The CRZ-1 Transcription Factor Regulates Hsp 80 Involves in the Acquisition of Thermotolerance, and NCA-2 for Calcium Stress Tolerance in Neurospora Crassa

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Keywords: Heat shock proteins 60 and 80, Calcineurin subunit B, CRZ-1, NCA-2

DOI: https://doi.org/10.21203/rs.3.rs-548634/v1

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Abstract

Heat shock proteins (Hsps) are molecular chaperones and required for survival of organisms under heat stress conditions. In this study, we studied Hsp80, a member of the Hsp90 family, in Neurospora crassa. The expression of hsp80 was severely reduced in the N. crassa calcineurin B subunit RIP-mutant (cnb-1RIP) strains under the heat shock conditions. Furthermore, the expression levels of cnb-1, hsp60, hsp80, and the calcineurin-regulated transcription factor crz-1 were increased, but expression levels were reduced in the presence of the calcineurin inhibitor FK506 under the heat shock stress in the N. crassa wild type. Therefore, the calcineurin-crz-1 signaling pathway transcriptionally regulates hsp60 and hsp80 under the heat shock stress condition in N. crassa. In addition, the transcript levels of trm-9 and nca-2, a Ca\(^{2+}\) sensor and a Ca\(^{2+}\) ATPase, respectively, were increased under the heat shock stress condition. Moreover, the expression of the hsp80, but not the hsp60, was reduced in the Δtrm-9, Δnca-2, and the Δtrm-9 Δnca-2 double mutants. These results suggested that hsp80, trm-9, and nca-2 play a role in coping the heat shock stress in N. crassa. We found that CRZ-1 binds to 5'-CCTTCACA-3' and 5'-AGCGGAGC-3' 8 bp nucleotide sequences, located about 1075 bp and 679 bp upstream of the ATG start codon, respectively, of hsp80. We also found that CRZ-1 binds to an 8 bp nucleotide sequence 5'-ACCGCGCC-3', located 234 bp upstream of the ATG start codon of nca-2 under Ca\(^{2+}\) stress condition. Thus, cnb-1, hsp60, hsp80, and crz-1 are involved in the heat shock stress response in N. crassa. Moreover, CRZ-1 upregulates the expressions of hsp80 and nca-2 under the heat shock stress and Ca\(^{2+}\) stress conditions, respectively, in N. crassa.

Introduction

Heat shock proteins (Hsps) are molecular chaperones classified into different families on the basis of molecular weight (Tiwari and 2015). Hsps are primarily responsible for survival of organisms under heat stress conditions. Among the family of heat shock proteins, hsp60 and hsp90 have been well characterized in eukaryotes for their role in phosphorylation of Cdc37 having essential role in cell wall formation, cell division, and viability in Saccharomyces cerevisiae and other higher eukaryotes via protein kinase C (Hawle et al. 2007). Hsp60 and Hsp90 regulate the glycerol pathway in Saccharomyces cerevisiae (Hawle et al. 2007). Hsp90 is involved in sporulation, conidiation, growth and germination in Aspergillus fumigatus (Lamoth et al. 2012). Furthermore, HSP90 inhibits the Ras/PKA pathway and negatively regulates morphogenesis in Candida albicans (Shapiro and Cowen 2012). Hsp90 is also involved in the regulation of cell cycle and helps to maintain the cell in the G0 phase in Hep3B and HuH7 hepatocellular carcinoma (HCC) cell lines (Watanabe et al. 2009). The Hsp90 homolog Hsp90\(\beta\) is important for placental development in mice (Christians et al. 2003).

Hsp90 also regulates different co-chaperones in response to stress and antifungal drug treatment (Gu et al. 2016). In C. albicans, Hsps have been targeted for antifungal drug therapy in relation to their role in regulation of the calcineurin-CRZ-1 pathway via direct interaction of Hsp90 with Cna1 (Singh et al. 2009; Imai and Yahara, 2000). In fungi, calcineurin activates CRZ-1, also known as nuclear factor of activated T
cells (NFAT) in mammals, an important transcription factor (Thewes 2014). CRZ-1 is required for the expression of a number of stress response genes such as PMC1, PMR1, PMR2A, and FKS2, necessary for the Ca\(^{2+}\), Mn\(^{2+}\), Na\(^{+}\), stress responses, respectively, in *S. cerevisiae* (Matheos et al. 1997). In *A. fumigatus*, the ΔcrzA mutant showed sensitivity to heat shock stress (Soriani et al. 2008). In the filamentous fungus *Neurospora crassa*, CRZ-1 upregulates the expression of *ncs-1* under the high Ca\(^{2+}\) condition for survival (Gohain and Tamuli 2019). Calcineurin has a role survival under the heat shock stress in *N. crassa* (Kumar et al. 2019). In *C. albicans*, deletion of CRZ-1 increases the susceptibility to antifungal azoles (Onyewu et al. 2004). The increasing global temperature and emergence of drug resistance in fungi have become a threat to public health, and therefore, understanding novel pathway for targeted drug therapy is necessary for effective treatment of fungal infections. In pathogenic filamentous fungi, calcineurin-CRZ-1 pathway is a promising target for antifungal therapies to overcome drug resistance (Onyewu et al. 2004; Juvvadi et al. 2017).

