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Two zinc finger transcription factors, CrzA and SltA, are involved in cation homeostasis and detoxification in *Aspergillus nidulans*

Running title: Cation response transcription factors in *Aspergillus nidulans*

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Abbreviations used: AFP, antifungal protein; CDD, calcineurin docking domain; CDRE, calcineurin-dependent response element; DTT, dithiothreitol; GFP, green fluorescent protein; ORF, open reading frame; WMM, watch minimal medium;

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Abstract

To investigate cation adaptation and homeostasis in *Aspergillus nidulans*, two transcription factor-encoding genes have been characterised. The *A. nidulans* orthologue *crzA* of the *Saccharomyces cerevisiae* *CRZ1* gene, encoding a transcription factor mediating gene regulation by Ca^{2+} , has been identified and deleted. The *crzA* deletion phenotype includes extreme sensitivity to alkaline pH, Ca^{2+} toxicity and aberrant morphology connected with alterations of cell wall-related phenotypes such as reduced expression of a chitin synthase gene, *chsB*. A fully functional C-terminally GFP-tagged form of the CrzA protein is apparently excluded from nuclei in the absence of added Ca^{2+} but rapidly accumulates in nuclei upon exposure to Ca^{2+} . In addition, the previously identified *sltA* gene, having no identifiable homologues in yeasts, was deleted and the resulting phenotype includes considerably enhanced toxicity by a number of cations other than Ca^{2+} and also by alkaline pH. Reduced expression of a homologue of the *S. cerevisiae* P-type ATPase Na^+ pump gene *ENAI* might partly explain cation sensitivity of null *sltA* strains. Up-regulation of the homologue of the *S. cerevisiae* vacuolar $\text{Ca}^{2+}/\text{H}^+$ exchanger gene *VCXI* might explain the lack of Ca^{2+} toxicity to null *sltA* mutants whereas down-regulation of this gene might be responsible for Ca^{2+} toxicity to null *crzA* mutants. Both *crzA* and *sltA* encode DNA-binding proteins and the latter exerts both positive and negative gene regulation.

19
20

Key words:

Aspergillus nidulans, nuclear localisation, calcium signalling, cation homeostasis, cell wall integrity, transcription factors

23

1 Introduction

2
3 Rapid adaptation to different environmental conditions, including appropriate adaptive
4 responses to various cations, is crucial for the survival and proliferation of microorganisms.
5 The Ca^{2+} /calcineurin/Crz1p signalling pathway is necessary for adaptation to diverse
6 environmental conditions including high cation levels and alkaline pH, and considerable
7 characterization of it has been achieved in the yeasts *Saccharomyces cerevisiae*,
8 *Schizosaccharomyces pombe*, *Torulaspota delbrueckii* and *Candida albicans* [1-4]. Sudden
9 exposure of *S. cerevisiae* to these stress conditions induces a transient increase in cytosolic
10 Ca^{2+} levels which triggers activation of the phosphatase calcineurin through binding of the
11 Ca^{2+} -binding protein calmodulin. *S. cerevisiae* calcineurin, like that of mammals is composed
12 of both catalytic and regulatory subunits [5]. Activated calcineurin mediates
13 dephosphorylation of the cytoplasmically localised transcription factor Crz1p, resulting in its
14 translocation to the nucleus ([6]; reviewed in [3]).

15 The nuclear localised zinc finger Crz1p transcription factor then activates expression
16 of genes whose promoters contain physiologically relevant calcineurin-dependent response
17 elements (CDREs) [7] 5'-GNGGC(G/T)CA-3, and up-regulates expression of various target
18 genes, including some encoding cation transporters acting at the plasma membrane or other
19 membranous organelles [8-10]. Previously we found that alkaline pH-induced up-regulation
20 of the *Aspergillus giganteus* *afp* gene encoding the antifungal protein can be prevented by
21 FK506, an inhibitor of calcineurin [11]. To pursue *afp* regulation in the context of cation
22 adaptation and homeostasis, we have chosen to investigate calcium-mediated signalling in
23 *Aspergillus nidulans*, an experimentally more amenable species than *A. giganteus*.

24 Calcium-mediated signalling has been less investigated in filamentous fungi than in
25 yeasts although some notable work was done on calcineurin (reviewed in [12]) which has
26 been revisited recently [13]. It was initially reported that the *A. nidulans* gene encoding the
27 calcineurin catalytic subunit is essential and required for cell cycle progression [14;15].
28 However, very recently an *A. nidulans* ΔcnaA , deleted for the calcineurin catalytic subunit,
29 mutant strain was found to be viable and display a phenotype similar to that observed for the
30 corresponding *Aspergillus fumigatus* (ΔcalA) mutant strain, which has severe defects in
31 growth extension, branching and conidiophore development [13]. In addition to having
32 abnormal morphogenesis, the *A. fumigatus* ΔcalA mutant is also impaired in virulence
33 [16;17]. In *Aspergillus oryzae* calcineurin is required for adaptation to elevated temperature,
34 high Na^+ levels and alkalisation [18]. Crz1p orthologues have been recently characterised in
35 the filamentous fungi *A. fumigatus* and *Botrytis cinerea*, where they play major roles in
36 tolerance to certain cations [13;19].

37 Another transcription factor important for cation adaptation and homeostasis is SltA.
38 An *A. nidulans* mutation, designated *sltA1*, resulting in toxicity by high levels of Na^+ and K^+
39 was described a number of years ago [20]. Subsequently, cloning of the *sltA* gene by
40 complementation of the *sltA1* mutation showed that it encodes a protein containing three
41 Cys₂His₂-type zinc fingers and exhibiting significant similarity to the Ace1 transcriptional
42 repressor of cellulase and xylanase genes in *Hypocrea jecorinia* (*Trichoderma reesei*) [21].
43 Although O'Neil et al (2002) showed that the *sltA1* mutation results from a premature stop
44 codon just downstream of the third zinc finger of the predicted protein, they did not
45 investigate the role of the SltA protein in cation tolerance, nor did they determine whether
46 *sltA1* has a null phenotype.

47 Here we characterise aspects of the role of the *A. nidulans* transcription factors CrzA,
48 a homologue of *S. cerevisiae* Crz1p, along with SltA, which has no identifiable *S. cerevisiae*
49 homologue, but, like CrzA, is involved in cation adaptation and homeostasis. We show that
50 both CrzA and SltA are capable of binding DNA and that SltA acts positively on transcription
51 of the Ena1p-like sodium pump *enaA* and negatively on transcription of the putative vacuolar

1 $\text{Ca}^{2+}/\text{H}^{+}$ exchanger *vcxA*. The negative regulation of *vcxA* by SltA is opposed by its
2 transcriptional activation by CrzA and the positive regulation by CrzA is the over-riding
3 factor as *vcxA* mRNA levels in a mutant lacking both of the transcription factors are
4 indistinguishable from those in a mutant lacking only CrzA. Exposure to calcium elicits
5 nuclear localization of CrzA.

Stage 2(a) POST-PRINT

1 Experimental

2 *Strains, growth conditions, molecular techniques and phenotype analysis*

3 *A. nidulans* strains used are listed in Table 1. *Escherichia coli* strains DH5 α (Gibco BRL) and
4 XL10-Gold $\text{\textcircled{R}}$ (Stratagene) were used as hosts for the maintenance of the plasmids listed in
5 Supplementary Table 1. General cloning procedures in *E. coli* were followed. Standard
6 growth media for *A. nidulans*, genetic techniques and phenotype testing followed those
7 described previously ([22] and references therein). Gene symbols are described in [23].
8 Reversion alleles of *sltA1* were selected in a UV-mutagenised strain of genotype *halA24*
9 *inoB2 sltA1* as enabling growth on appropriately supplemented complete medium containing
10 (final concentration) 1 M NaH $_2$ PO $_4$, and their phenotypes were determined in a selection of
11 outcrossed strains. *A. nidulans* transformation followed ([22] and references therein). *A.*
12 *nidulans* total RNA and genomic DNA preparation as well as Northern and Southern analyses
13 followed standard procedures.
14

15 *Identification of the crzA gene*

16 The CrzA homologue in the *A. nidulans* genome was identified by Blastp searches using the
17 *S. cerevisiae* Crz1p sequence. On the basis of sequence conservation and apparently similar
18 function, AN5726 was named *crzA*. The position of the predicted intron within the *crzA* gene
19 was verified by RT-PCR and is in agreement with GenBank entry AB259568. Strain J734 was
20 grown in liquid minimal medium and exposed for two hours to 1 M NaCl after which total
21 RNA was obtained. Total RNA treated with DNase I (FPLC pure, Amersham Biosciences)
22 was used as template for the RT-PCR reaction. First strand synthesis used primer CrzA-9 rev
23 (Supplementary Table 2) and ArrayScript TM Reverse Transcriptase (Ambion). The following
24 PCR performed with primers CrzA-8 fw and CrzA-9 rev yielded full length *crzA* cDNA
25 which was cloned into plasmid pGEM-T-Easy (Promega) giving plasmid pGEM-T-
26 CrzAcDNA. The insert was subsequently sequenced using primer walking.
27

