determined by incubating a serial dilution of the conidia on YAG medium, n at 37°C. Mice were anaesthetized by halothane inhalation and infected by

intranasal instillation of 5.0×10^5 conidia in 20 μ l of PBS. As a negative control, a group of five mice received PBS only. Mice were weighed every 24 h from the day of infection and visually inspected twice daily. In the majority of cases, the end of the survival experiment was determined when a 20% reduction in body weight was recorded. The mice were then sacrificed. The statistical significance of the comparative survival values was calculated using log rank analysis and the Prism statistical analysis package. Additionally, at 3 days post infection, two mice per strain were sacrificed and the lungs removed, fixed, and processed for histological analysis.

Lung histopathology and fungal burden

After sacrifice, the lungs were removed and fixed for 24 h in 3.7% formaldehyde-PBS. Samples were washed several times in 70% alcohol before dehydration in a series of alcohol solutions of increasing concentrations. Finally, the samples were diafanized in xylol and embedded in paraffin. For each sample, sequential 5- μ m-thick sections were collected on glass slides and stained with Gomori methenamine silver (GMS) or haematoxylin and eosin (HE) stain following standard protocols (Kim *et al.*, 2010). Briefly, sections were deparaffinized, oxidized with 4% chromic acid, stained with methenamine silver solution, and counterstained with picric acid. For HE staining, sections were deparaffinized and stained first with haematoxylin and then with eosin. All stained slides were immediately washed, preserved with mounting medium, and sealed with a coverslip. Microscopic analyses were performed using an Axioplan 2 imaging microscope (Zeiss) at the stated magnifications under bright-field conditions.

To investigate fungal burden in murine lungs, mice were immunosuppressed with cyclophosphamide (150 mg kg⁻¹ of body weight), which was administered intraperitoneally on days -4 and -1, while hydrocortisonacetate was injected subcutaneously (200 mg kg⁻¹) on day -3. Five mice per group (wild-type, Δ *sskB*, and Δ *sskB::sskB*⁺ strains and PBS control) were intranasally inoculated with 1×10^6 conidia per 20 μ l of suspension. A higher inoculum, in comparison to the survival experiments, was used to increase fungal DNA detection. Animals were sacrificed 72 h post infection, and the lungs were harvested and immediately frozen in liquid nitrogen. Samples were homogenized by vortexing with glass beads for 10 min, and DNA was extracted via the phenol-chloroform method. DNA quantity and quality were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific). At least 500 μ g of total DNA from each sample was used for quantitative real-time PCRs. A primer and a Lux probe (Invitrogen) were used to amplify the 18S rRNA region of *A. fumigatus* (primer, 5'-CTTAAATAGCCCGGTCCGCATT-3'; probe, 5'-CATCACAGACCTGTTATTGCCG-3') and an intronic region of mouse GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (primer, 5'-CGAGGGACTTGGAGGACAC AG-3'; probe, 5'-GGGCAAGGCTAAAGGTCAGCG-3'). Six-point standard curves were calculated using serial dilutions of gDNA from all the *A. fumigatus* strains used and the uninfected mouse lung. Fungal and mouse DNA quantities were obtained from the threshold cycle (*C_T*) values from an appropriate standard curve. Fungal burden was determined as the ratio between picograms of fungal and micrograms of mouse DNA.

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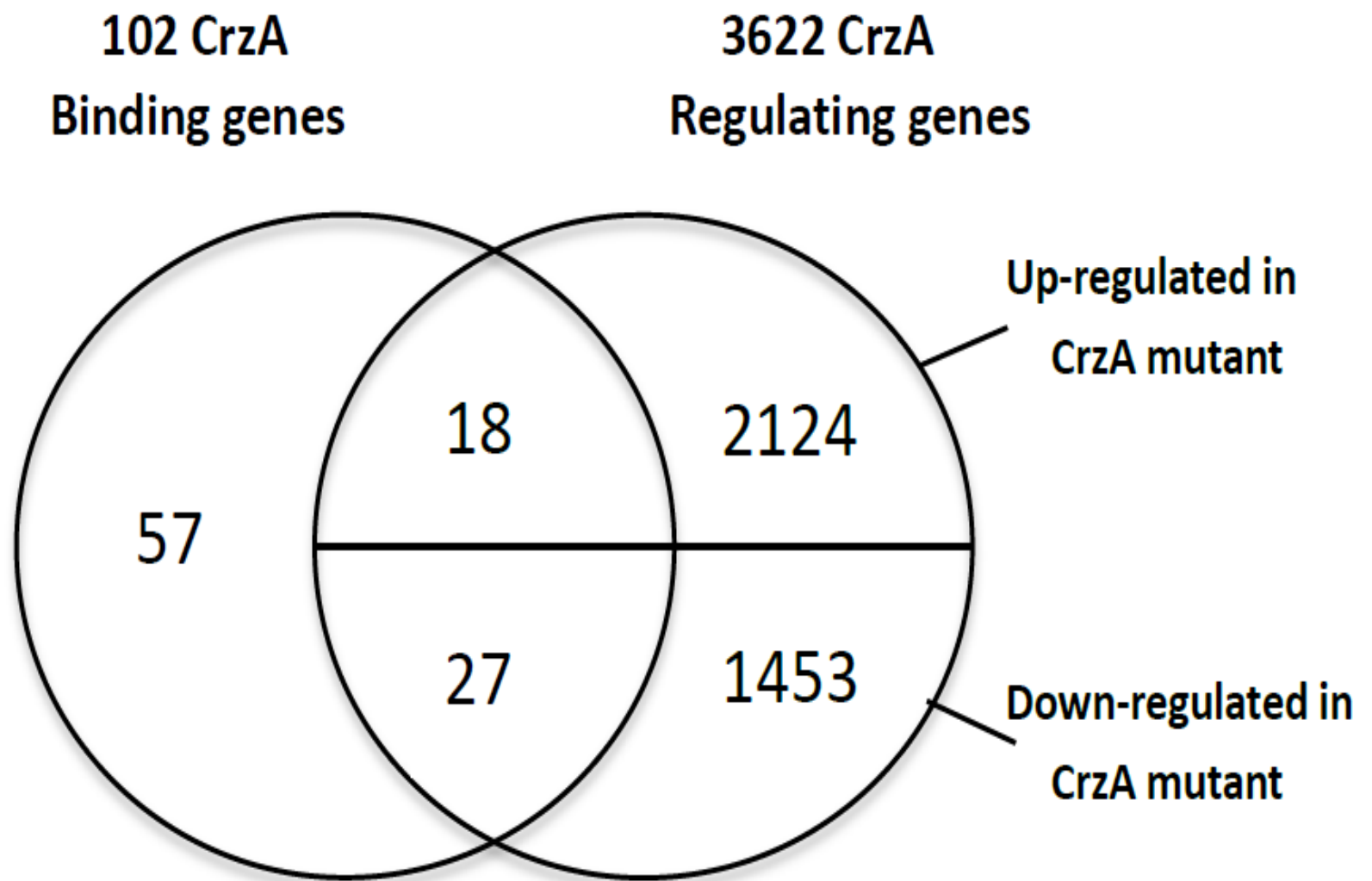
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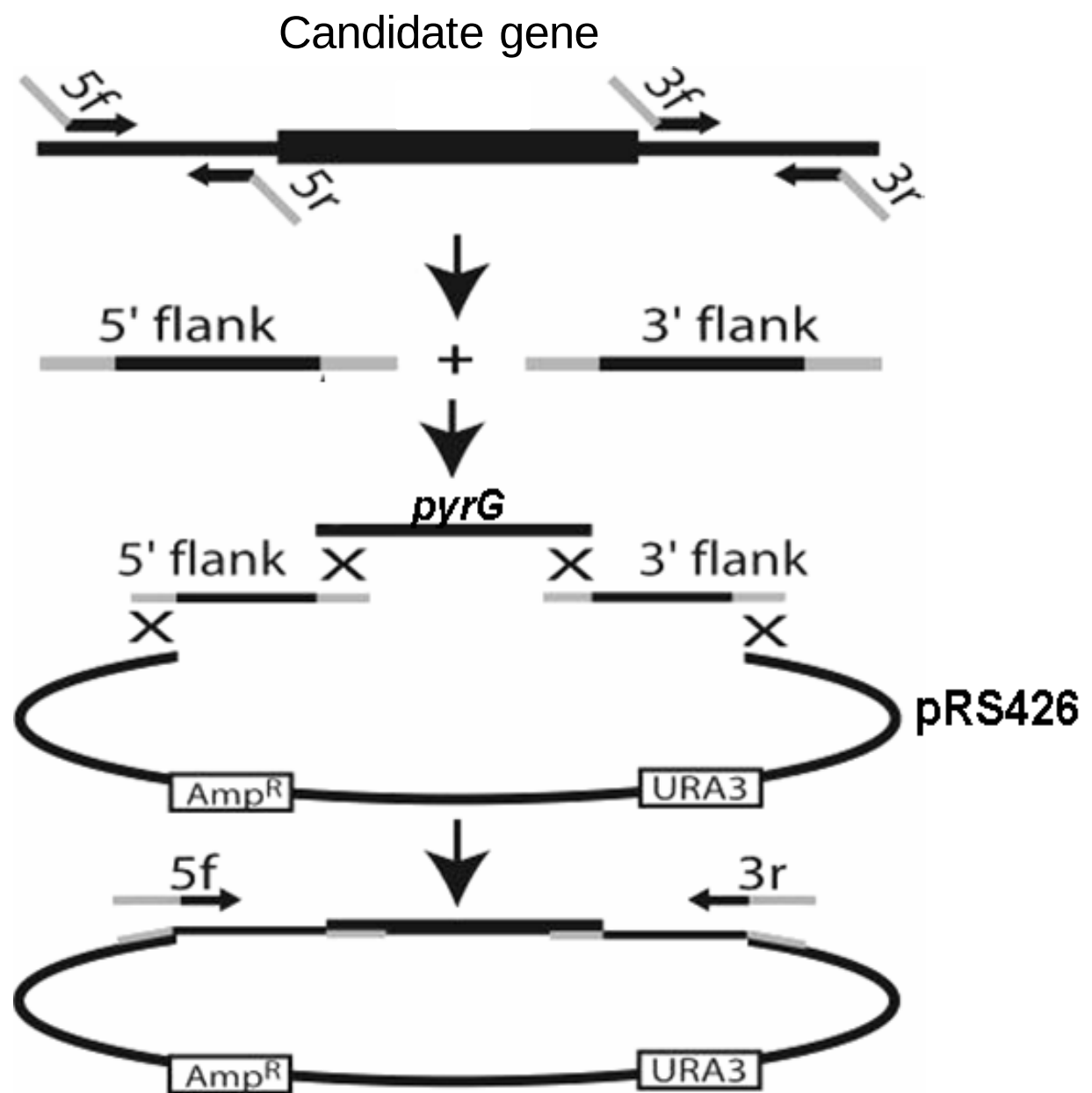
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Additional supporting information may be found in the online version of this article at the publisher's web-site.