Calcineurin mediated regulation of HSP90 is still unclear in fungi. Till date, the heat shock factor 1 (*hsf1*) in *C. albicans* is the only well-known transcriptional regulator of Hsp90 under the heat stress condition (Sorger 1990; Nicholls et al. 2009; Leach et al. 2016). Moreover, the interaction of Hsp90 with calcineurin was shown important for the antifungal drug resistance (Gong et al. 2017) and thermotolerance (Rocha et al. 2020) in *C. albicans* and *A. fumigatus*. Therefore, unravelling the mechanism of the calcineurin-mediated regulation of the Hsp90 might be promising for antifungal drug development. In *N. crassa*, calcineurin RIP mutants showed reduced viability on exposure to lethal heat shock temperature of 52 °C (Kumar et al. 2019). Till date, the Hsps have been identified in *N. crassa* (Kapoor et al. 1995; Borkovich et al. 2004), but information regarding the specific family of Hsps involved in the heat shock stress response has remained limited.

**Materials And Methods**

**Strains growth conditions**

*N. crassa* wild type strains, Δnca-2 and Δtrm-9 mutants (Table 1) were obtained from Fungal Genetics Stock Center (FGSC), University of Missouri, Kansas City MO 64110 (McCluskey 2003;McCluskey et al. 2010). The other cnb-1RIP mutant strains 599, 600, 602, and P*tcu-1::crz-1::5xGly::V5::gfp* strains were generated in the laboratory of Prof. Katherine A. Borkovich (University of California, Riverside). The Δtrm-9Δnca-2 double mutant was generated in our laboratory (Laxmi and Tamuli 2015). For vegetative growth on solid medium, the strains were cultured in 1 X Vogel’s minimal medium N (VM; Vogel, 1956, 1964) containing 1.5% D-glucose as carbon source and 2% agar (VGM). The pantothenate auxotrophs were grown on media supplemented with 0.01 mg/ml pantothenic acid and bathocuproinedisulfonic acid (BCS). A potent inhibitor of calcineurin FK506 was supplemented to the media wherever mentioned at a concentration of 1 µg/ml.

**RNA isolation from N. crassa strains and expression analysis using quantitative real time PCR (qRT-PCR)**
For gene expression studies under the heat shock conditions, ~1 X 10^6 conidia were inoculated in two 250 ml conical flasks containing 25 ml VG liquid media and incubated at 28 °C with shaking at 180 rpm for 14 h. Then, one of the flasks was transferred to 48 °C for about 1 h to induce heat shock proteins. In addition, for the transcriptional studies under Ca^{2+} stress conditions, the conidial suspension of ~1 X 10^6 conidia were grown in two 250 ml conical flasks containing 25 ml VG liquid media with or without supplementation with 0.2 M CaCl_2 and incubated at 30 °C with shaking at 180 rpm for 16 h. The mycelia from these cultures were harvested by filtration and powdered using liquid nitrogen in a mortar and pestle. Then, RNA was isolated from the mycelial powders using Trizol reagent (Life Technologies, USA), and synthesis of cDNA from total RNA was done using Verso™ cDNA Synthesis Kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. To perform qRT-PCR, gene specific primers (Table 2), SYBR Select Master Mix (Life Technologies, USA) and 7500 Real Time PCR system (Applied Biosystems, USA) as per the manufacturer protocol. The expression of the above target genes was calculated by 2^{-\Delta\Delta C_T} (Livak and Schmittgen 2001). The expression of β-tubulin was considered as the endogenous control.

In silico analysis of the promoter elements

Promoter analysis was performed for the hsp80 gene using an online database Genomatix MatInspector (Quandt et al. 1995; Cartharius et al. 2005).

Protein isolation and purification

For protein isolation of CRZ-1::5xGly::V5::GFP and 5xGly::V5::GFP, the P_{tcu-1}::crz-1 (559) and pccg-1_GFP (Table 1) strains were used. The strains were cultured in 50 ml of VG liquid medium using required supplements at 30 °C with shaking @ 180 rpm for 16 h. Mycelial mass was harvested using filter and was crushed into fine powder using liquid nitrogen in a mortar and pestle in 2 ml microfuge tubes and it was further suspended in 400 µl native protein extraction buffer [50 mM Tris-HCl (pH-7.5), 1 mM EDTA, 6mM MgCl_2, 2.5 mM phenylmethylsulphonyl fluoride (PMSF) and 0.1% fungal protein inhibitor cocktail (FPIC, Sigma Aldrich, USA)]. Crude protein was extracted by centrifugation at 8000 X g for 8 min at 4 °C. Further to pull down the protein, 50 µg of Dynabeads™ Protein A magnetic beads (Life Technologies, USA) conjugated and crosslinked with mouse anti-V5 monoclonal antibody (Life Technologies, USA) was incubated along with the crude protein samples and kept overnight at 4 °C on a rocking platform. Protein was eluted from the beads by addition of 30 µl of elution buffer [50 mM of glycine (pH- 2.8)] followed by addition of 5 µl of 1M Tris-HCl (pH- 7.5) for neutralization of acidic pH. An aliquot of 5 µl of the protein sample was run on an 10% SDS-PAGE gel and stained with the Coomassie Brilliant Blue dye (Himedia, India) to check the purity. Quantification of the protein concentration was done using Bradford method (Bradford reagent, Himedia, India).