28 *Deletion of crzA*

29 The *crzA* deletion cassette was constructed as gene replacement cassette comprising the *pyr-4*
30 gene of *Neurospora crassa* as selection marker flanked by 1.5 kb of 5' and 3' untranslated
31 regions of the *crzA* gene. The 5' untranslated region of *crzA* was amplified by PCR using
32 genomic DNA from strain J734 and primers crznulo-1 and crznulo-2, which introduced a *NotI*
33 and a *BamHI* restriction site, respectively. The resulting PCR fragment was digested with *NotI*
34 and *BamHI* and inserted into pBluescriptSK(+) (Stratagene) giving plasmid pBSKpromCrzA.
35 The *pyr-4* selection marker obtained from plasmid pFB6 [22] by *BglIII* digestion was inserted
36 into *BamHI*-linearized pBSKpromCrzA yielding plasmid pBSKpromCrzApyr4-fw. Correct
37 orientation of the *pyr-4* gene was verified by PCR and restriction analysis. The 3' untranslated
38 region of *crzA* was amplified by PCR using genomic DNA from strain J734 and primers
39 crznulo-3 and crznulo-4. The resulting PCR fragment was inserted into pGEM-T-Easy and
40 excised using *EcoRI* digestion. The *EcoRI* fragment was inserted in the correct orientation
41 into pBSKpromCrzApyr4-fw via the *EcoRI* restriction site. The final plasmid harbouring the
42 6.2 kb *crzA* deletion cassette was named pBSKdeltaCrzA. This cassette was amplified by
43 PCR using primers crzAnulo1 and crzAnulo-4 and used to transform *A. nidulans* strain
44 MAD1425. Pyrimidine prototrophic transformants were screened for the *crzA* deletion by
45 Southern and PCR analysis and strain BER02 was selected for further work.
46

47 *Deletion of sltA*

48 A cassette comprising the *A. fumigatus riboB* gene fused to the upstream and downstream
49 untranslated regions (UTRs) of *sltA* was used for transformation to delete the *sltA* coding
50 region. A 1.5kb 5'-UTR region of *sltA* was amplified using primers sltnul1 and sltnul2, which
51

1 included a *NotI* and a *BamHI* restriction site, respectively. The PCR fragment was digested
2 with *NotI* and *BamHI* and cloned into the same restriction sites in the pBluescriptSK(+)
3 (Stratagene) plasmid to give pBS-SltA5'. A 1.5kb 3'-UTR region of *sltA* was amplified using
4 primers sltnul3 (inserting an *EcoRI* restriction site) and sltnul4 (carrying a *XhoI* site). The
5 PCR fragment was digested with *EcoRI* and *XhoI*, and ligated to pBS-SltA5' to give pBS-
6 SltA5'3'. The *EcoRI* 1.9 kb fragment containing the *A. fumigatus riboB* coding region plus 5'
7 and 3' UTRs was obtained from plasmid p1548, and ligated to plasmid pBS-SltA5'3' digested
8 with *EcoRI* to give pBSdeltaSltA. pBSdeltaSltA was digested with *NotI* and *XhoI* to yield the
9 deletion cassette. The *sltA* null strain was made using MAD1427 as recipient strain selecting
10 for transformants able to grow in the absence of riboflavin. Riboflavin prototrophs were
11 analysed by Southern blot and one transformant carrying the desired insertion was used for
12 further work.

13

14 **GFP tagging of CrzA**

15 Construction of an *A. nidulans* strain expressing CrzA::GFP followed the methodology
16 described in [24], taking advantage of recently available strains deficient in heterologous
17 recombination [25]. 1.5 kb from the most 3' end of the gene and the 3'-UTR region were
18 amplified with primers pairs CrzA1/CrzA2 and CrzA3/CrzA4, respectively, (see
19 Supplementary Table 2) and fused in a 3-way PCR with a fragment, amplified with primers
20 CrzA-GFP1 and CrzA-GFP2, containing the *gfp* gene together with the selectable *pyrG* gene
21 of *A. fumigatus* [24]. The fusion cassette was transformed into MAD1732 following the
22 protocol described in ([22] and references therein). Pyrimidine prototrophs were selected and
23 homokaryotic transformants were analysed by Southern blot for single copy integration of the
24 gene replacement cassette.

25

26 **Gene expression analyses**

27 Steady state mRNA levels of the *Aspergillus nidulans ENA1*-like genes were detected using
28 specific, PCR-amplified, genome probes. For AN6642 a probe of 1357 bp using primers
29 An6642rev and An6642fw covering 40% of the ORF was used. For AN1628 a probe of 948 bp
30 using primers An1628rev and An1628fw covering 29% of the ORF was used. For AN10982 a
31 probe of 1295 bp using primers An10982rev and An10982fw, and covering 37% of the ORF
32 was used. To detect AN0471 (designated *vcxA*), a 926 bp fragment using primers An0471rev
33 and An0721fw, covering 65% of the ORF, was synthesised. For *chsB* detection (AN2523), a
34 fragment of 719 bp was amplified using primers An2523rev and An2523fw, covering 30% of
35 the ORF. Actin transcript detection used as probe the fifth exon, amplifying the genomic
36 DNA with primers Anid-AcnA and Anid-AcnB. For oligonucleotide sequences see
37 Supplementary Table 2.

38 Northern analyses used total RNA extracted from wild type and mutant strains grown in
39 minimal medium (MMA) adjusted to pH 6.5, containing 1% glucose and 10 mM NH₄⁺ [as the
40 (+)-tartrate] as main carbon and nitrogen sources, respectively. For investigating the various
41 cation effects, after 18 hrs of culture at 37°C the indicated additions and pH alterations were
42 made with further incubation as specified in the figure legends.

43 Quantification of *enaA* and *acnA* transcription was done exposing the filters to a
44 Phosphorimager screen (Molecular Dynamics) and developing with a FLA-5100 Reader
45 (Fujifilm Global). Band intensities were measured using Multi-Gauge V3.0 (Fujifilm Global)
46 software.

47

48 **Construction of GST::CrzA and GST::SltA fusion proteins**

49 The plasmid pGEX-CrzA123(469-603) was constructed to express a GST fusion protein
50 containing the complete zinc-finger region of CrzA (aa 469 - 603). The zinc-finger region of
51 *crzA* was amplified by PCR using genomic DNA of strain J734 as template and primers

1 CrzA-ZF-fw-BamHI and CrzA-ZF-rev-EcoRI. Both oligonucleotides introduced the
2 restriction sites for *Bam*HI and *Eco*RI that allowed in-frame ligation of the PCR fragment into
3 pGEX-2T (Amersham Biosciences). The final plasmid was named pGEX-CrzA(469-603).
4 The plasmid expressing a GST::SltA(381 - 549) fusion protein was constructed by cloning a
5 PCR amplicon obtained from J734 genomic DNA with oligonucleotides SltA-ZF-fw-BamHI
6 and SltA-ZF-rev-EcoRI (amplifying the entire zinc finger region of the *sltA* gene and
7 introducing *Bam*HI and *Eco*RI sites) into pGEX-2T. The resulting plasmid was named pGEX-
8 SltA(381-549). Fidelity and correct orientation of the inserts were verified by sequencing.
9 Fusion proteins were expressed in *E. coli* DH1 and purified by glutathione affinity
10 chromatography according to the manufacturer's instructions (Amersham Biosciences).

11

12 **Electromobility shift assays**

13 Synthetic double-stranded oligonucleotides were generated as described in [26]. *A. giganteus*
14 sites *afp*-CDRE-5 and *afp*-SLTA-1 were obtained annealing oligonucleotides CDRE-5 with
15 CDRE-5(-) and SltA-1 with SltA-1(-), respectively. *T. reesei* probes 2C and 2C4 were
16 obtained annealing oligonucleotides Ace1-2C-1 with Ace1-2C-2 and Ace1-2C4-1 with Ace1-
17 2C4-2, respectively. For the *A. nidulans chsB* CDRE-1 site, oligonucleotides Chs-CDRE-1fw
18 and Chs-CDRE-1rev, were annealed (for oligonucleotide sequences see Supplementary Table
19 2).