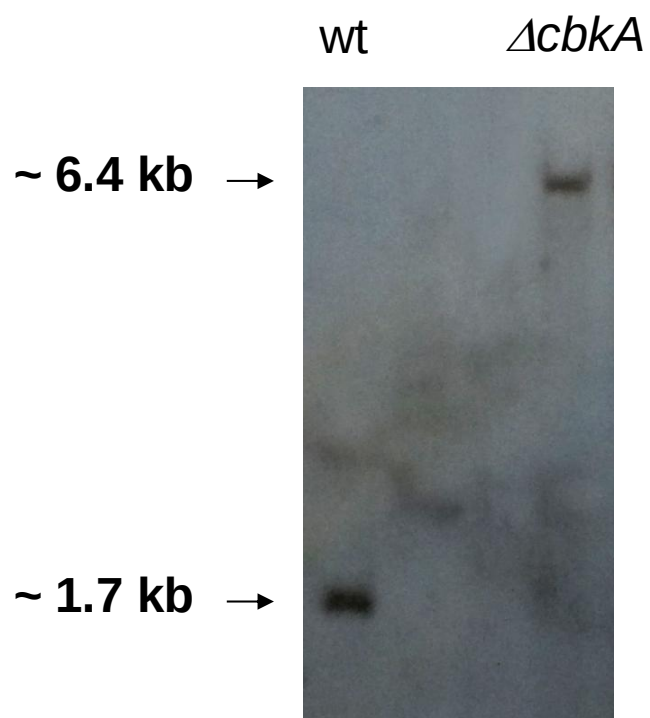
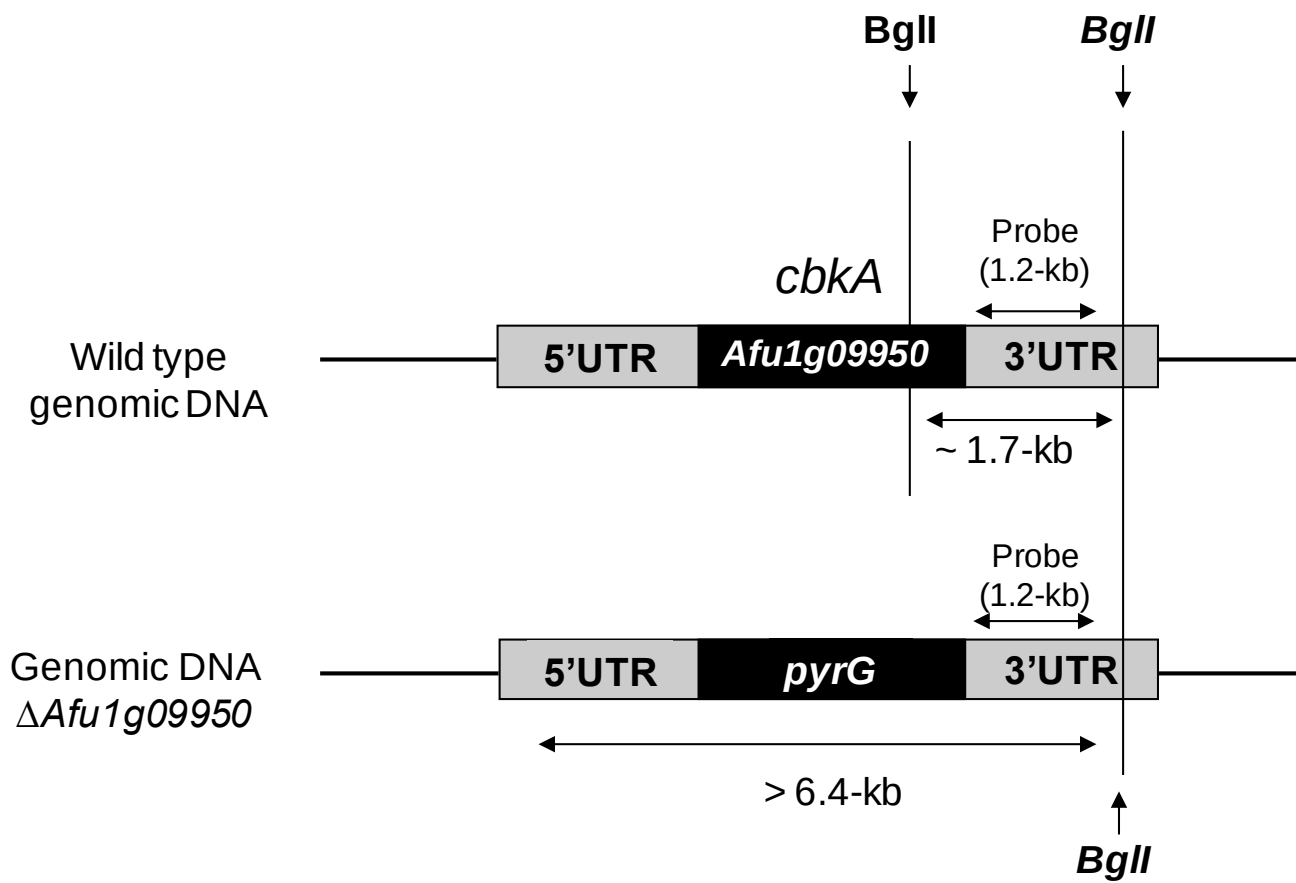


Supplementary Figure S1 – Venn diagram of the CrzA-binding and CrzA-regulated genes. Genes transcriptionally regulated by CrzA were categorized into those which were up-regulated or down-regulated in *crzA* mutant.