Chromatin immunoprecipitation and sequencing
Chromatin immunoprecipitation (ChIP) for heat shock conditions was performed using 15 h old germlings of *N. crassa*. 1 X 10^6 conidia ml^{-1} was inoculated in two 250 ml conical flasks containing 50 ml VG liquid media and incubated at 28 °C for 14 h in shaking condition at 180 rpm and then one of the flasks was subjected to heat shock at 48 °C with 180 rpm in shaking for 1 h. For Ca^{2+} stress conditions 1 X 10^6 conidia were grown in two 250 ml conical flasks consisting of 50 ml VG liquid media with or without supplementation with 0.2 M CaCl_2 at 30 °C with shaking @ 180 rpm for 5 h and with this 5 h old germlings ChIP analysis was performed. For both the above conditions, media was supplemented with 50 µM of BCS and 10 µg ml^{-1} Next, for fixing the cells about 1 % formaldehyde was added and kept incubated at 28 under shaking at 180 rpm for 1 h. For crosslinking about 125 mM glycine was added and kept for shaking under the similar condition for 30 mins. The culture was pelleted by centrifugation at 3000 g for 5 mins at 4. Then, the pellet was washed with 1X PBS at 3000 g for 5 mins in cold condition. Finally, the pellet was resuspended in 1.2 ml ChIP lysis buffer [50 mM of HEPES (pH- 7.5), 1 mM EDTA, 140 mM NaCl, 0.1% sodium deoxycholate, 1% Triton X-100, 1 mM PMSF and 0.1% fungal protease inhibitor cocktail (FPIC, Sigma Aldrich, USA)] and samples were sheared in a sonicator (Vibra cell soncis, USA) using the parameters- 33 % amplitude, 8s ON pulse, 10s OFF pulse, 120 cycles and 20 mins time. Following that the lysate was centrifuged at 12000 g at 4 for 5 mins. The supernatant containing the DNA was quantified using spectrophotometer (BioSpectrometer kinetic, Eppendorf, Germany). Further immunoprecipitation of CRZ-1::5xGly::V5::GFP bound to hsp80 promoter, the sheared chromatin was incubated with anti GFP antibody (Life Technologies, USA) about 1 µg of antibody was used for 25 µg of DNA. Both the antibody treated and the control (without antibody) were incubated with 50 ul of pre-blocked Protein A magnetic beads (Life Technologies, USA) overnight on a rocking platform in cold room. RIPA buffer [2 mM EDTA, 50mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.1% sodium deoxycholate, 1mM PMSF and 0.1% of FPIC (Sigma Aldrich, USA)] was used to wash the beads. Next, the beads were washed once with high salt, then with low salt and finally with LiCl wash buffer. Elution of the bound chromatin was done using elution buffer (100 mM NaHCO_3, 1% SDS). De-crosslinking of the chromatin was done overnight at 65 using 5M NaCl in a circulating water bath. Then the decrosslinked chromatin was treated with RNAse A for 1 h at 65 followed by proteinase K for 1 h 45. Finally, the chromatin was purified using PCR purification kit (Qiagen, Germany). The DNA was then quantified in Nanophotometer. PCR primers 3F and 4R, 4F and 3R, 2F and 5R, 5F and 2R and 1F and 1R (Table 2) were used in appropriate pairs and using Phusion High Fidelity DNA Polymerase (New England Biolabs, USA) to perform the PCR and determine the binding position of CRZ-1 to hsp80 promoter. The PCRs were performed using reaction conditions 98 for 2 mins, 25 cycles of 98 for 10 s, 63 for 30 s and 72 for 18 s and then 72 for 10 min. The PCR products were run on 1.2 % agarose gel containing EtBr (0.5 µgml^{-1}) and visualization was done using Gel Doc (Bio-Print ST4, Vilber Lourmat, France).

**Electrophoretic mobility shift assay (EMSA)**

Electrophoretic mobility shift assay was performed using Molecular Probes EMSA kit (Thermo Scientific, USA) following manufacturer's protocol. DNA probes were amplified from *N. crassa* wild type genomic DNA as template with primers- 3F, 4R, 5F, EMSA_hsp80 1R, EMSA_hsp80 1F, 2R, Chip NCA-2 1F, Chip
NCA-2 2R, Chip NCA-2 1F, EMSA NCA-2 Rv, EMSA NCA-2 Fw, and Chip NCA-2 2R (Table 2) in appropriate pairs using Phusion High Fidelity DNA Polymerase (New England Biolabs, USA). Further, the PCR products were analysed using 1.2 % agarose gel and then they were purified using QIAquick Gel Extraction kit (Qiagen, Germany). The 30 bp duplex DNA probes were prepared from the complementary primer pairs- Duplex Hsp80_1, Duplex Hsp80_1 comp, Duplex Hsp80_2, Duplex Hsp80_2 comp, Duplex Hsp80_1 Mut, Duplex Hsp80_1 Mut comp, Duplex Hsp80_2 Mut, Duplex Hsp80_2 Mut comp, Duplex NCA-2_1 and Duplex NCA-2_1C, Duplex NCA-2_1, Duplex NCA-2_2C, Duplex NCA-2_Mut 1 and Duplex NCA-2_Mut 1C in appropriate pairs. These primers were initially denatured at 95 °C for 45 min, and then annealed at 55 °C for 50 min using a buffer [10 mM HEPES (pH 7.5), 0.1 mM EDTA, 0.1 M NaCl, and 5 mM MgCl\textsubscript{2}], in a thermal cycler (Arktik, Thermo scientific, USA). To detect the binding the individual DNA probes (5 µM) were incubated with the purified CRZ1::5xGly::V5::GFP protein (50 µM). To resolve protein-DNA complexes, 20 % non-denaturing polyacrylamide (30:0.8)/1 X TBE gel was used. Electrophoresis was performed at 200 volts for 1 h in 1X TBE running buffer. The gels were visualized in a gel documentation system (Bio-Print ST4, Vilber Lourmat, France).