20 Synthetic probes were labelled by end-filling using Klenow polymerase in the presence of a
21 mixture containing dGTP and [α -³²P]dCTP. Binding reactions were performed as described
22 previously [26]. The binding reaction mixture (final volume 20 μ l) contained binding buffer
23 (25 mM HEPES-KOH, 50 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 20%
24 glycerol; pH 7.9), 1 ng labelled double-stranded oligonucleotides, 3 μ g non-specific
25 competitor [poly(dI-dC) or poly(dA-dT)] and variable amounts of proteins, as purified GST or
26 GST::CrzA(469-603) fusion, or as *E. coli* protein extracts for GST::SltA(381-549). Various
27 amounts, as indicated in figures or legends, of unlabelled double-stranded oligonucleotides as
28 specific competitors, were added to the reaction mixtures before the addition of protein.
29 Reaction mixtures were kept on ice for 30 min and DNA and DNA-protein complexes were
30 separated on native 5% poly-acrylamide gels. Gels were dried and autoradiographed.

31

32 **AFP susceptibility testing**

33 Determination of the sensitivity of *A. nidulans* strains to the antifungal protein AFP followed
34 the protocol of [27].

35

36 **Microscopy**

37 Conidia of *A. nidulans* strains were grown in appropriately supplemented liquid minimal
38 medium placed on cover slips. Germlings adhering to cover slips were observed using an
39 Axioskop 50 (Zeiss) equipped with a Camedia Digital Camera C-4000 Zoom (Olympus,
40 Hamburg). Light images were captured with a 40 x objective. Images were processed using
41 Adobe Photoshop 6.0 (Adobe Systems Inc.). Observations of *Aspergillus* fluorescent strains
42 were done in "watch minimal medium" (WMM) as described in [28]. The *Aspergillus* strain
43 expressing CrzA::GFP was cultured on the surface of glass bottom petri dishes immersed in
44 2.5 ml of appropriately supplemented WMM at 25°C during 16 h. Media were replaced by
45 fresh WMM containing either with or without 10 mM CaCl₂ and the green fluorescence was
46 observed using a Leica DMI6000b inverted microscope, equipped with a GFP specific filter
47 (Ex.470nm; Em. 525nm), and recorded with an Orca-ER camera (Hamamatsu) driven by
48 Metamorph (Universal Image Co.)

1 Results

2 3 Identification of the *crzA* gene.

4 The putative *A. nidulans* homologue of *S. cerevisiae* CRZ1, AN5726.3 denoted *crzA*,
5 was identified by Blastp searches using the full-length Crz1p protein sequence. The *in silico*
6 predicted cDNA sequence of *crzA* was verified by sequencing a RT-PCR product obtained
7 from total RNA.

8 CrzA and Crz1p share 66% identity within the putative DNA binding domain which
9 comprises three zinc fingers, the first two of which conform to the Cys₂-His₂ consensus
10 [(Y/F)XCX₂₋₄CX₃(Y/F)X₅LX₂HX₃₋₄H] (Figure 1A). This degree of sequence conservation
11 suggests that CrzA and Crz1p might bind the same DNA target sequence. The third finger of
12 Crz1p and its homologues, including CrzA, appears to have an atypical Cys₂-His-Cys
13 structure with unusual spacing (Figure 1A) and is currently under study. The 730-residue
14 CrzA protein, in common with its homologues, contains the DNA binding domain in its C-
15 terminal moiety. CrzA contains two overlapping putative calcineurin docking domains (CDD)
16 (Figure 1B) whereas Crz1p which is activated by dephosphorylation by the calcineurin
17 phosphatase is thought to contain a single specific recognition sequence PxIxIT [29-31].
18 Putative Crz1p orthologues are widely distributed amongst the fungi with sequence
19 conservation notably high amongst filamentous fungi (Figure 1C and Supplementary Figure
20 1). The *crzA* transcript appears to be expressed at a very low level by northern analysis but is
21 increased somewhat by exposure to 100 mM Ca²⁺ for periods of 5-30 min (data not shown).
22 In contrast, no increase in *A.fumigatus* *crzA* transcript levels was reported in response to 10 or
23 60 min exposure to 200 mM Ca²⁺ [13]. The very modest increase in *crzA* transcript levels in
24 response to Ca²⁺ suggests that regulation might be mainly post-transcriptional, particularly in
25 view of the effect of Ca²⁺ on CrzA localisation (see below).

26 27 The phenotype of a *crzA* null mutation.

28 To assess the physiological role of CrzA, a strain deleted for *crzA* was constructed by
29 gene replacement using the *Neurospora crassa* *pyr-4* gene as selectable marker, as described
30 in Experimental. The original transformant and outcrossed Δ *crzA* strains are, like the
31 corresponding deletion mutants of *A. fumigatus* and *B. cinerea* [13;19], strongly inhibited by
32 elevated Ca²⁺ concentrations and alkaline pH (Figure 2), thus resembling the behaviour of
33 *crz1* mutants [9;32] but much more extreme.

34 Strains deleted for *crzA* have delayed germination in minimal medium in which Ca²⁺ is
35 present only as a trace contaminant (Figure 3A). The presence of 100 mM Ca²⁺ causes hyphal
36 hyperbranching in Δ *crzA* strains (Figure 3B). Similar morphological abnormalities have been
37 noted for the corresponding *A. fumigatus* and *B. cinerea* deletion mutants [13;19].
38 Interestingly Δ *crzA* strains show significant resistance to the toxicity of the antifungal protein
39 AFP (Figure 3C), recently shown to inhibit chitin biosynthesis, very probably as a result of
40 inhibition of chitin synthases of classes III and V [33]. In contrast, Δ *crzA* strains show slight
41 hypersensitivities to other cell wall perturbing compounds such as caspofungin and congo red
42 (data not shown), further suggesting a role for CrzA in determining cell wall structure. Cell
43 wall abnormalities have also been reported for the orthologous *B. cinerea* *crz1* deletion
44 mutant [19]. The similarities in phenotype between null *crzA* and null *chsB* *A. nidulans*
45 strains, which lack a class III chitin synthase [34;35], suggested that *chsB* might be under
46 CrzA control. Figure 3D shows that whereas transfer to medium containing 100 mM Ca²⁺
47 elevates transcript levels of *chsB* in wild type, it fails to do so in a Δ *crzA* strain, indicating that
48 up-regulation of *chsB* in response to Ca²⁺ is CrzA-dependent. In agreement, the *chsB*
49 promoter contains two putative CDRE sites (data not shown) conforming to the Crz1p binding

1 site consensus [7]. In contrast to the effect of Ca^{2+} , 300 mM Li^+ appears to have no effect on
2 *chsB* transcript levels (Figure 3D).

3 Another target of CrzA regulation is AN0471.3, denoted *vcxA*^{AN0471}, which we
4 identified as the closest *A. nidulans* homologue of *S. cerevisiae* *VCXI* using tBlastn and
5 Blastp searches. In *S. cerevisiae* the vacuolar $\text{Ca}^{2+}/\text{H}^+$ exchanger Vcx1p mediates import of
6 excess cytosolic Ca^{2+} into the vacuole [36]. *VCXI* transcription is negatively regulated by
7 calcineurin in a Crz1p-independent manner [8;37]. Figure 4A shows that *vcxA*^{AN0471} transcript
8 levels are increased by 100 mM Ca^{2+} in a CrzA-dependent manner. As a large number of
9 calcium-regulated *S. cerevisiae* genes show a greater transcriptional response at an early time
10 after alkalinisation provokes a transient rise in cytosolic calcium levels than at a later time [6],
11 *vcxA*^{AN0471} transcript levels were monitored at both 30 and 60 min following calcium
12 exposure. Indeed, steady-state transcript levels were lower at the later time point (Figure 4A).
13 Expression of *VCXI* homologues in *A. fumigatus* and *B. cinerea* is also dependent on the
14 respective CRZ1 orthologues [13;19].
15

16 Subcellular localisation of CrzA.

17
18 To analyse the subcellular distribution of CrzA, a mutant *crzA* allele expressing a C-
19 terminally GFP-tagged CrzA was constructed by gene replacement using previously described
20 procedures [24;25;38]. The protein CrzA::GFP encoded by the tagged allele appears to be
21 fully functional as no Ca^{2+} sensitivity or growth impairment at alkaline pH is observable (data
22 not shown). In the absence of Ca^{2+} addition and any form of stress, CrzA::GFP is exclusively
23 cytosolic, apparently excluded from nuclei (Figure 5). Upon addition of CaCl_2 to a final
24 concentration of 10 mM, CrzA rapidly accumulates in nuclei, eventually depleting the
25 cytoplasm of fluorescence (Figure 5 and data not shown). Thus, as previously described for
26 the CrzA orthologue Crz1p ([6;31;39] and reviewed in [3]), CrzA appears to be subject to an
27 active nuclear export and import machinery, with an increase of cytosolic Ca^{2+} concentration
28 eliciting its nuclear import (Figure 5). Presumably, as in *S. cerevisiae* [31], Ca^{2+} activation of
29 calcineurin results in dephosphorylation of CrzA and consequent loss of nuclear export
30 capability. *A. fumigatus* CrzA also localises to the nucleus in response to Ca^{2+} [13] as does *B.*
31 *cinerea* CRZ1 when expressed heterologously in *S. cerevisiae* [19].
32

33 Further characterization of *sltA* mutations.

34 In view of the sensitivity of $\Delta crzA$ strains to toxicity of the cation Ca^{2+} , further
35 characterization of mutations in the *sltA* gene where the *sltA1* allele confers sensitivity to the
36 cations Na^+ and K^+ [20;21;40] seemed warranted. Although SltA homologues are widely
37 distributed in filamentous fungi, we have been unable to detect any in searches of yeast
38 genomes. The *sltA* gene encodes a 698-residue protein containing three Cys₂-His₂ zinc fingers
39 and the *sltA1* mutation changes tryptophan codon 502, the second codon following that of the
40 last histidine of finger 3, to a stop codon (Supplementary Figure 2A) [21].