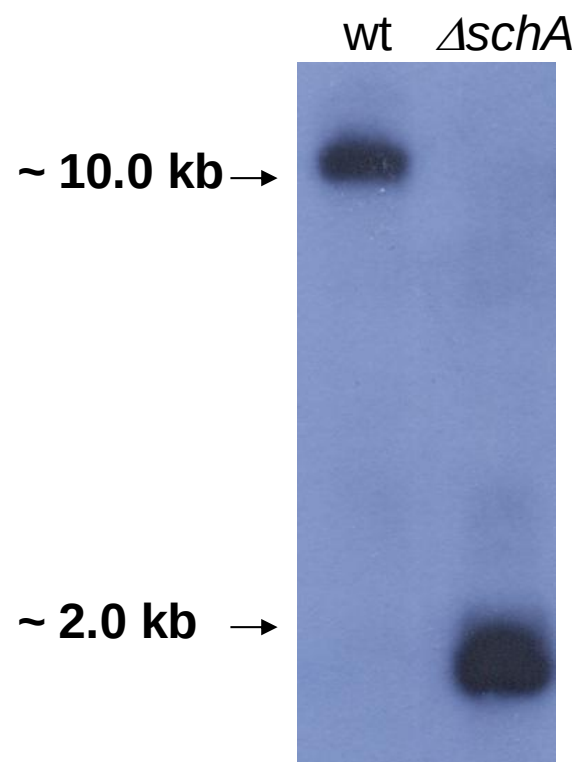
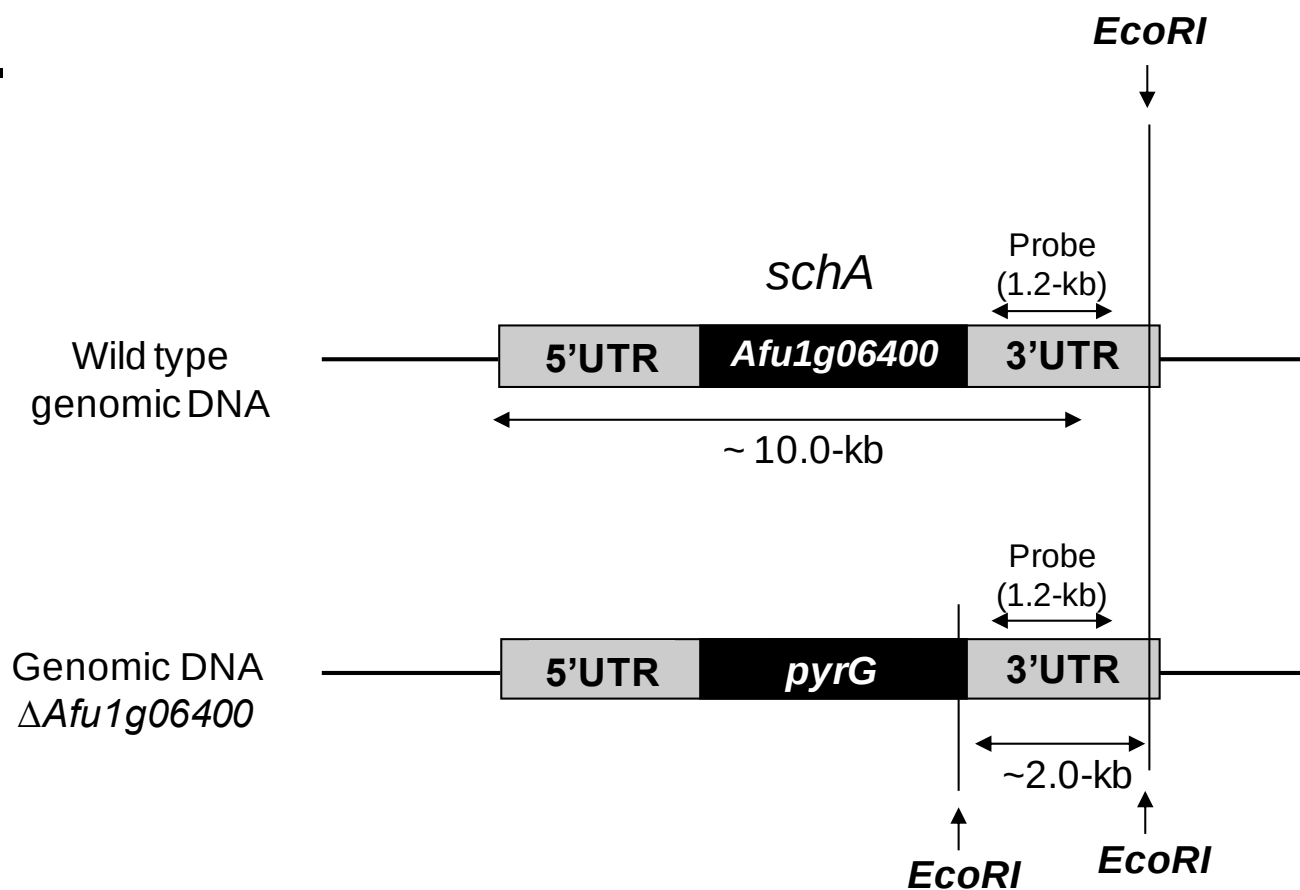
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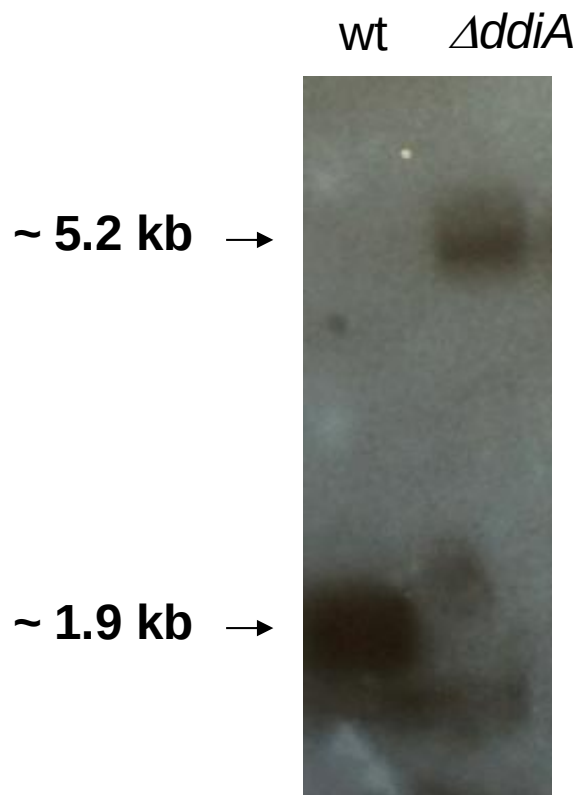
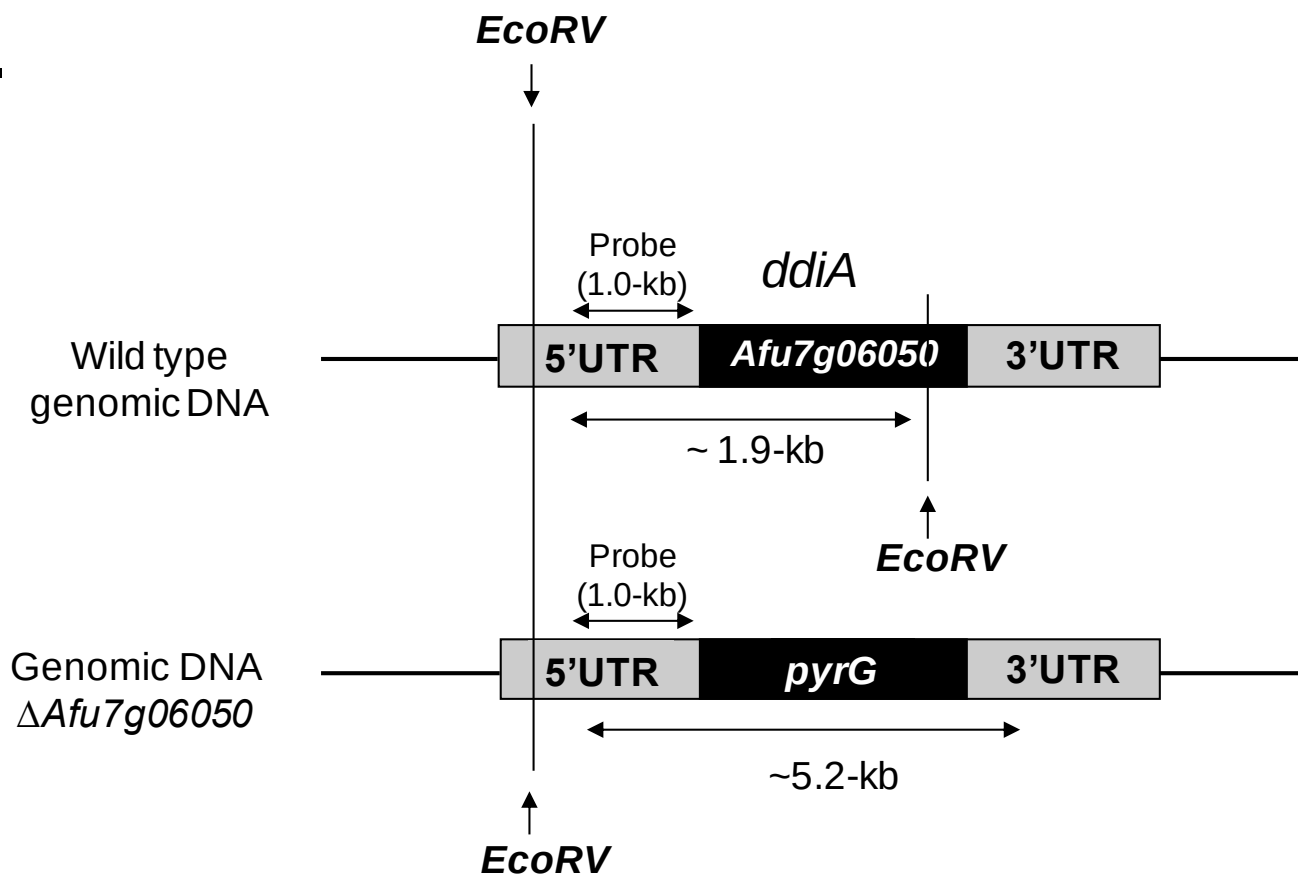
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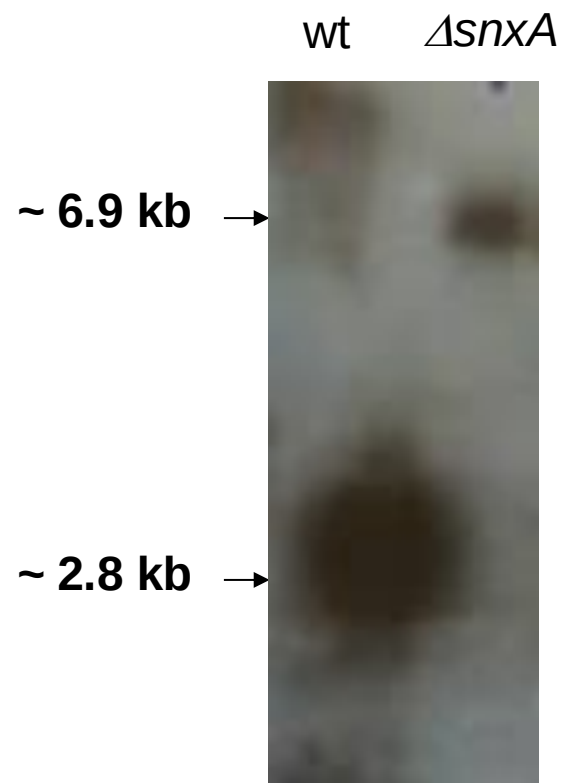
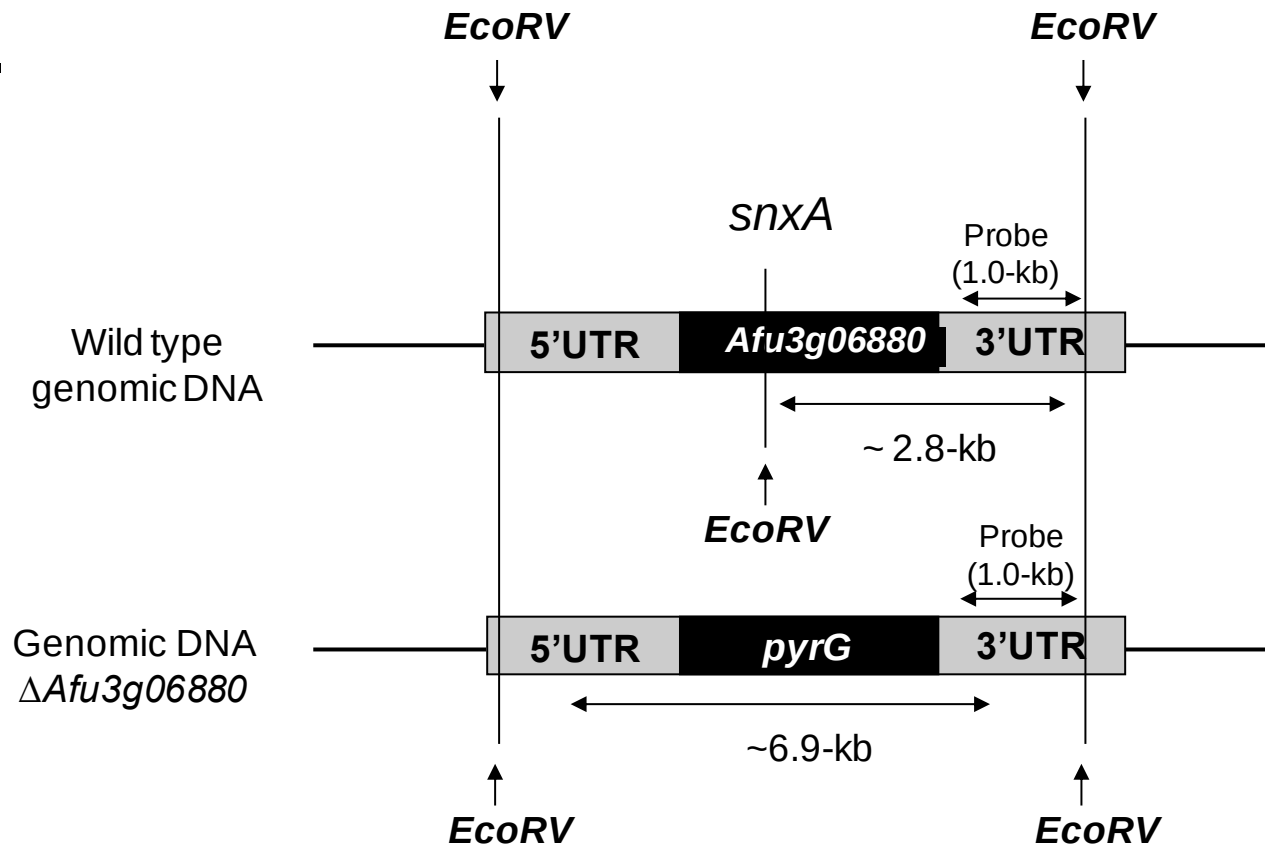
C.