Results

Hsp 80 is necessary for acquisition of thermotolerance in \textit{N. crassa}

To test if Hsp80, belonging to the family of Hsp90 hsps, is involved in the heat shock stress tolerance, we analysed the expression of \textit{hsp80} and \textit{hsp60} in the \textit{cnb-1}\textsuperscript{-}\textit{RIP} mutant strains under the heat shock conditions. The expression of \textit{hsp80}, but not \textit{hsp60}, was reduced by almost ~0.4, 0.3, and 0.2-fold in the 599, 600 and 602 mutant strains in comparison with the wild type under the heat shock conditions (Fig. 1B and 1A). Furthermore, the expression levels of \textit{cnb-1}, \textit{hsp60}, \textit{hsp80}, and \textit{crz-1} were studied in the wild type strain in the VG liquid media with or without supplementation with FK506 both with and without heat shock conditions. The results indicated that expression levels of \textit{cnb-1}, \textit{hsp60}, \textit{hsp80}, and \textit{crz-1} were increased by ~1.7, 2.1, 1.9-fold, but expression levels were reduced by about ~0.8, 0.2, 0.6, and 0.8-fold on supplementation with FK506 under the heat shock stress (Fig. 1C). Therefore, the above results suggested that \textit{hsp60} and \textit{hsp80} are transcriptionally regulated by the calcineurin-crz-1 signaling pathway under the heat shock stress condition.

Previous studies in our laboratory revealed that double mutants of \textit{trm-9} and \textit{nca-2} results in decreased cell survival on exposure to heat shock stress condition as compared to the wild type (Laxmi and Tamuli 2015). Therefore, to analyse the involvement of \textit{trm-9} and \textit{nca-2} genes in the regulation of the heat shock stress conditions, expressions of \textit{trm-9} and \textit{nca-2} were determined in the wild type strain under het shock conditions. The transcript levels of \textit{trm-9} and \textit{nca-2} were increased by ~2 and 3-fold (Fig. 2C), suggesting a requirement of \textit{trm-9} and \textit{nca-2} for the heat shock stress tolerance, and further support the phenotypic results observed previously (Laxmi and Tamuli 2015). Furthermore, to test if the \textit{hsp80} was involved in the \textit{trm-9} and \textit{nca-2} mediated heat shock response, we determined the expressions of both \textit{hsp60} and \textit{hsp80} in the \textit{trm-9} and \textit{nca-2} mutant strains under the heat shock conditions. The expression of the \textit{hsp80}, but not the \textit{hsp60}, in the \textit{Dtrm-9, Dnca-2}, and the \textit{Dtrm-9 Dnca-2} double mutant was reduced by
~0.2, 0.15 and 0.6-fold (Fig. 2B) compared to the wild type strain. Therefore, Hsp80 is necessary for coping up the heat shock stress in *N. crassa*.

**CRZ-1 binds to the promoter of hsp80 under thermotolerance conditions**

To identify the consensus sequences for transcriptional regulation, *hsp80* promoter analysis was performed using an online database Genomatix MatInspector (Quandt et al. 1995; Cartharius et al. 2005). This analysis identified a putative 8 bp sequence for CRZ-1 binding with consensus 5'-GTGGCTG-3' (Table 3), located at about ~1208 bp upstream of the start codon.