41 To determine the phenotype of a null *sltA* allele and to see whether *sltA1* has the null
42 phenotype a deletion allele was constructed by gene replacement using the *Aspergillus*
43 *fumigatus* *riboB* gene as selectable maker (Experimental; Supplementary Figure 2B). We have
44 detected no phenotypic differences between $\Delta sltA$ and *sltA1*, suggesting that at least some
45 portion of the C-terminal 197 residues is essential to SltA function (data not shown). As seen
46 in Figure 2, the absence of functional SltA results in elevated sensitivity to Li^+ , Cs^+ and Mg^{2+}
47 in addition to Na^+ and K^+ , inability to grow at alkaline pH and hypersensitivity to neomycin
48 toxicity. In plate tests $\Delta crzA \Delta sltA$ double mutants have phenotypes which are the sum of the
49 single mutant phenotypes (Figure 2).

50 Although overall conservation of the C-terminal sequence beyond the third zinc finger
51 of SltA is low among putative homologues, tryptophan-502 and the residues immediately

1 downstream from it are highly conserved, suggesting this region might be required for full
2 functionality (Supplementary Figure 2A). To test this possibility, revertants of a *sltA1* strain
3 were obtained, after ultraviolet mutagenesis, as allowing growth in the presence of 1 M
4 NaH_2PO_4 . As well as reversion in codon 502 to the wild type tryptophan codon, partially
5 functional revertants with codon 502 encoding arginine, leucine, phenylalanine, lysine or
6 cysteine were also obtained (Table 2). Unsurprisingly, the reversion to phenylalanine appears
7 to give the greatest degree of functionality. These reversion data indicate that tryptophan-502
8 is itself an important residue and that one or more additional important residues is/are located
9 C-terminal to it.

10
11 **SlmA acts negatively in the control of *vcxA*^{AN0471} and positively in the control of**
12 ***enaA*^{AN6642}**

13 The Δ *sltA* mutant showed an elevated response to Ca^{2+} in *vcxA*^{AN0471} transcript levels
14 at both 30 and 60 min and the response is more pronounced at the latter time point (Figure 4B;
15 data not shown for 30 min). However, Δ *crzA* is epistatic to Δ *sltA* in the control of *vcxA*^{AN0471}
16 as in the Δ *crzA* Δ *sltA* double mutant there is no response to Ca^{2+} in *vcxA*^{AN0471} transcript levels
17 (Figure 4B), correlating with the fact that Ca^{2+} inhibition of Δ *crzA* Δ *sltA* double mutants is
18 very similar to that of Δ *crzA* single mutants (Figure 2).

19 Using *S. cerevisiae* Ena1p, Ena2p and Ena5p in Blastp and tBlastn searches of the *A.*
20 *nidulans* genome
21 (http://www.broad.mit.edu/annotation/genome/aspergillus_group/Blast.html) three loci whose
22 predicted protein products are closely related to the Ena P-type ATPase sodium pumps,
23 AN6642.3, designated *enaA* [41], AN1628.3 and AN10982.3, were identified (data not
24 shown). We have designated these genes *enaA*^{AN6642}, *enaB*^{AN1628} and *enaC*^{AN10982}. In *S.*
25 *cerevisiae* the Ena transporters are involved in the salt stress response and are required for
26 growth at alkaline pH [42;43].

27 Expression of *enaA*^{AN6642} requires a combination of elevated $[\text{Na}^+]$ and alkaline pH
28 (Figure 6). We have been unable to detect transcripts for *enaB*^{AN1628} and *enaC*^{AN10982} in the
29 conditions or genetic backgrounds shown in Figure 6. Neither 300 mM Li^+ nor 100 mM Ca^{2+}
30 is sufficient to induce expression of any of the *ena* genes (data not shown). Figure 6 shows
31 that SlmA plays a significant, positive-acting role in *enaA*^{AN6642} expression. If CrzA plays a
32 role, it is much more marginal.

33
34 **CrzA and SlmA are both DNA-binding proteins.**

35 The putative DNA-binding domains, containing the entire zinc finger regions, residues
36 469-603 for CrzA and residues 381-549 for SlmA were expressed in *E. coli* as glutathione-S-
37 transferase (GST) fusion proteins. Although purification of GST::CrzA(469-603) using
38 glutathione affinity chromatography was feasible, we did not succeed in purifying
39 GST::SlmA(381-549) despite trying several elution conditions (data not shown). Consequently
40 bacterial protein crude extract were used in DNA binding studies.

41 To characterise the DNA binding ability of GST::CrzA(469-603) probes conforming
42 to the *S. cerevisiae* Crz1p consensus site CDRE [7] were synthesised (Figure 7A). Two
43 putative CDRE sites were identified in the *A. nidulans* *chsB* promoter and five such sites were
44 identified in the *Aspergillus giganteus* *afp* promoter, previously suggested to be under the
45 control of the Ca^{2+} -calcineurin signalling pathway [11]. CrzA binds to CDRE-1 from the *chsB*
46 promoter and to CDRE-5 from the *afp* promoter (Figures 7B and C), confirming that binding
47 sequences for CrzA and Crz1p are very similar or identical.

48 Because of the sequence similarity of SlmA to *Trichoderma reesei* ACEI in the zinc
49 finger region ([21]), the ability of GST::SlmA(381-549) to bind to the previously described 2C
50 and mutant 2C4 probes [44] was tested. In similarity to ACEI binding, SlmA binds to the 2C

1 but not the 2C4 probe (Figure 7D). SltA also binds to the SLTA-1 site from the *afp* promoter,
2 which conforms to the 5'-AGGCA ACEI consensus (Figures 7A, D and E).

3 As the trinucleotide 5'-GGC is common to sequences bound by CrzA and SltA, we
4 tested whether either of the fusion proteins might bind to a site bound by the other. However,
5 we found no evidence for binding of SltA to *afp* CDRE-5 (Figure 7D) or of CrzA to *afp*
6 SLTA-1 (Figure 7E).

7
8

Stage 2(a) POST-PRINT

1 Discussion

2 The two putative transcription factors investigated here are both involved in
3 preventing toxicity of certain cations. In the absence of functional SltA, *A. nidulans* is
4 abnormally sensitive to toxicity by Na⁺, K⁺, Li⁺ Cs⁺ and Mg²⁺ but not by Ca²⁺ whereas the
5 absence of CrzA results in toxicity of Ca²⁺. Transcript levels of the putative vacuolar Ca²⁺/H⁺
6 exchanger gene *vcxA* in response to high Ca²⁺ levels provide a possible explanation for the
7 Ca²⁺ toxicity to Δ *crzA* strains and the lack of Ca²⁺ toxicity in Δ *sltA* strains. Whereas Δ *sltA*
8 strains show an elevated response to Ca²⁺ in *vcxA* transcript levels, putatively allowing greater
9 vacuolar storage and thus detoxification of Ca²⁺, Δ *crzA* strains apparently show no Ca²⁺
10 response at all, putatively resulting in elevated cytosolic Ca²⁺ levels. The VcxA homologue
11 Vcx1p of *S. cerevisiae* mediates the vacuolar import of excess cytosolic Ca²⁺ [36]. One
12 possible factor in cation toxicity in *sltA* null strains is the reduction in induced levels of the
13 putative cation exporter EnaA^{AN6642}. The homologous *S. cerevisiae* plasma membrane
14 exporter Ena1p enables a detoxification response to Na⁺, Li⁺ and alkalinity [42;43].