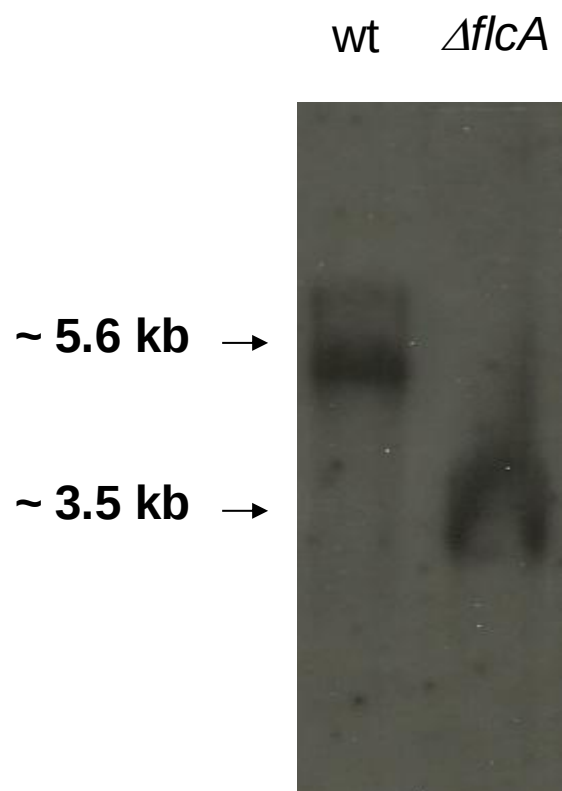
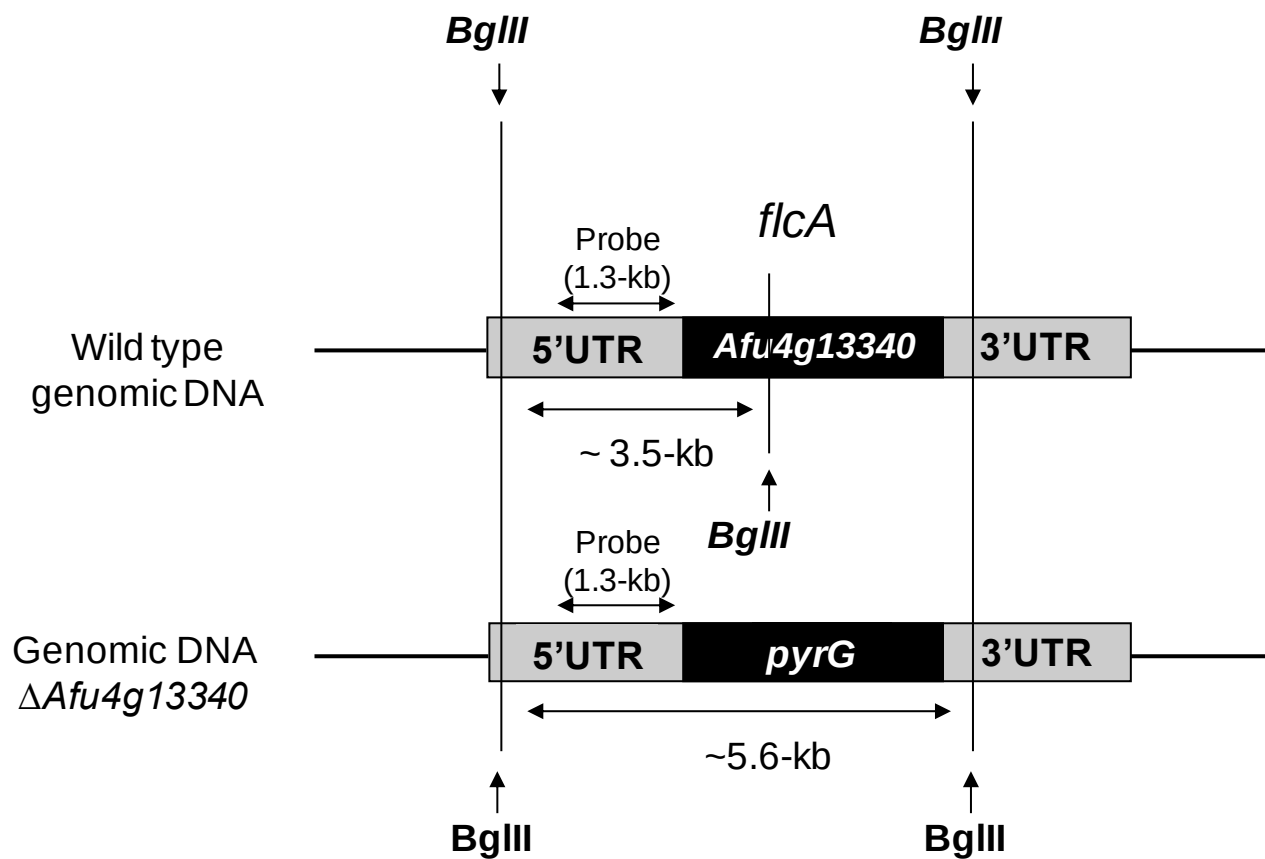
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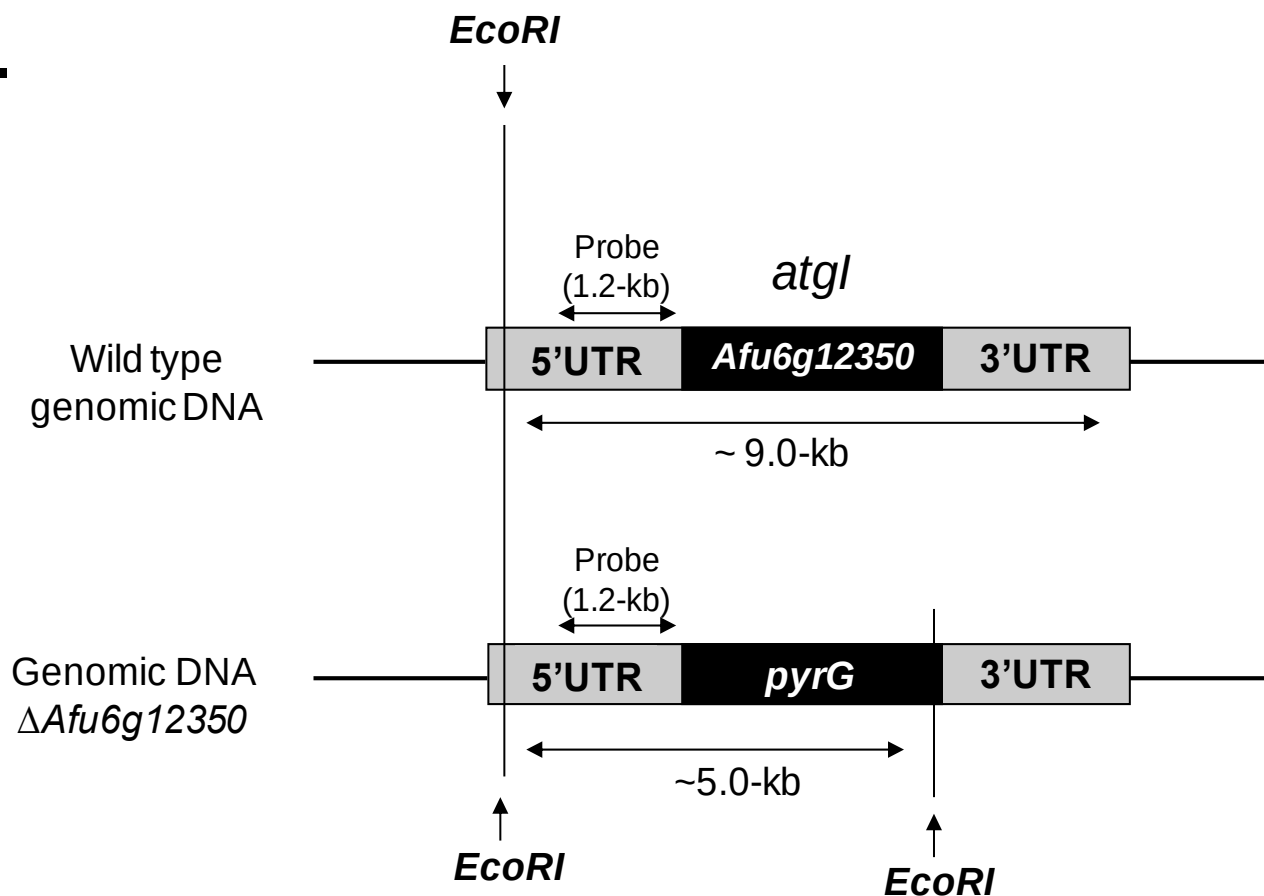
E.