The expression of the *crz-1* was increased by ~2.5-fold under heat stress conditions and also the decrease in the fold change of *hsp80* in the wild type strain supplemented with FK506 (Fig. 1C). To determine CRZ-1 binding to the *hsp80* promoter, chromatin immunoprecipitation (ChIP) assay was performed using a tagged version of *crz-1 N. crassa* strain (Table 1) grown in VG liquid media with and without heat shock conditions. To map the CRZ-1 binding region, five pairs of overlapping primers were designed covering 1263 bp of the *hsp80* promoter region. Binding of the CRZ-1 to the *hsp80* was found at two regions, a 174 bp fragment amplified using primers 3F and 4R, and another fragment of 331 bp amplified using 5F and 2R primers (Fig. 4A). The binding intensity of CRZ-1 to the promoter of *hsp80* was increased under the heat shock conditions (Fig. 4B). To further unravel the binding site for CRZ-1 in the promoter of *hsp80*, electrophoretic mobility shift assay (EMSA) was performed. The P*tcu-1::crz-1::5xGly::V5::gfp* (559) strain was grown on VG liquid media to isolate CRZ-1::5xGly::V5::GFP protein and EMSA was performed using four different PCR amplified fragments covering the 174 bp and 331 bp candidate regions, identified in the ChIP assay, in the promoter of hsp80 (Fig. 5A). The EMSA results suggested the binding of CRZ-1 recombinant protein to 174 bp and 161 bp regions of the 331 bp fragment in the promoter of *hsp80* (Fig. 5B). Further, to determine the exact nucleotide sequences in the 174 and 161 bp CRZ-1 binding regions, binding sites were predicted using online prediction tool for DNA binding sites in the Cys\(^2\)His\(^2\) zinc finger proteins (http://zf.princeton.edu; Persikov et al. 2009; Persikov and Singh 2014). Based on the predicted results (Table S1), two 30 bp DNA duplex probes were designed consisting of the first two predicted nucleotide sequences having the highest support vector machine (SVM) score and lowest P-value. EMSA was performed using the DNA probes and it was observed that the recombinant CRZ-1 proteins bind to two probes containing 5\(^\prime\)-CCTTCACA-3\(^\prime\) and 5\(^\prime\)-AGCGGAGC-3\(^\prime\) nucleotide sequences (Figure 5C). However, no gel shift was observed when the mutated version of 5\(^\prime\)-CCTTCACA-3\(^\prime\) and 5\(^\prime\)-AGCGGAGC-3\(^\prime\) nucleotide sequences were used (Fig. 5C), confirming that CRZ-1 specifically binds to the two different 8 bp nucleotide sequences. Therefore, CRZ-1 binds to 5\(^\prime\)-CCTTCACA-3\(^\prime\) and 5\(^\prime\)-AGCGGAGC-3\(^\prime\) 8 bp nucleotide sequences located about 1075 bp and 679 bp upstream of the ATG start codon, and upregulates the expression of *hsp80* under the heat shock stress condition in *N. crassa*.

**Calcineurin-CRZ-1 signaling pathway regulates Ca\(^{2+}\) stress tolerance in *N. crassa***
The cnb-1RIP mutants showed sensitivity to Ca\(^{2+}\) stress when grown in VG agar media supplemented with higher concentration of CaCl\(_2\) about 0.4 M (Kumar et al. 2019). The previous work in our laboratory showed that single and double mutants of the Ca\(^{2+}\) sensor ncs-1 and a Ca\(^{2+}\) ATPase nca-2 were sensitive to Ca\(^{2+}\) stress (Deka and Tamuli 2013) in \textit{N. crassa}. Furthermore, for the tolerance to high Ca\(^{2+}\), CRZ-1 upregulates NCS-1, but CRZ-1 mediated regulation of NCA-2 was not known (Gohain and Tamuli 2019). To test if CRZ-1 binds to the nca-2 promoter, a pair of primers covering 400 bp of the nca-2 promoter were used for ChIP analysis (Fig. 6A). In the ChIP assay, a distinct band of about 400 bp was observed, and intensity of CRZ-1 binding to the nca-2 promoter was increased on addition of 0.2 M CaCl\(_2\) (Fig. 6B). To further unravel the binding site for CRZ-1 in the promoter of nca-2, EMSA was performed using the P\textsubscript{tcu-1}::crz-1::5xGly::V5::gfp (559) strain grown on VG liquid media to isolate CRZ-1::5xGly::V5::GFP protein, and three different PCR amplified fragments covering the 400 bp candidate region in the nca-2 promoter (Fig. 7A). The EMSA results suggested the binding of CRZ-1 fusion protein to a 220 bp region of the 400 bp fragment in the nca-2 promoter (Fig. 7B). Furthermore, to determine the exact nucleotide sequences in the 220 bp region that renders the binding of CRZ-1 to nca-2, putative binding sites were predicted using online prediction tool for DNA binding sites in the Cys\(_2\)His\(_2\) zinc finger proteins (http://zf.princeton.edu; Persikov et al. 2009; Persikov and Singh 2014). From the predicted results (Table S2) two 30 bp DNA duplex probes were designed consisting of the first two predicted nucleotide sequences having the highest support vector machine (SVM) score and lowest P-value. EMSA was performed using the DNA probes and it was observed that the CRZ-1 fusion protein binds to the probe containing 5’-ACCGCGCC-3’ nucleotide sequence (Fig. 7C). However, no gel shift was observed either for the mutated version of 5’-ACCGCGCC-3’ or the DNA duplex having 5’-TGCGCAGC-3’ nucleotide sequence (Fig. 7C), located in the 180 bp region (Fig. 7A) where no binding for CRZ-1 has been observed when EMSA was performed for the binding of CRZ-1 to the promoter of nca-2. Therefore, CRZ-1 specifically binds to an 8 bp nucleotide sequence 5’-ACCGCGCC-3’, located 234 bp upstream of the ATG start codon, and upregulates the expression of nca-2 under the Ca\(^{2+}\) stress condition in \textit{N. crassa}.