15 The presence of three putative SltA/ACEI binding sites 5' to the *enaA* coding region
16 and one such site 5' to the *vcxA* coding region is consistent with SltA regulation of transcript
17 levels of these genes. Five putative CDRE sites lie upstream of the *vcxA* coding region
18 consistent with CrzA transcriptional regulation of *vcxA*. One CDRE site lies upstream of the
19 *enaA* coding region but, in view of the lack of solid evidence that CrzA regulates *enaA*, its
20 significance, if any, is unclear. In any case, further work will be necessary to determine any
21 physiological relevance of these 5' sites.

22 This work has highlighted significant differences between *A. nidulans* and *S.*
23 *cerevisiae*. *S. cerevisiae* lacks an identifiable SltA homologue and whereas *S. cerevisiae*
24 Crz1p is not involved in *VCX1* expression but plays a crucial role in *ENA1* expression [9], *A.*
25 *nidulans* CrzA plays a crucial role in *vcxA* expression (in similarity to findings in *A. fumigatus*
26 and *B. cinerea*) but is hardly involved, if at all, in *enaA*^{AN6642} regulation. The role of CrzA in
27 calcium-induced *chsB* expression finds a parallel in *Candida albicans* where calcium
28 stimulation of promoter activity for four different chitinase genes is Crz1p-dependent [45].

29 A further point of interest is that SltA appears to play both a positive (in *enaA*^{AN6642}
30 regulation) and a negative (in *vcxA* regulation) regulatory role with the caveat that we have
31 not yet shown that SltA acts directly on the regulation of the genes in question. The PacC
32 transcription factor, mediating control of gene expression by ambient pH in *A. nidulans* acts
33 as both an activator and a repressor (reviewed in [46]) but its *S. cerevisiae* homologue
34 Rim101p exerts positive regulation by repressing transcription of genes encoding other
35 transcriptional repressors [47]. Finally we note that at least three transcription factors are
36 necessary for growth of *A. nidulans* at alkaline pH: PacC (reviewed in [46]), and, as
37 established here, SltA and CrzA. In the case of SltA, at least one pertinent factor is its role in
38 *enaA*^{AN6642} expression.

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1 **Tables and Figures**

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4

TABLE 1: *A. nidulans* strains used in this work

Strain	Genotype	Source
J734	<i>biA1</i>	Imperial College collection
MAD1425	<i>pyroA4, pyrG89, argB2, ΔnkuA::argB</i>	Reference [25]
MAD1427	<i>pyrG89, pabaA1, riboB2, argB2, ΔnkuA::argB</i>	Reference [25]
MAD1732	<i>pyroA4, pyrG89, inoB2, wA4, ΔnkuA::BAR, Myc3xPacC(5-678)</i>	Madrid collection
MAD2055	<i>pyroA4, pyrG89, inoB2, wA4, ΔnkuA::BAR, Myc3xPacC(5-678), crzA::gfp/Af pyrG</i>	Madrid collection
BER02	<i>pyroA4, pyrG89, argB2, ΔnkuA::argB, ΔcrzA::pyr-4</i>	This work
HHF17a	<i>pabaA1, yA2, argB2::argB+ afp::lacZ</i>	This work
HHF17d	<i>pabaA1, yA2, argB2::argB+ afp::lacZ, ΔcrzA::pyr-4, pantoB100</i>	This work
HHF17e	<i>pabaA1, yA2, argB2::argB+ afp::lacZ, ΔsltA::Af riboB, pantoB100</i>	This work
HHF17f	<i>argB2::argB+ afp::lacZ, ΔsltA::Af riboB, ΔcrzA::pyr-4, pantoB100</i>	This work

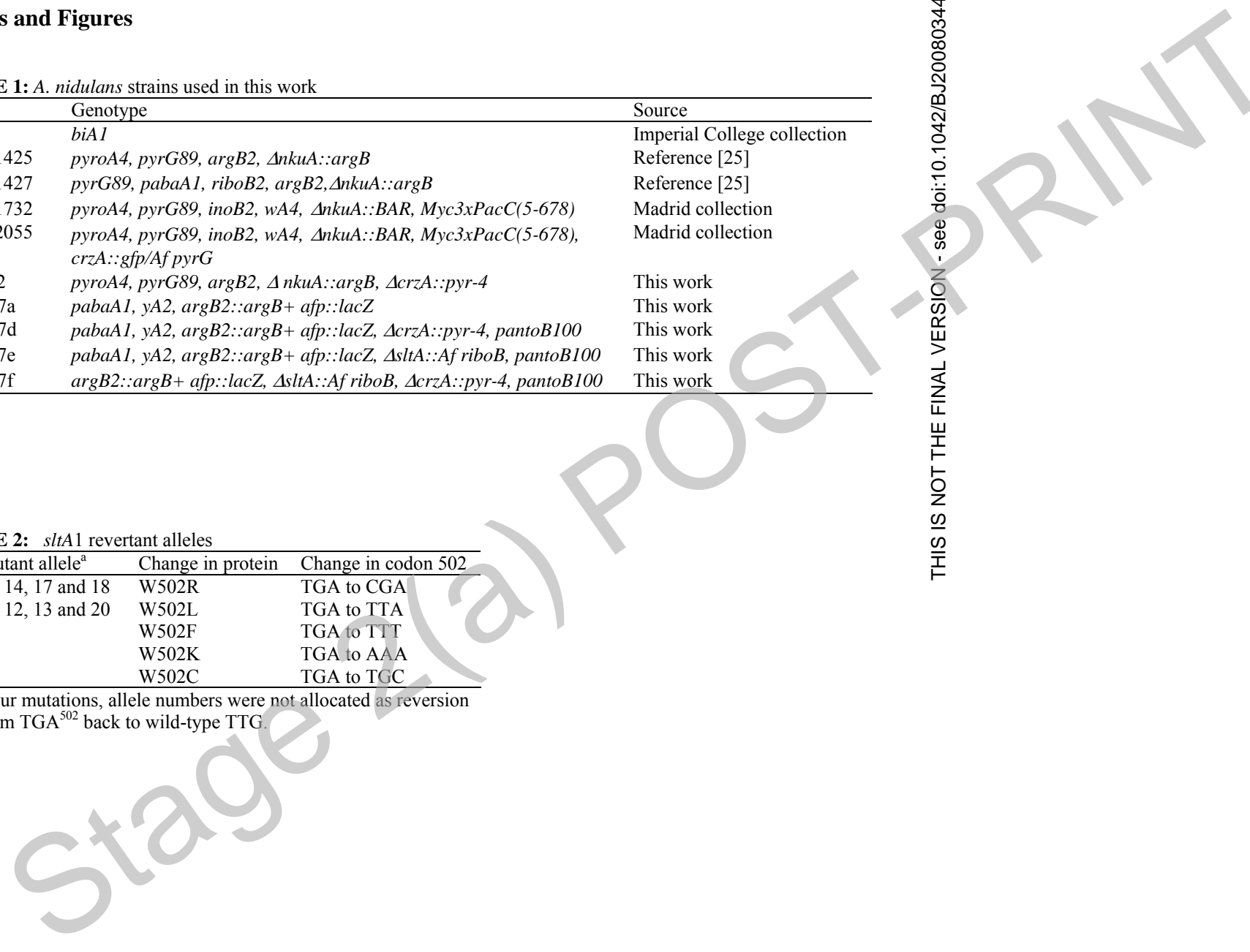
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TABLE 2: *sltA1* revertant alleles

<i>sltA</i> mutant allele ^a	Change in protein	Change in codon 502
<i>sltA</i> 10, 14, 17 and 18	W502R	TGA to CGA
<i>sltA</i> 11, 12, 13 and 20	W502L	TGA to TTA
<i>sltA</i> 15	W502F	TGA to TTT
<i>sltA</i> 16	W502K	TGA to AAA
<i>sltA</i> 19	W502C	TGA to TGC

^a For four mutations, allele numbers were not allocated as reversion was from TGA⁵⁰² back to wild-type TTG.

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1 Figure Legends

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3 **Figure 1 Sequence analysis of CrzA.** (A) Multiple alignment of the zinc finger domain of
4 predicted CrzA homologues from yeast and filamentous fungi. Shown is the zinc finger region
5 containing three C₂H₂ zinc fingers (zf), each indicated by an open bar. Two paralogues were
6 found in *Candida albicans* (Calb(1) and (2)). Protein alignments were done using the program
7 ClustalX [48]. (B) Alignment of a selected number of Crz homologues showing the putative
8 calcineurin docking domain (CDD). Open rectangles above the alignment indicate the limits
9 of the two putative CDDs in CrzA orthologues from filamentous fungi, which are compared at
10 the right with the CDD present in Crz1p and the consensus established from the CDDs present
11 in the NFAT protein [29]. (C) Phylogenetic tree obtained from the full alignment of the
12 Crz1p/CrzA homologues. The phylogenetic tree was built using MEGA 3.0 [49]. The
13 bootstrap value obtained after 10,000 replicates is shown on top of each branch. The scale bar
14 indicates relative evolutionary distance.