F.



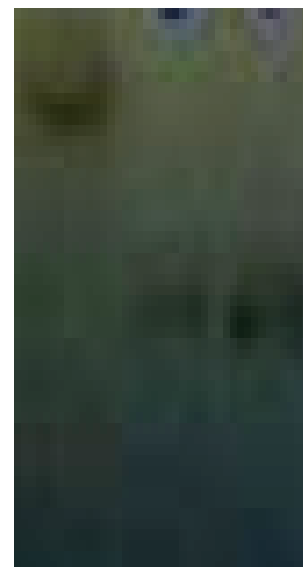
G.



wt Δ *atgl*

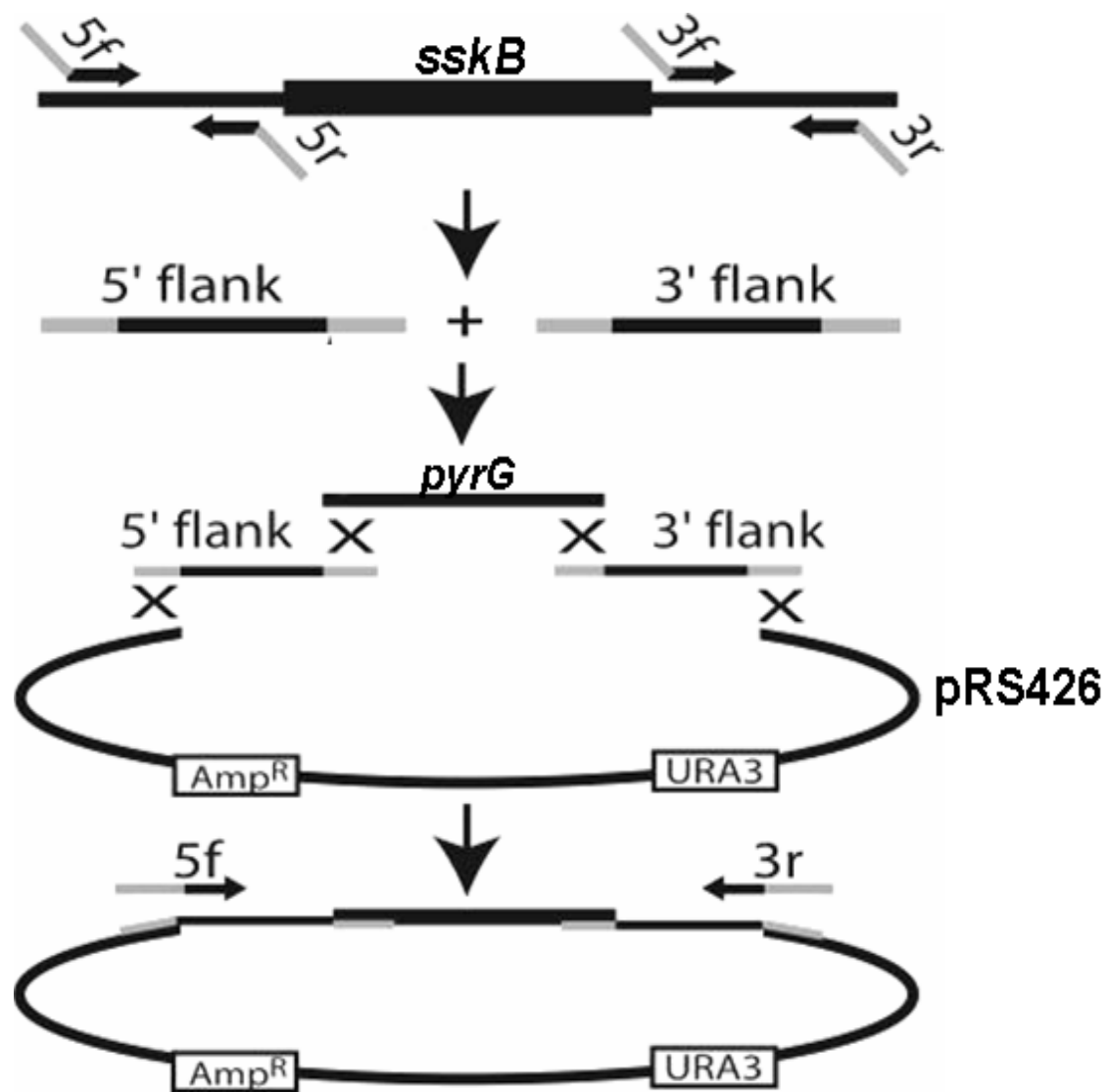
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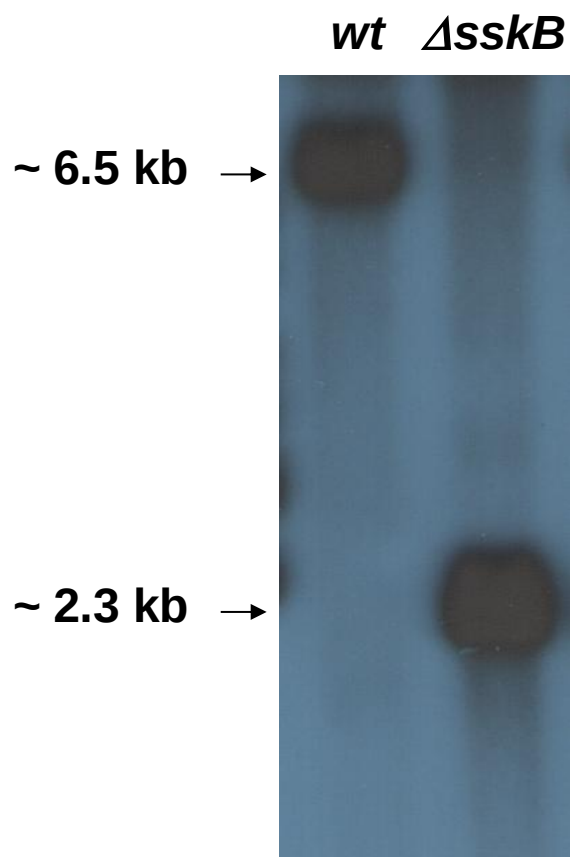
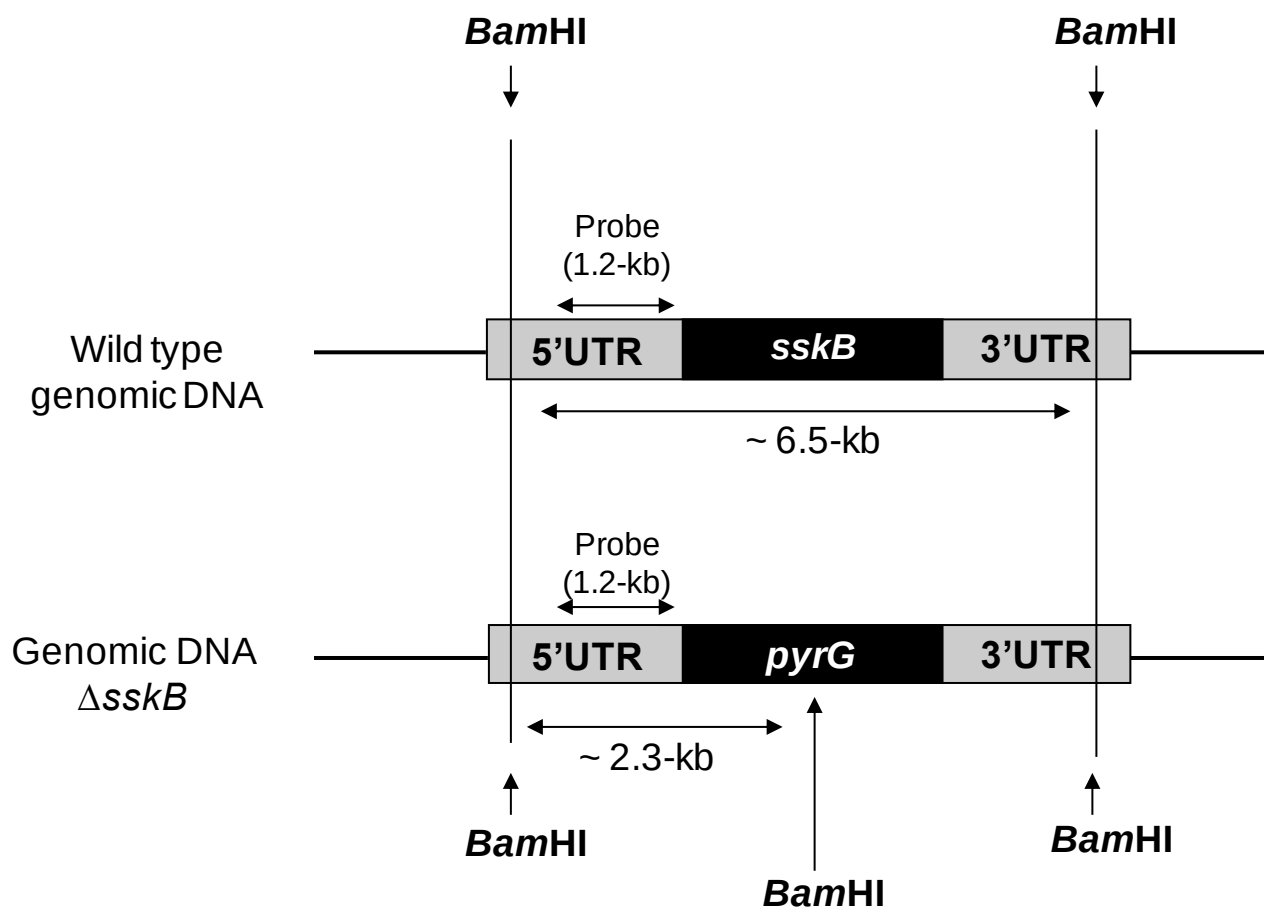


Supplementary Figure S2 – (A) Schematic representation of the deletion cassette assembly. (B–G) Southern blot analyses for all the deleted strains.