**Discussion**

Earlier studies from our laboratory have shown the role of cnb-1RIP mutants in acquisition to thermotolerance (Kumar et al. 2019). Since, different families of Hsps are there so it will be important to decipher overexpression of which hsp family responsible for the acquisition to thermotolerance in \textit{N. crassa}. Expression studies of hsp60 and hsp80 in the wild type strain along with cnb-1RIP mutants and in the \textit{trm-9}, nca-2 and their double mutants suggested that hsp80 has a role in the survival of \textit{N. crassa} under heat shock conditions. Till date, the interaction of hsp90 with cna-1 have been established in \textit{C. albicans} and this pathway have been targeted for antifungal drug development (Gong et al. 2017). Still, the mechanism behind the regulation of hsp80 is limited. Therefore, to unravel this molecular mechanism, analysis of the hsp80 promoter sequence revealed the presence of a 5’-GTGGCTG-3’ 8 bp consensus sequence responsible for CRZ-1 binding. Next, the expression studies depicted the reduced expression of crz-1 under heat shock condition in the wild type strain when the growth medium was
supplemented with FK506, a potent inhibitor of calcineurin. Also, the expression of cnb-1 and hsp80 was reduced in the wild type strain in the presence of FK506 under the heat shock stress. The above observations along with the promoter analysis indicates that CRZ-1 might have a role in the regulation of hsp80 under thermotolerance conditions. Henceforth, to confirm the molecular mechanism behind the calcineurin- CRZ-1 mediated activation of hsp80, we have designed primers to map the promoter of hsp80. The ChIP assay using the CRZ-1 tagged strain under the heat shock condition confirmed the binding of CRZ-1 to the promoter of hsp80. CRZ-1 probably, binds to two specific regions of about 174 bp and 331 bp upstream of hsp80, which are amplified by the primers 3F and 4R, and 5F and 2R, respectively. Thus, to further confirm the exact nucleotide sequences responsible for the binding of CRZ-1, EMSA was performed that revealed that 5´-CCTTCACA-3´ and 5´-AGCGGAGC-3´ 8 bp sequences results in the binding of CRZ-1 to the promoter of under heat shock stress conditions in N. crassa. In general, transcription factors may have multiple binding sites within the promoter of specific target genes, thereby regulates different cell functions (Harbison et al. 2004). In organisms such as S. cerevisiae, computational studies revealed that a single transcription factor can have more than one binding sites in the promoter of the target gene and further, sometimes the specific pattern of the consensus is not followed across the different binding sites (Harbison et al. 2004). In S. cerevisiae, the Reb1 transcription factor binds to many genes with a deviation in the consensus sequence, and more the number of binding sites on the same promoter, greater is the deviation (Bilu and Barkai 2005). A similar trend was observed in CRZ-1 binding sites on the promoter of hsp80, and altogether the binding sequences differed from GTGGCTG, the consensus sequence found in the promoter analysis of hsp80. Also, no calcineurin dependent response element (CDRE) sequence − 5´-AGCCTC-3´ specific for N. crassa (Kumar et al. 2006) was observed in the promoter of hsp80. Therefore, it indicates that the binding sites for the same transcription factor may vary across the genes within the same species.

Previous work from our laboratory showed how the calcineurin activated CRZ-1 binds to the promoter of ncs-1 to upregulate its expression in response to the high concentrations of Ca\(^{2+}\) in the growth medium (Gohain and Tamuli 2019). N. crassa comprises of a group of Ca\(^{2+}\) transporters in the Ca\(^{2+}\) signalling machinery that ensures tight regulation of the Ca\(^{2+}\) entry and exit into the various internal stores (Borkovich et al. 2004; Zelter et al. 2004; Tamuli et al. 2012). Crz1p in S. cerevisiae is known to regulate the Pmc1p and Pmr1p ATPases via calcineurin signaling pathway (Matheos et al. 1997; Hirayama et al. 2003). The homologs of the Pmc1p and Pmr1p ATPases are NCA-2 and PMR-1, respectively, in N. crassa former being localized to both the plasma membrane and vacuoles (Bowman et al. 2012) while the later to the Golgi bodies (Rudolph et al. 1989). Earlier studies shown that both nca-2 and ncs-1 are required for the Ca\(^{2+}\) stress tolerance in N. crassa (Deka and Tamuli 2013). Further, it was shown that CRZ-1 upregulates the expression of ncs-1 under Ca\(^{2+}\) stress (Gohain and Tamuli 2019), but the information about the regulation of nca-2 mediated by calcineurin-CRZ-1 signaling pathway is still limited. In this study, we mapped the promoter of nca-2 for the binding of CRZ-1 binding using ChIP assay. In S. cerevisiae, Crz1p, the homolog of NCA-2, is involved in the induction of Pmc1p in a calcineurin dependent manner (Hirayama et al. 2003). The CRZ-1 binding to an 8 bp sequence 5´-ACCGCGCC-3´ upstream of nca-2 under Ca\(^{2+}\) stress condition, suggesting that similar to the S. cerevisiae PMC1 (Rudolph et al. 1989;
Cunningham and Fink 1994, 1996; Matheos et al. 1997), NCA-2 is transcriptionally regulated via calcineurin signaling pathway. In *S. cerevisiae*, regulation of *PMC1* by CRZ-1 is still not known; however, our study confirmed that CRZ-1 binds to a sequence different from that of the calcineurin-dependent response element (CDRE) sequence 5'-AGCCTC-3' in the *nca-2* promoter (Kumar et al. 2006). Therefore, there could be some unique binding sequence for CRZ1 other than CDRE present in the upstream of *nca-2* which remains unique to *N. crassa*.