15 For panels A and B, Residue shading follows BLOSUM32 similarities. Protein sequences are
16 as follows: Anid, *Aspergillus nidulans* BAE94327; Aory, *Aspergillus oryzae* BAE57003;
17 Afum, *Aspergillus fumigatus* EAL88401; Mgri, *Magnaporthe grisea* XP_359644; Ncra,
18 *Neurospora crassa* EAA32849; Cglo, *Chaetomium globosum* EAQ88414; Gzea, *Gibberella*
19 *zeae* EAA68022; Cimm, *Coccidioides immitis* EAS33001; Pnod, *Phaeosphaeria nodorum*
20 EAT87393; Dhan, *Debaryomyces hansenii*, CAG84727; Tdel, *Torulasporea delbrueckii*
21 AAZ04388; Agos, *Ashbya gossypii* AAS51722; Scer, *Saccharomyces cerevisiae* CAA95889;
22 Klac, *Kluyveromyces lactis* CAG99429; Ylip, *Yarrowia lipolytica* CAG80473; Calb(1),
23 *Candida albicans* EAK97605; Calb(2), *Candida albicans* EAK93473; Cgla, *Candida*
24 *glabrata* CAG62620; Spom, *Schizosaccharomyces pombe* Q09838.

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26

27 **Figure 2 Phenotypic analysis of *A. nidulans* strains defective for CrzA and/or SlTA**
28 **function.** Spores were point-inoculated on agar plates containing minimal medium (MMA,
29 pH 6.5; 1% glucose as carbon source) supplemented with different salts, as indicated, or
30 neomycin (625 ng/ml). Alkaline pH plate (pH ~8) contained 100 mM Na₂HPO₄. 200 mM
31 NaCl is a Na⁺ control for the alkaline pH medium. Plates were incubated for two days at
32 37°C. wt: wild-type (strain HHF17a), Δ crzA (strain HH17d), Δ sltA (strain HH17e), Δ crzA
33 Δ sltA (strain HH17f).

34

35 **Figure 3 Effect of deletion of crzA on germination, morphology, AFP sensitivity and**
36 **expression of the chsB chitin synthase gene.** (A) Conidia from strains MAD1425 (wt) and
37 BER02 (Δ crzA) were grown on cover slips at 37°C for 6 or 12 h. (B) Strains HHF17a (wt),
38 HHF17d (Δ crzA) HHF17e (Δ sltA) and HHF17f (Δ crzA, Δ sltA) were grown on MMA agar
39 plates in the presence or absence of 100 mM CaCl₂. Microscope photographs, using 10 x
40 magnification, were taken of the colony edges. (C) 1000 conidia obtained from strains
41 MAD1425 (wt) and BER02 (Δ crzA) were cultivated at 28°C in 150 μ l complete medium
42 (YPG) in the presence of different AFP concentrations. After 48 h, the optical densities were
43 measured. Mean values of triplicate experiments are shown. (D) *chsB* transcript levels were
44 determined in strains MAD1425 (wt) and BER02 (Δ crzA) treated for 60 min with 100 mM
45 CaCl₂ or LiCl. The untreated controls are indicated by (-). Strains carrying either wt *crzA*⁺ or
46 the Δ crzA allele were cultivated in liquid MMA (pH 6.5) for 18 h at 37°C after which CaCl₂
47 was added to a final concentration 100 mM. After additional cultivation for 30 or 60 min, total
48 RNA was isolated, blotted and hybridised with a *chsB* probe. Methylene blue-stained 18S
49 rRNA served as loading control.

50

1 **Figure 4 Effects of various genotypes and elevated Ca²⁺ levels on *vcxA* expression.** (A)
2 *vcxA* transcript levels were determined after 30 and 60 min in the presence (+) or absence (-)
3 of CaCl₂. wt: strain MAD1425, Δ *crzA*: strain BER02. (B) Expression of *vcxA* was determined
4 at 30 and 60 min (only shown for the latter) after Ca²⁺ addition in the following strains:
5 HHF17a (wt), HHF17d (Δ *crzA*) HHF17e (Δ *sltA*) and HHF17f (Δ *crzA* Δ *sltA*). The growth and
6 treatment protocol followed that described for Figure 3.

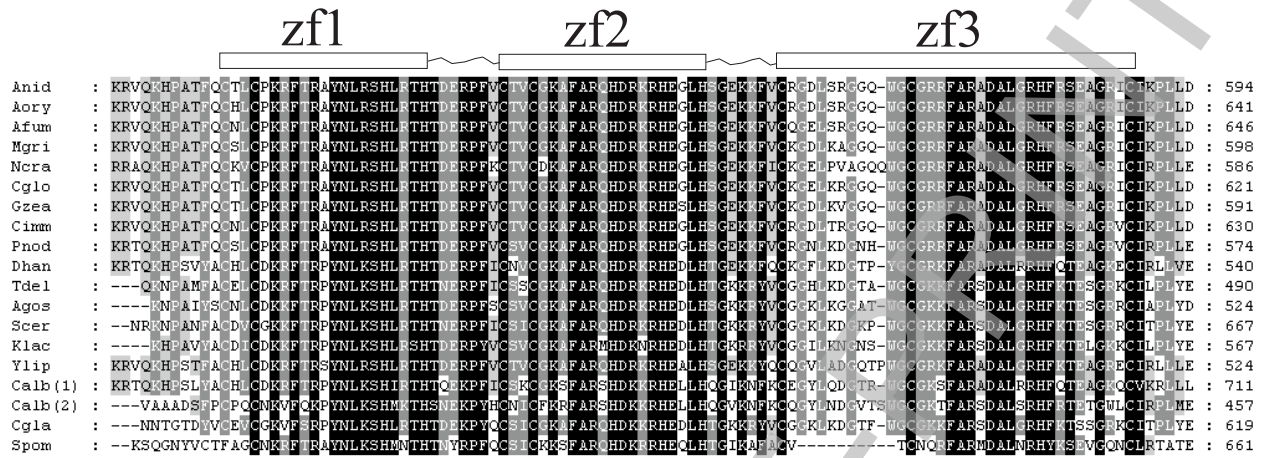
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8 **Figure 5 Subcellular distribution of CrzA.** An *A. nidulans* strain, MAD2055, expressing
9 CrzA::GFP was grown in WMM at 25°C in glass bottom petri dishes for microscopy. Mycelia
10 were transferred to fresh medium with or without 10 mM CaCl₂ and photographed after 10
11 min. incubation at 25°C. Nuclei devoid of fluorescence, indicated by arrows, are visible in
12 cells growing in medium without added Ca²⁺. In the presence of Ca²⁺, the nuclei become
13 strongly fluorescent. Images at the bottom show in more detail the fluorescence distribution
14 within the hyphae under both growth conditions. The bar scales indicate 5 μ m.

15
16 **Figure 6 Effects of various treatments and genotypes on *enaA* expression.** Strains
17 carrying Δ *crzA*, Δ *sltA*, both or neither were cultivated in liquid MMA (pH 6.5) for 18 h at
18 37°C after which NaCl was added (final concentration 1 M) and/or the pH of the culture broth
19 was adjusted to pH 8 (using NaOH). After an additional cultivation for 60 min, total RNA was
20 isolated, blotted and hybridised with *enaA* and *actA* (actin) probes. The following strains
21 were used in this experiment: HHF17a (wt), HHF17d (Δ *crzA*) HHF17e (Δ *sltA*) and
22 HHF17f (Δ *crzA* Δ *sltA*). Levels of *enaA* transcripts were determined and expressed relative to
23 those of the actin mRNAs.