A.



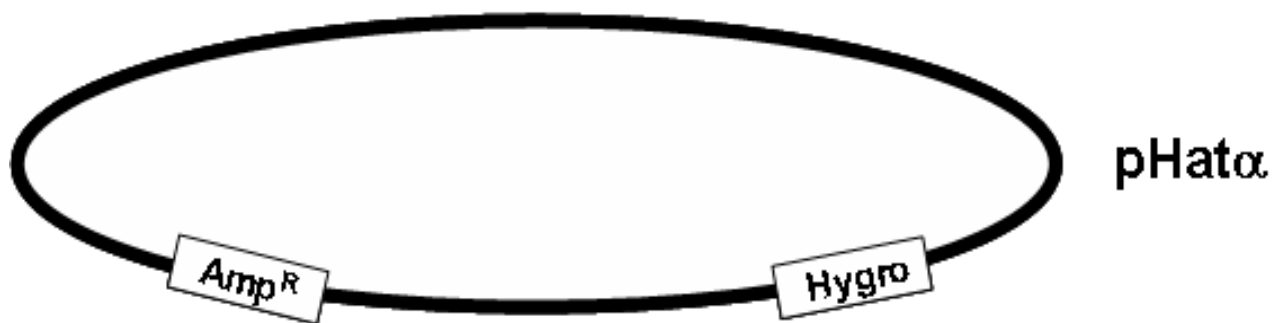
B.



C.

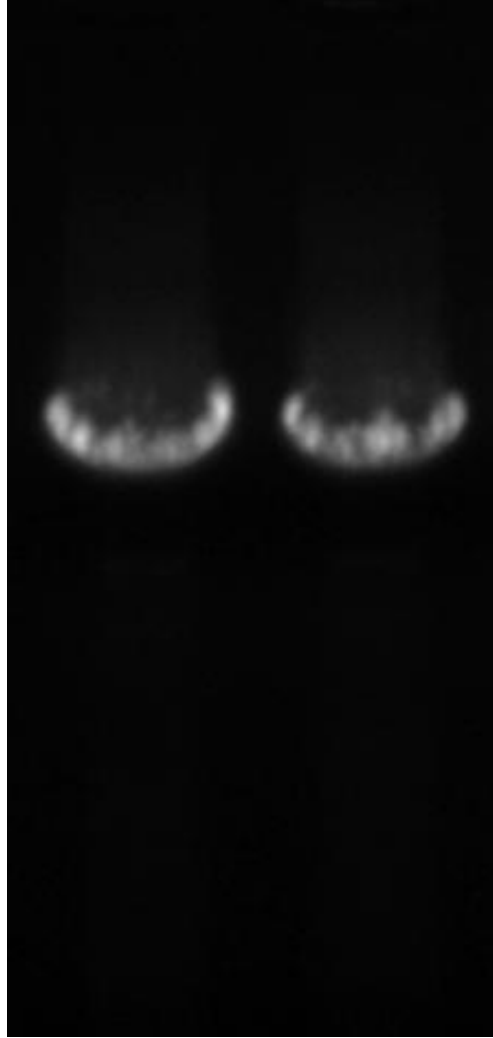


+



D.

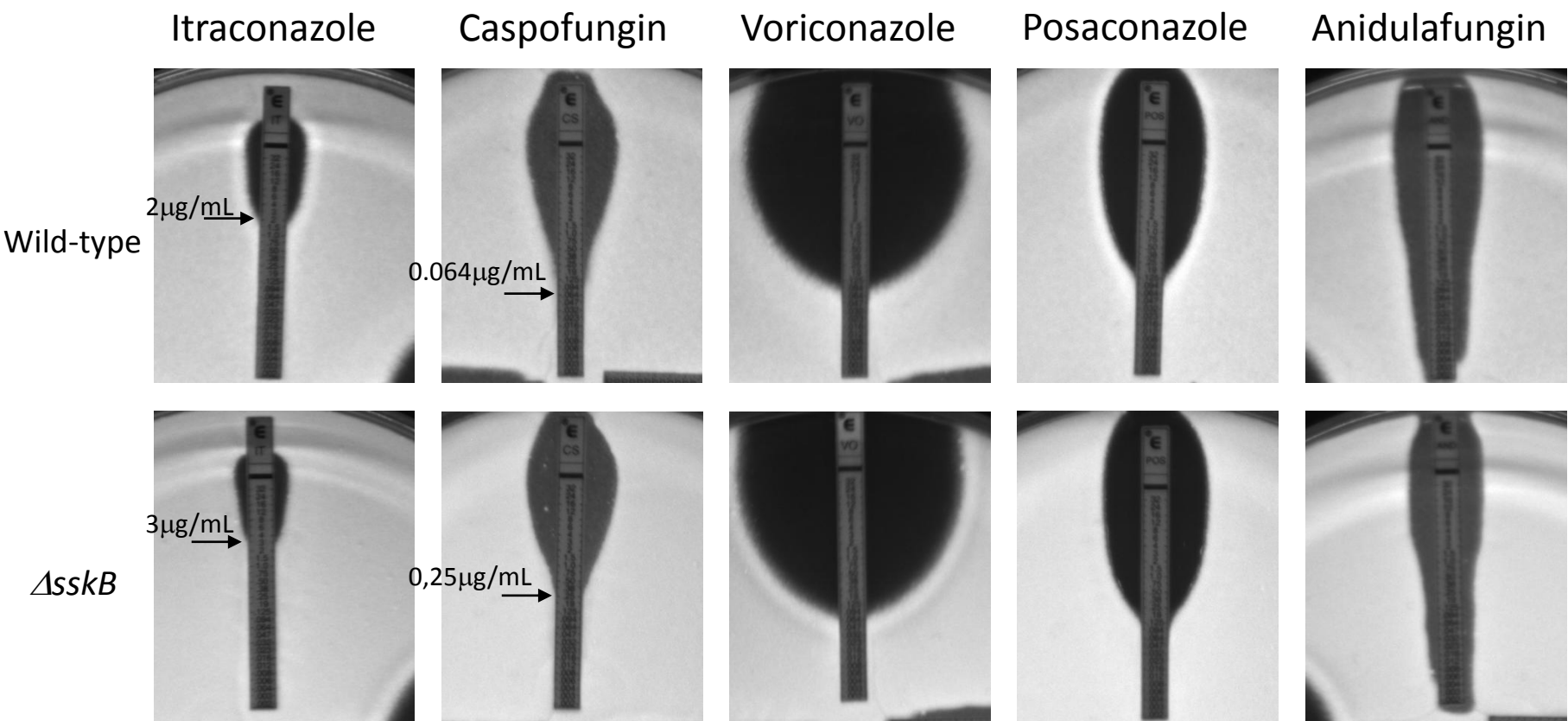
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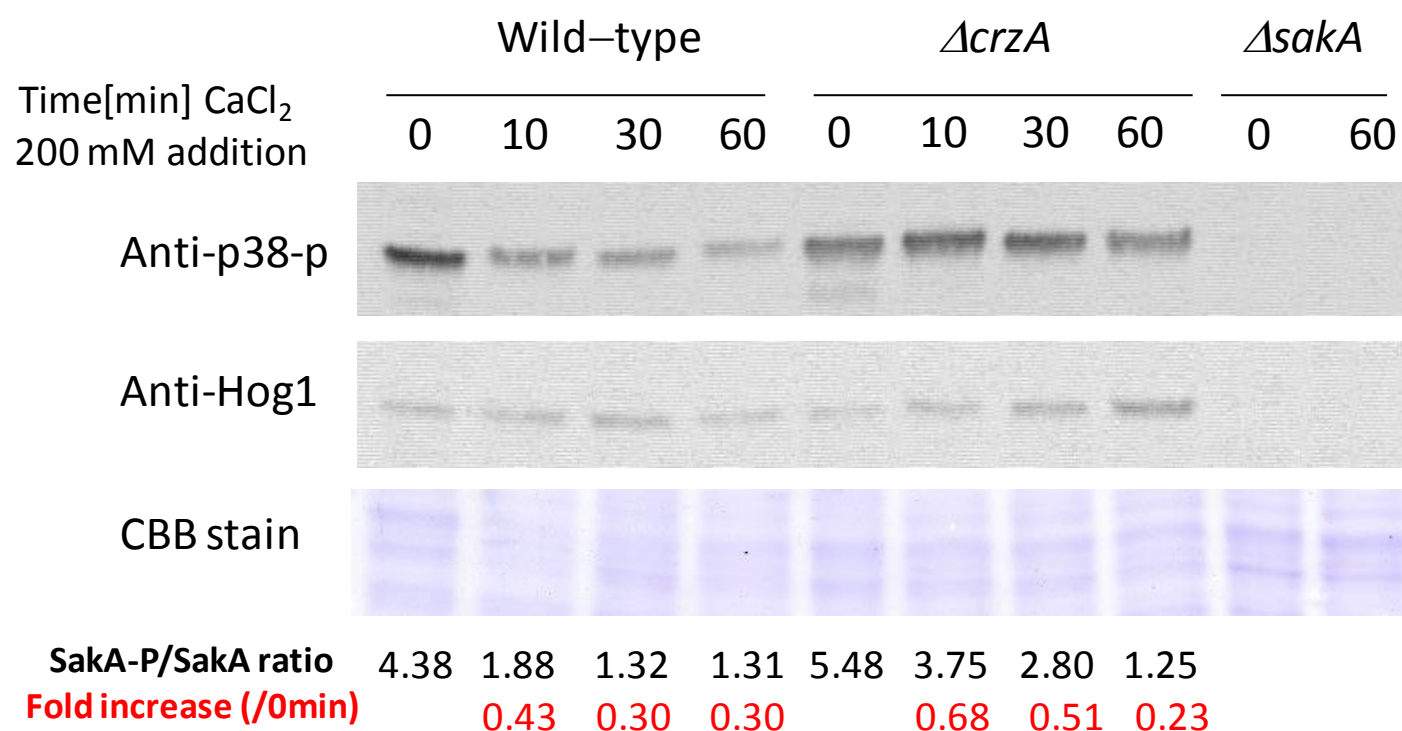
$\Delta sskB::sskB^+$

Wild-type

Supplementary Figure S3 – (A) Schematic representation of the deletion cassette assembly. Southern blot analysis for (B) $\Delta sskB$ (D) Schematic representation of the complementation assembly. (E) The complementation was confirmed by PCR amplifying of a 2kb fragment within the ORF.