We proposed a model governing the regulation of *hsp80* and *nca-2* via calcineurin-CRZ-1 pathway (Fig. 8). During stress conditions, the influx of Ca$^{2+}$ ions increases in the cytoplasm via the Ca$^{2+}$-ATPase transporters and Ca$^{2+}$ channel proteins such as NCA-2. When the [Ca$^{2+}$]$_c$ concentration rises above the threshold level of about 1 mM, then the Ca$^{2+}$ signaling machinery is activated and the Ca$^{2+}$ ion binds to one of the Ca$^{2+}$ sensor CaM which thereafter activates calcineurin complex. The activated calcineurin dephosphorylates its downstream target CRZ-1, which shuttles into the nucleus from the cytoplasm to bind the promoter of both *hsp80* and *nca-2*. The HSP80 protein is involved in the survival of cell against the thermotolerance condition, while NCA-2 transports the excess Ca$^{2+}$ ions into the internal stores, including vacuoles and also exports excess Ca$^{2+}$ to extracellular environment to maintain the Ca$^{2+}$ homeostasis in the cell. Also, when the growth medium is supplemented with FK506 that inhibits calcineurin, the downstream signaling cascade is disrupted and results in reduced expressions of *cnb-1*, *hsp80* and *crz-1* (Fig. 1C). In *C. albicans*, *A. fumigatus*, and *Cryptococcus neoformans*, hsp90 have been reported to be important among different hsps to confer antifungal drug resistance (de Aguiar Cordeiro et al. 2016; Lamoth et al. 2016; Chatterjee and Tatu 2017), therefore, hsp90 could be a new target for increasing the efficacy of antifungal drugs. In *N. crassa*, hsp80 belongs to the hsp90 family of heat shock proteins and shares about 100% similarity structurally to hsp90-domain containing protein (Galagan et al. 2003). Therefore, our study shade light on the regulation of hsp80 in *N. crassa*. Therefore, targeting the calcineurin-CRZ-1 pathway, which activates hsp80 in *N. crassa*, in different pathogenic fungal model systems might unravel new strategies to combat the increasing drug resistance a big challenge in future.

**Declarations**

**Availability of data and materials (data transparency)**

All data generated or analysed during this study are included in this published article (and its supplementary information files). Additional datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

**Competing interests/Conflict of interest** (data transparency)

The authors have no conflicts of interest.

**Funding** (information that explains whether and by whom the research was supported).
The RT laboratory is supported by a DBT-NER twinning grant BT/PR24473/NER/95/737/2017, from DBT, Govt. of India. However, the funder has no role in this publication.

Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Avishek Roy and Ranjan Tamuli. The first draft of the manuscript was written by Avishek Roy and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Acknowledgments

We thank Professor Katherine A. Borkovich and Dr. Shouqiang Ouyang, Department of Plant Pathology & Microbiology, University of California Riverside, USA, for generation of the $P_{tcu-1}::crz-1::5\times Gly::V5::gfp$, $P_{tcu-1}::cnb-1\text{RIP}::5\times Gly::V5::gfp$ and pccg-1_GFP strains. The FGSC generously waived charges for strains. The FGSC was supported by NSF grant BIR-9222772. AR were supported by Research Fellowships from the Ministry of Human Resource Development, Government of India. We thank IIT Guwahati for partial financial support. The RT laboratory is supported by a DBT-NER twinning grant BT/PR24473/NER/95/737/2017, from DBT, Govt. of India.

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| SI No. | Strain    | Strain type or NCU no. | Genotype | Reference             |
|-------|-----------|------------------------|----------|-----------------------|
| 1     | 74-OR23-IVA | Wild type            | Wild type; mat A | FGSC 2489             |
| 2     | ORS-SL6a | Wild type            | Wild type; mat a | FGSC 4200             |
| 3     | 13040    | 04898              | Δtrm-9::hph, mat A | FGSC 13040         |
| 4     | 20       | 20                 | Δtrm-9::hph, mat a | (Laxmi and Tamuli, 2015) |
| 5     | 23       | 23                 | Δnca-2::hph, mat a | (Laxmi and Tamuli, 2015) |
| 6     | 12       | 12                 | Δtrm-9::hphΔnca-2::hph; mat A | (Laxmi and Tamuli, 2015) |
| 7     | 19       | 19                 | Δtrm-9::hphΔnca-2::hph; mat a | (Laxmi and Tamuli, 2015) |
| 8     | 559      | Homokaryotic        | Δcrz-1::hph; Δpan-2::P_{tcu-1}::crz-1::5xGly::V5::gfp, mat a | (Gohain and Tamuli, 2019) |
| 9     | pccg-1_GFP | Homokaryotic        | Δpan-2::P_{ccg-1}::5xGly::V5::gfp, mat a | (Ouyang et al., 2015) |
| 10    | 599 A    | Homokaryotic RIP    | Δcnb1::hph; Δpan2::P_{tcu-1}::cnb-1\_RIP::5xGly::V5::gfp, mat A | (Tamuli et al., 2016) |
| 11    | 600 A    | Homokaryotic RIP    | Δcnb1::hph; Δpan2::P_{tcu-1}::cnb-1\_RIP::5xGly::V5::gfp, mat A | (Tamuli et al., 2016) |
| 12    | 602 A    | Homokaryotic RIP    | Δcnb1::hph; Δpan2::P_{tcu-1}::cnb-1\_RIP::5xGly::V5::gfp, mat A | (Tamuli et al., 2016) |