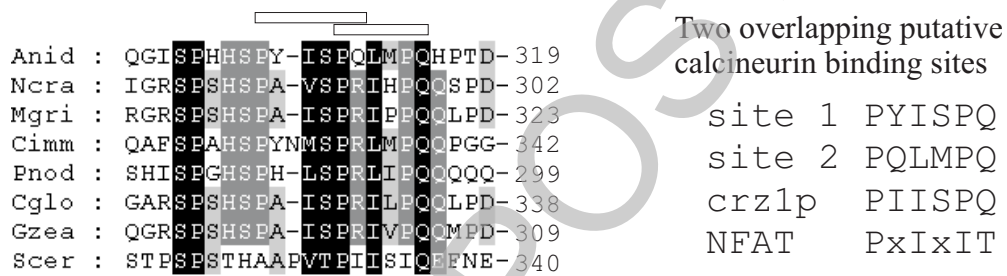
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25 **Figure 7 DNA binding capabilities and specificities of CrzA and SltA.** (A). Alignment of
26 Crz1p/CrzA and ACEI/SltA DNA binding sites. CDRE consensus sequences 1 and 2
27 correspond to those described in [10] and [7], respectively. The core sequences of these
28 binding sites are indicated. Probes CDRE-5 and SLTA-1 are synthetic sites from the
29 antifungal protein promoter (*afp*) of *A. giganteus*. Probe 2C is one of the ACEI binding sites
30 found in the *T. reesei* *cbhl* promoter and 2C4 is a 2C mutant probe as described in [44].
31 Probe CDRE-1 is a synthetic site from the *A. nidulans* *chsB* promoter. (B) CrzA binding to an
32 *afp*-CDRE-5 probe. (C) Binding of CrzA to a CDRE present in the *chsB* promoter. Binding to
33 this site is subject to competition by the same unlabelled probe or the *afp*-CDRE-5 probe. For
34 (B) and (C), numbers at the top of the gel refer to ng of purified protein used in the assay. (D)
35 Binding of SltA to the 2C probe. 1.5 μ g of *E. coli* extract expressing GST::SltA was used in
36 each reaction. Affinity and specificity of binding is shown by competition with 1000x (300ng)
37 of unlabelled probes SLTA-1, CDRE-5, 2C and 2C4. (E) Specific recognition of *afp*-SLTA-1
38 probe by SltA. Binding of SltA is shown using different amounts (μ g) of *E. coli* crude extract
39 expressing GST::SltA. Purified GST::CrzA does not bind to this probe.

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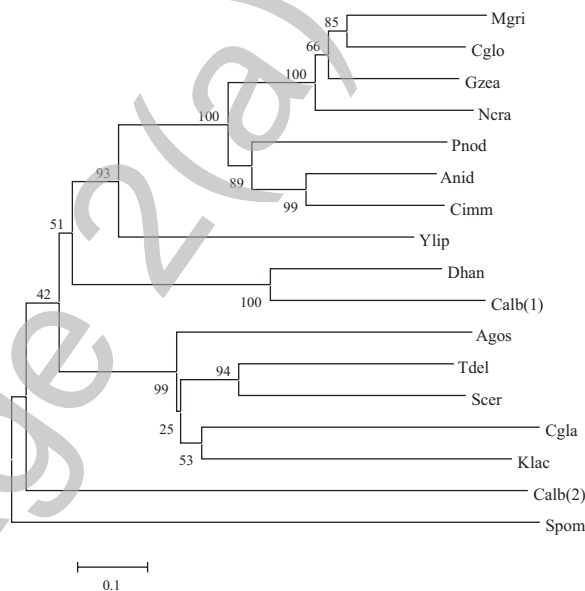
A



B



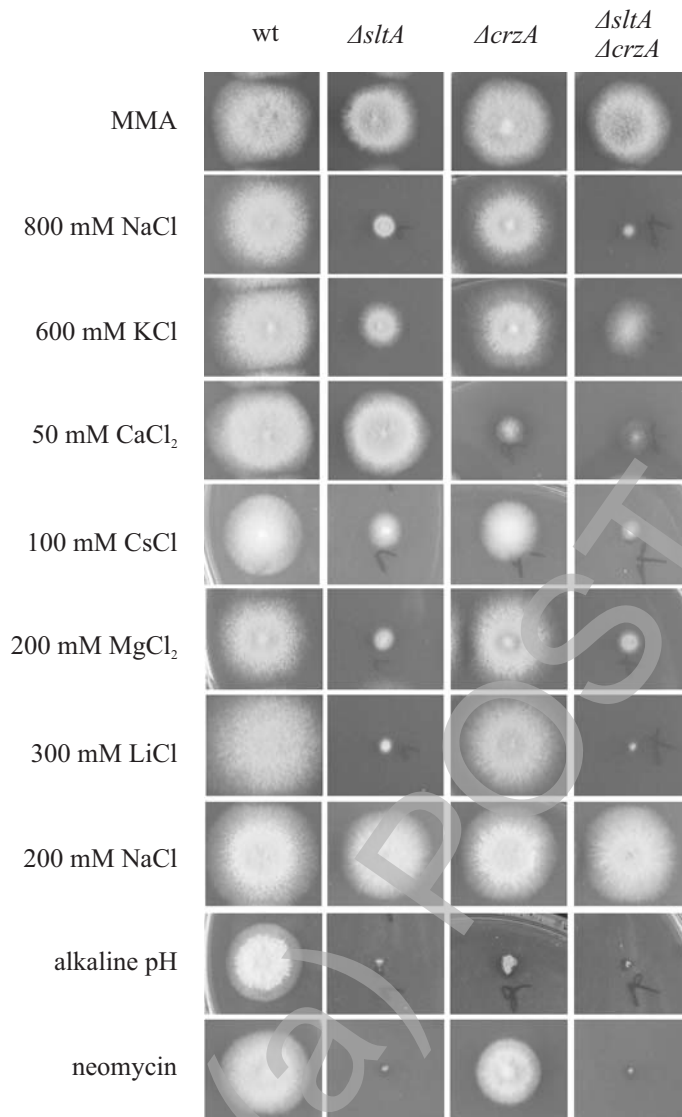
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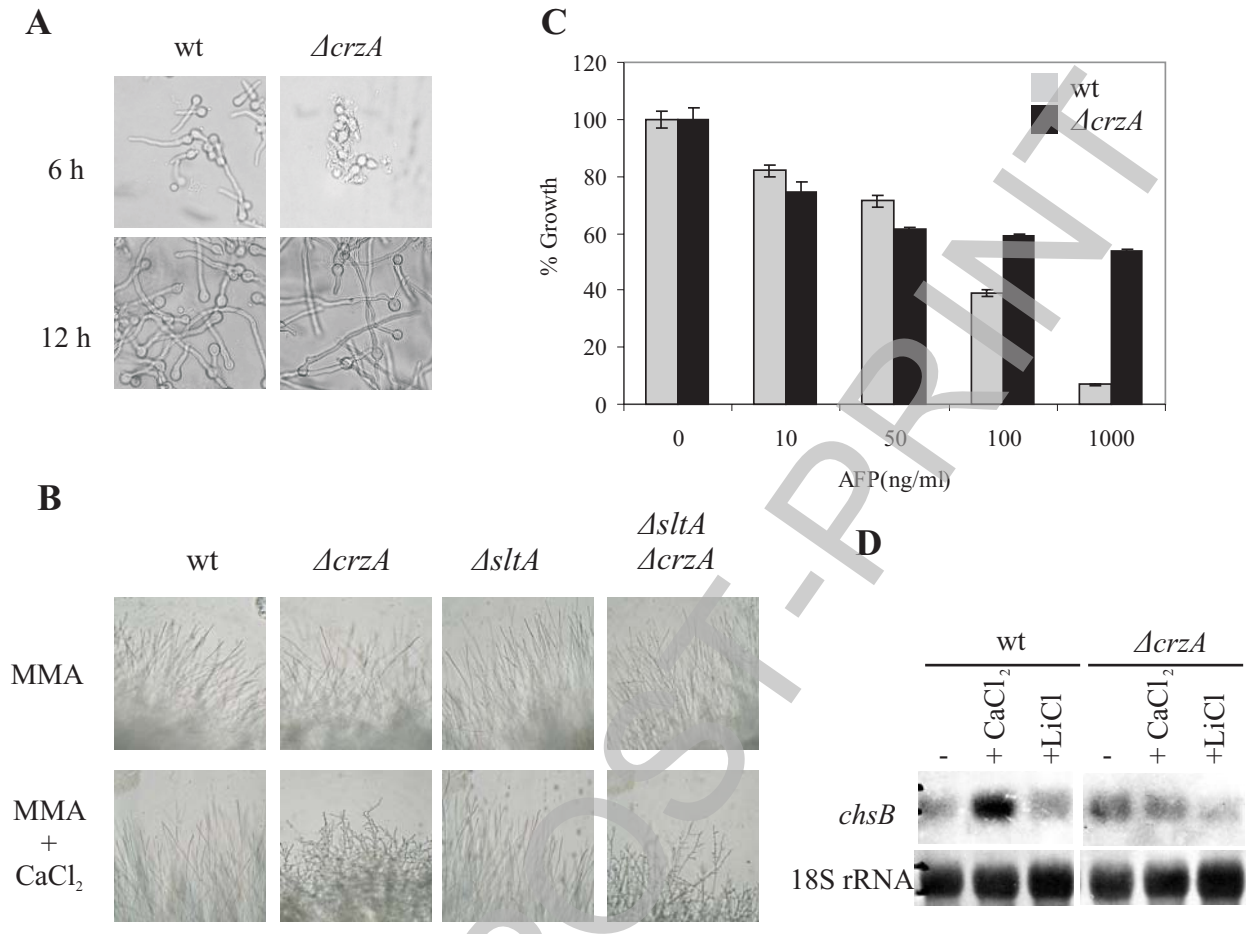
Spielvogel et al 2008. Figure 1.