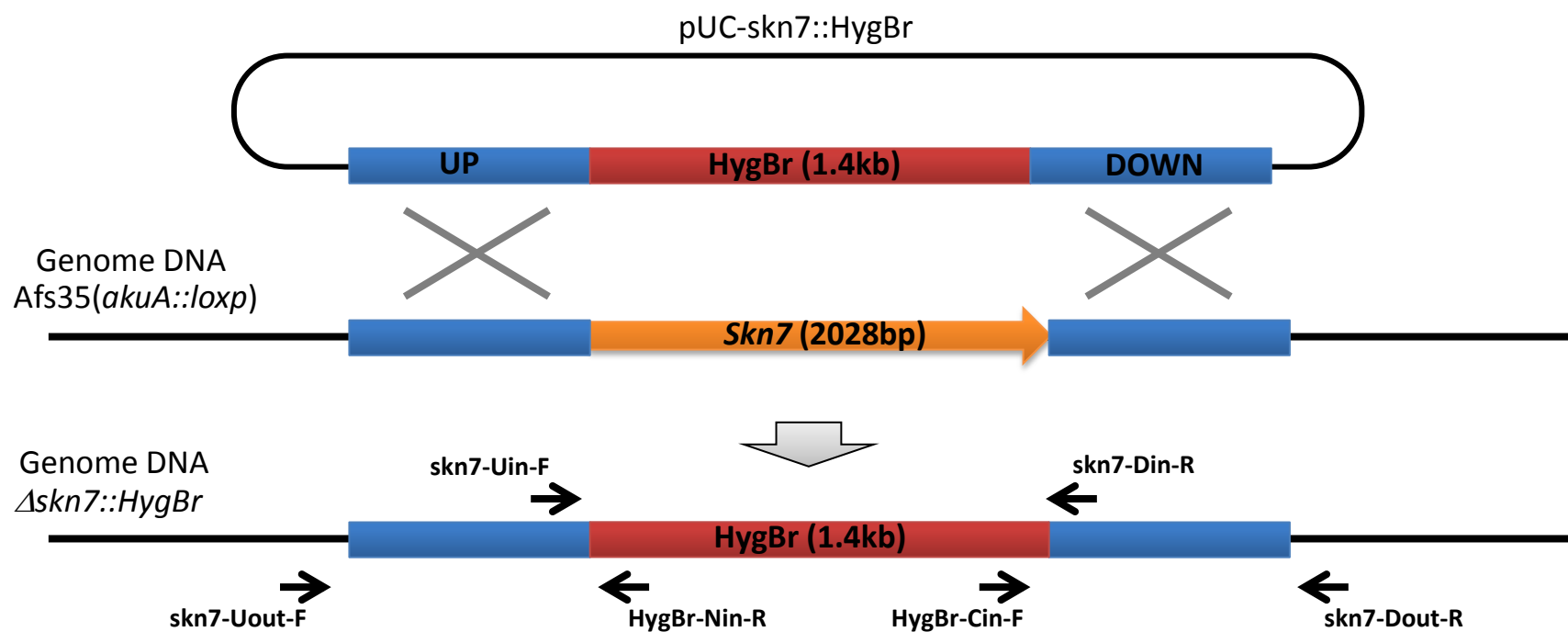


Supplementary Figure S4- Itraconazole, Caspofungin, Voriconazole, Posaconazole, Anidulafungin E-test for the wild-type and $\Delta sskB$ mutant.

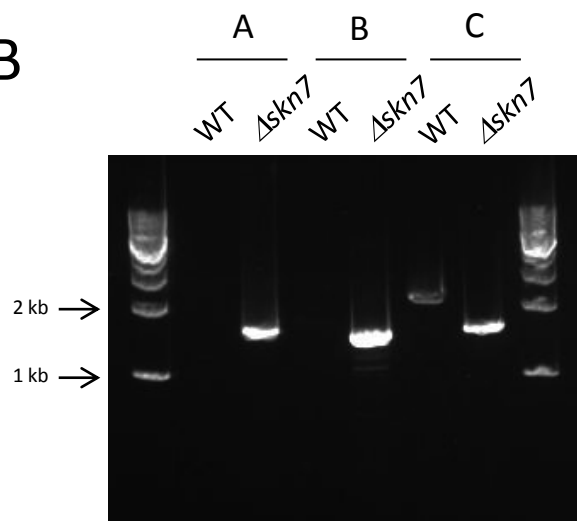


Supplementary Figure S5– Immunoblot analysis for phosphorylation of the SakA MAPK in response to calcium stress. The wild-type, $\Delta crzA$, and $\Delta sakA$ mutant strains were grown in YG medium for 18 h at 37°C. Then, CaCl₂ 200 mM was added for 0 to 60 minutes. The mycelium was harvested at the indicated times and total protein extracted. Anti-phospho-p38 was used to detect the phosphorylation of SakA, and anti-Hog1p was used to detect the total SakA protein. A Coomassie Brilliant Blue (CBB)-stained gel is shown as a loading control.

A



B

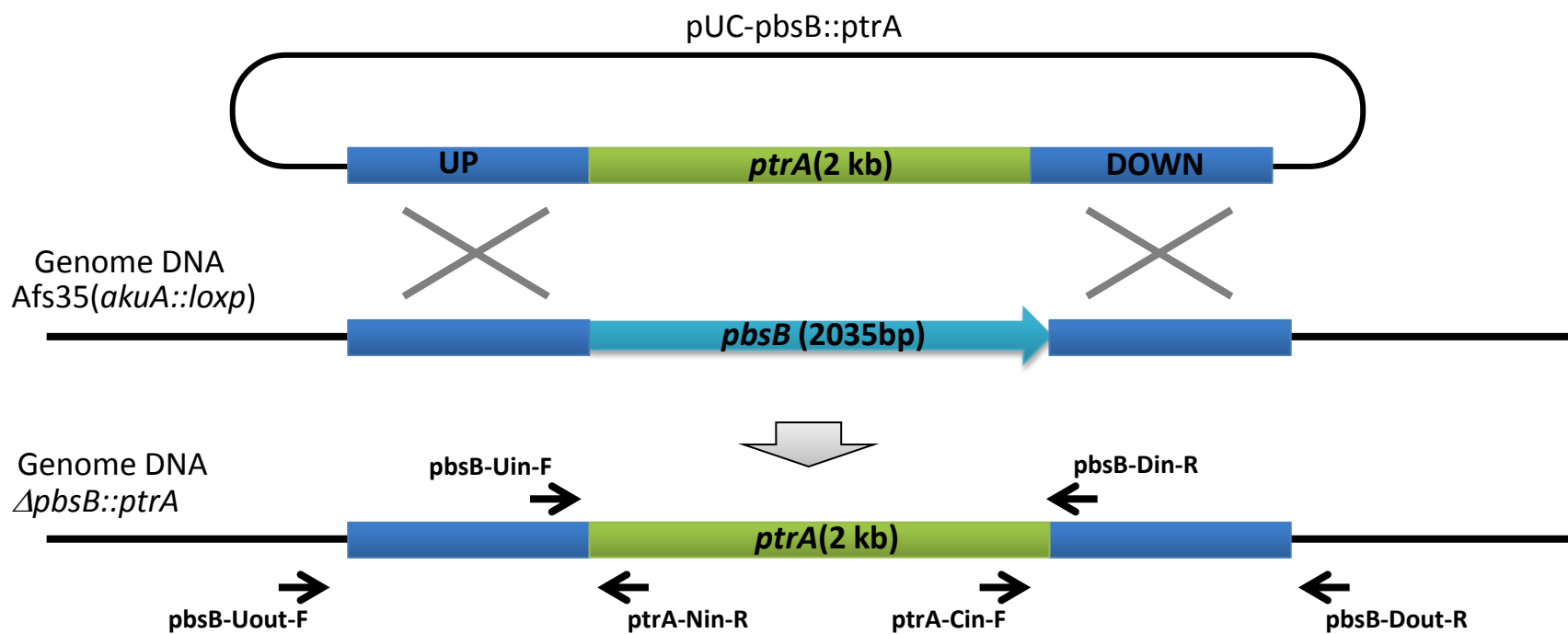


A: skn7-Uout-F/HygBr-Nin-R

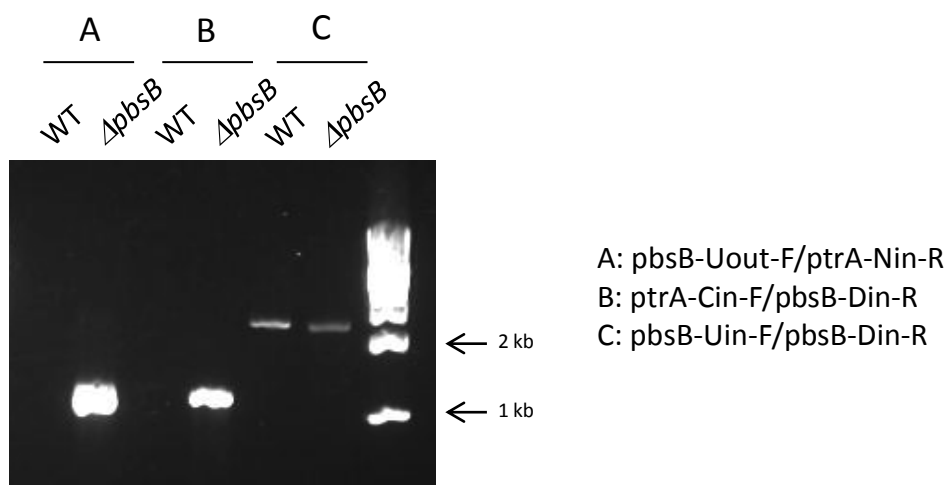
B: HygBr-Cin-F/skn7-Din-R

C: skn7-Uin-F/skn7-Din-R

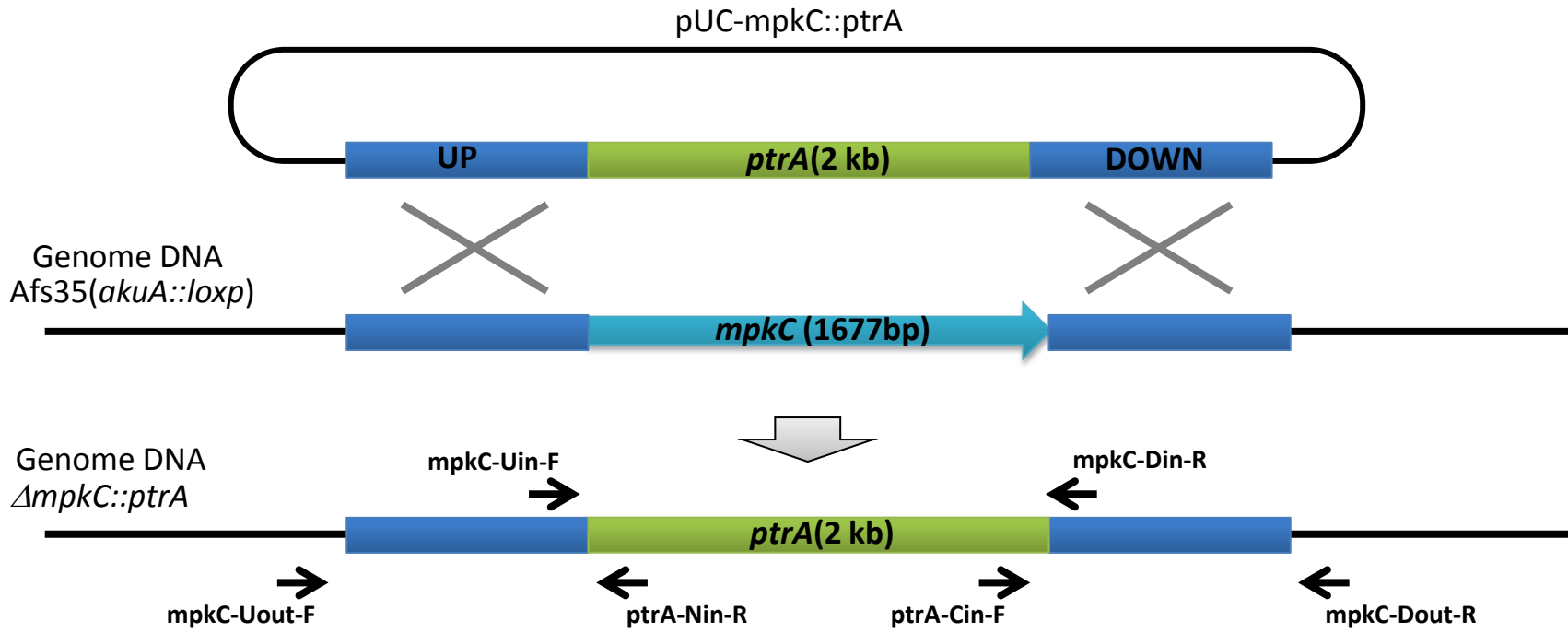
C



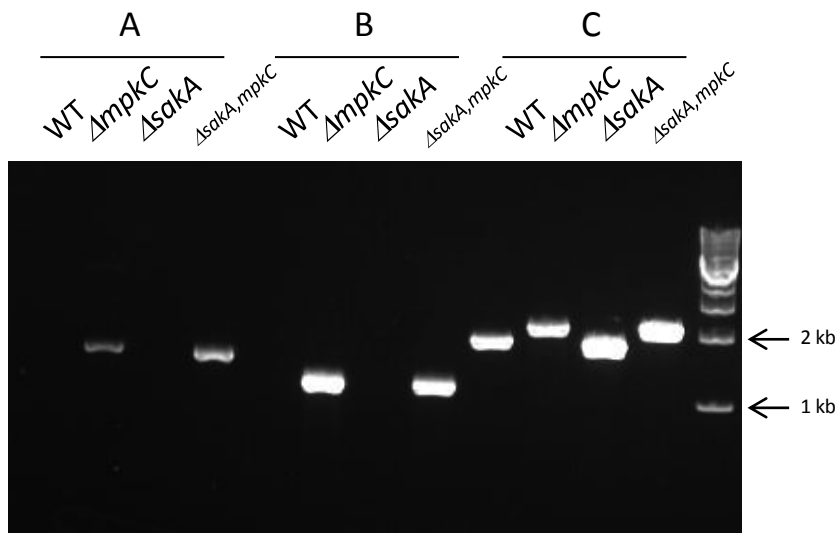
D



E



F



A: mpkC-Uout-F/ptrA-Nin-R

B: ptrA-Cin-F/mpkC-Din-R

C: mpkC-Uin-F/mpkC-Din-R

Supplementary Figure S6 - (A), (C), (E) Schematic representations of homologous recombination for replacing the target genes, *skn7* (A), *pbsB* (C), and *mpkC* (E), with a respective marker cassette. (B), (D), (F) Confirmation of the genome structure in respective transformants by PCR.

Supplementary Table S1 - ChIP-seq experimental design and description of the 6 libraries.

Library	Description	Group
TM3	Afu + Ca ²⁺ - no IP	1
TM4	Afu + Ca ²⁺ - IgG	
TM5	Afu + Ca ²⁺ - antiGFP	
TM6	Afu + Ca ²⁺ + FK506 - no IP	2
TM7	Afu + Ca ²⁺ + FK506 – IgG	
TM8	Afu + Ca ²⁺ + FK506 – antiGFP	

Supplementary Table S2 - Short reads alignment statistics.

Library	Aligned paired reads	Aligned single reads	Total pairs	Aligned percentage
TM3	1825085	4464988	7085327	57.27%
TM4	77145	95862	278801	44.86%
TM5	69543	144748	312954	45.35%
TM6	4613386	10646289	21723258	45.74%
TM7	61057	99744	239467	46.33%
TM8	183216	611480	1406681	34.75%

Supplementary Table S3 – The number of CrzA target genes identified and the location of their predicted binding regions.

Distance to TSS	Group 1	Group2
0-1kb upstream	50 genes	3 genes
1-2 kb upstream	30 genes	4 genes
2-3 kb upstream	19 genes	3 genes
3-4 kb upstream	6 genes	2 genes
Total	105 genes	12 genes

Supplementary Table S7 – Primers and probes used in this work.

Probe/Primer	Sequence
Afu1g10940_207FL	cggaaAGGGCAAGACCGTAGTTC?G
Afu1g10940_207FL/229RU	CGAGAGCAGCATCCTTGTGATT
Afu3g06880_230RL	cgggttATTACGTCGTCGCTGAAC?G
Afu3g06880_230RL/208FU	GAGAGTCACTATCCCGCCCTTG
Afu4g13340_178FL	cgggcCATCAACTTCGTCAATGC?G
Afu4g13340_178FL/197RU	CCTGGTTGGTTGAAGACCTCTG
Afu3g04240_361FL	cgggtcCAAAGACAATCTTTCACGAC?G
Afu3g04240_361FL/380RU	GCAGACAAAGGCGGAGGTATG
Afu3g09960_122RL	cggggGGGAGATTGTCTGAAGCC?G
Afu3g09960_122RL/73FU	TACTCCATGCCGGGTAATCCTG
Afu1g06400_390FL	cgggtgCAAGCTCAAGTCTGACAC?G
Afu1g06400_390FL/410RU	GTCAAGAGCGTTGGTGAACCTCG
Afu1g03210_159RL	cgggcTAGATGGGACTCAACTGC?G
Afu1g03210_159RL/129FU	CGAGCATTGAGACACGACGAG
Afu3g12530_451FL	cggggTAGGAATCAAGACATGGCC?G
Afu3g12530_451FL/485RU	CCGATGACCTCCTTCACCATC
Afu6g14100_159FL/182RU	CCGTCACGAGCCAGAGTCAT
Afu6g14100_159FL	cgggatTACAAGGAGGGTGGTATC?G
Afu3g14140_65FL/91RUa	GAAACCCAATCGCCGTCATC
Afu3g14140_65FL	cgtatgCAGCGCCATTAGCTCATA?G
Afu6g06640_45FL/65RU	GAGGGATGTGGTGCCGTGTT
Afu6g06640_45FL	cgaccTATATCCTCTCGTTTGTGGT?G
pRS426_5UTR Afu1g10940_F	GTAACGCCAGGGTTTTCCAGTCACGACGCCG TGGTTCATAATTCCCATTAT
5UTR Afu1g10940_pyrG_R	CGCATCAGTGCCTCCTCTCAGACAGAATACTT GCAACTAATAGCTAAGGCC
pyrG_3UTR Afu1g10940_F	GGTGAAGAGCATTGTTTGAGGCGAATTCACCA GCTATCCAGCCTTTTATTG
3UTR Afu1g10940_pRS426_R	GCGGTTAACAATTTCTCTCTGGAAACAGCGAG CGATTGTCCTCTTGGAGAGA
HygBr-FW	TTTTTGGGCTTGGCTGGAGCTAGTGG
HygBr-RV	TGGTTCCCGGTCGGCATCTACTCTATTC
ptrA-F	GGGGATCTGACAGACGGGCAATTG
ptrA-R	GCTAAGGCCCAACCCACCGCCG
skn7-U-F(pUC119B)	CAGGTCGACTCTAGAGCTCCTTGGGTCTATGT

Supplementary Text S1 – The promoter regions of the genes, Afu1g09950 and Afu3g06880, used for EMSA analysis. Nucleotides highlighted in yellow represent the probes used for both genes, while red represents the CDRE motif.

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