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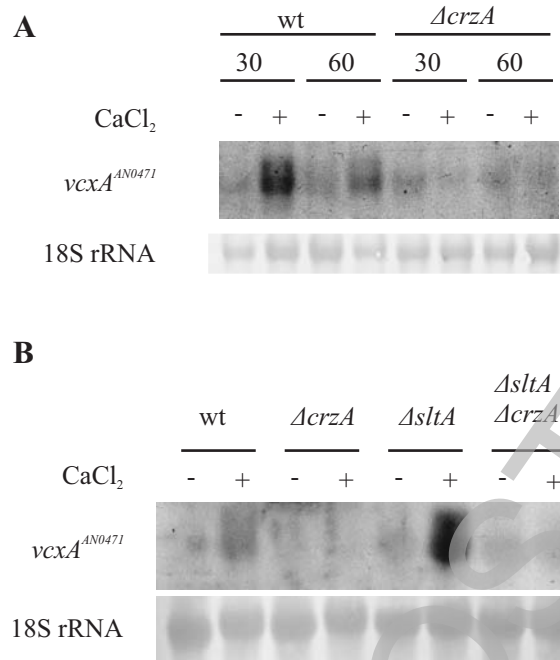


Spielvogel et al 2008. Figure 2

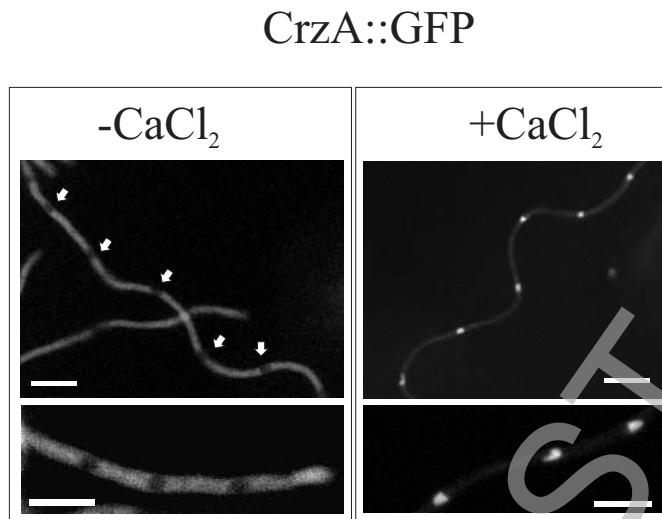


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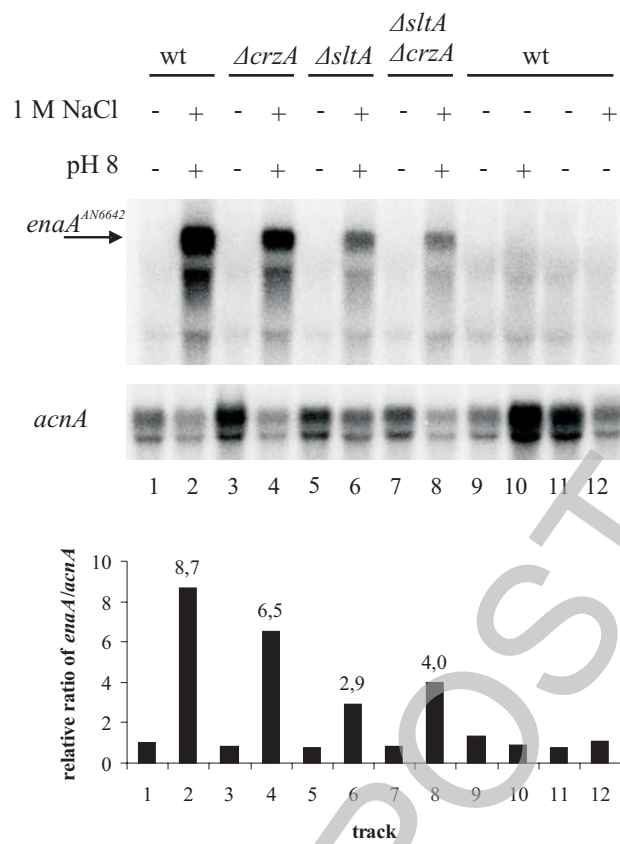
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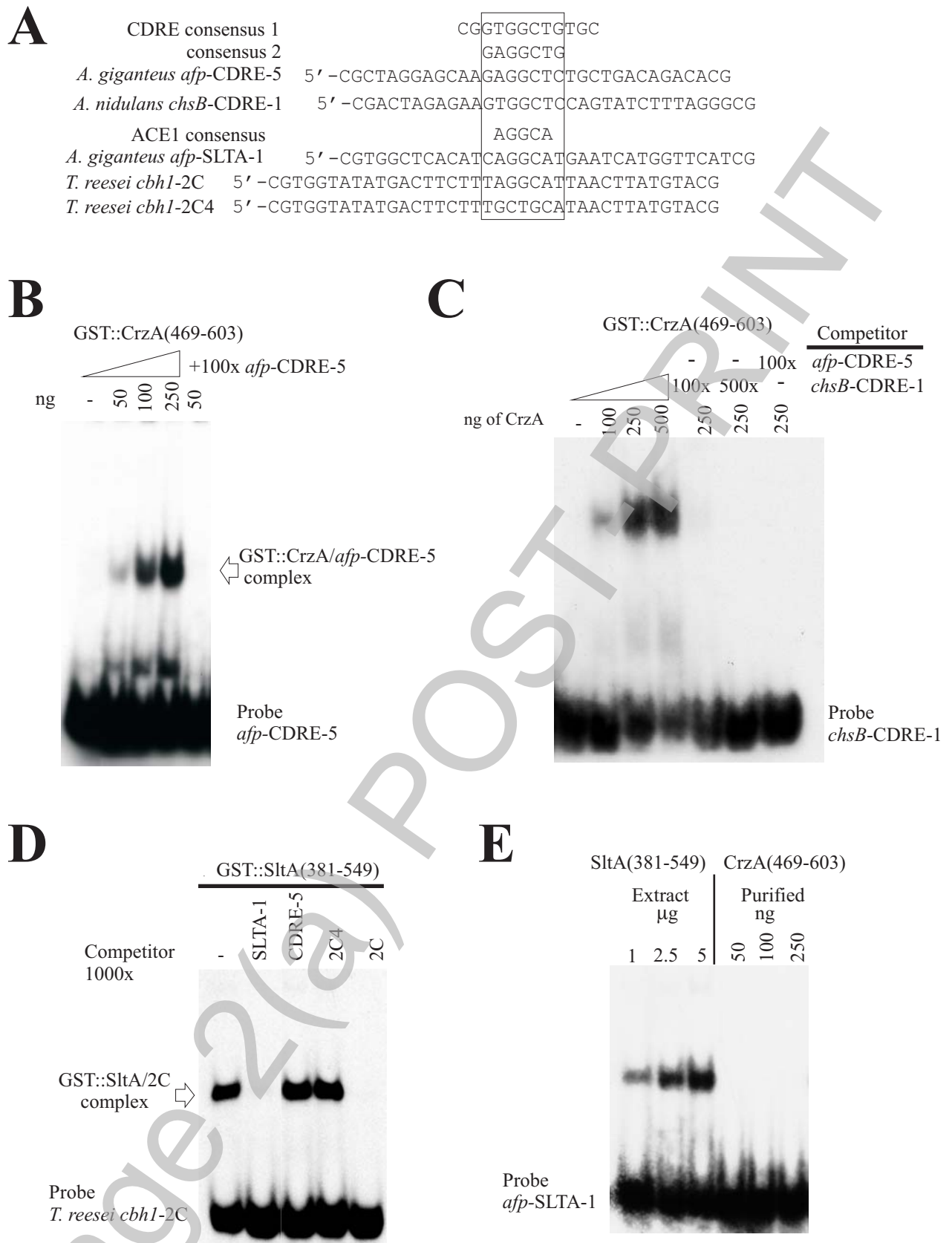
Spielvogel et al 2008 Figure 4



Spielvogel et al 2008. Figure 5



Spielvogel et al 2008. Figure 6



Spielvogel et al 2008. Figure 7