**Table 2** List of primers used in the study
| Sl No. | Name of the Primer | Sequence (5'→3') | Reference                  |
|-------|-------------------|------------------|----------------------------|
| 1     | Hsp60 ARrt Fw     | GTCCTCATCGAGTCCAGCTT | This study                 |
| 2     | Hsp60 ARrt Rv     | CCGAGGTTTCTCGAATTGTC | This study                 |
| 3     | Hsp80RT-Fw        | CGAACAAGACCTTCACATC | This study                 |
| 4     | Hsp80RT-Rv        | GAGCGGGCAATAGTACCAAG | This study                 |
| 5     | RT-CNB-1-F        | GGCAACAAGGAGCAGAAGCT | This study                 |
| 6     | RT-CNB-1-R        | CCTCCCATGATCGTTTGTGC | This study                 |
| 7     | RT-NCU07952-F     | GATGTTCTCTCGGTAGCCCA | (Gohain and Tamuli, 2019) |
| 8     | RT-NCU07952-R     | CGTCGACAGACTGAAGTTG | (Gohain and Tamuli, 2019) |
| 9     | RT-NCU04898-F     | CCTCTCTACCTCCACGCCTA | (Laxmi and Tamuli, 2017)   |
| 10    | RT-NCU04898-R     | CCAACAAAAAGGTCCATTCT | (Laxmi and Tamuli, 2017)   |
| 11    | RT-NCU04736-F     | GAGATGACTCCTCCAGTC  | (Laxmi and Tamuli, 2017)   |
| 12    | RT-NCU04736-R     | GGAGTTGCTGTGATTGG   | (Laxmi and Tamuli, 2017)   |
| 13    | q-B-tub-FW        | CCCAAGAACATGATGGCTGC | (Barman and Tamuli, 2017)  |
| 14    | q-B-tub-Rv        | TTGTCTGAGCAGTCCGAGTC | (Barman and Tamuli, 2017)  |
| 15    | 1F                | CATCGAATTCTGTGCTGCG | This study                 |
| 16    | 1R                | GAGAAGCGTGAGAGTCAGTC | This study                 |
| 17    | 2F                | ACCCGGTATTTGGAGTTGA | This study                 |
| 18    | 2R                | ATTGGGAAGACTGAGGGAA | This study                 |
| 19    | 3F                | AGGCTTCTTGAACACGGCAG | This study                 |
| 20    | 3R                | GGGAATGAAATGACTCCAGA | This study                 |
| 21    | 4F                | GCCTCGAGGATTCTGAGTC | This study                 |
| 22    | 4R                | CCCTGTGGTCTGTGTGTAGT | This study                 |
| 23    | 5F                | AAGAGGGACCTGCCCCCTC | This study                 |
|   |   |   |   |   |
|---|---|---|---|---|
| 24 | 5R | CCATCCGCATTTCCTCAGCT | This study |
| 25 | Chip 1F Hsp60 | AAATTTCATGCTAGGGGACG | This study |
| 26 | Chip 1R Hsp60 | TTTGGGGGTATTGTGTGAAG | This study |
| 27 | ChIP NCA-2 1F | gacagcaacagcgacctgac | This study |
| 28 | ChIP NCA 1R | gatagctggcactcaagggc | This study |
| 29 | EMSA_hsp80 1F | GGCCGCAAACGAATCAGAG | This study |
| 30 | EMSA_hsp80 1R | GTTGCTTGCTATCCCCACAT | This study |
| 31 | EMSA NCA-2 Fw | agcaacgcgtacattcgaaca | This study |
| 32 | EMSA NCA-2 Rv | CAGCTCAGGCGAAGCTCTGGT | This study |
| 33 | Duplex Hsp80_1 | GGACGTAGCACGAGCGACGAGGACGAC | This study |
| 34 | Duplex Hsp80_1 comp | CCTGCAATCGTGCGTGCTGGCTCCCGTG | This study |
| 35 | Duplex Hsp80_2 | ACCTGCCCTCAGCGAGCGATTTGGAGCG | This study |
| 36 | Duplex Hsp80_2 comp | TGGACGGGAGGTCGCTGGCTAAACCTGCC | This study |
| 37 | Duplex Hsp80_1 Mut | GGACGTAGCAATATATTACTAAGGAGGACGAC | This study |
| 38 | Duplex Hsp80_1 Mut comp | CCTGCAATCGTTATGATTGCTCCCGTG | This study |
| 39 | Duplex Hsp80_2 Mut | ACCTGCCCTCCTATTCTAGATTGGAGGGACG | This study |
| 40 | Duplex Hsp80_2 Mut comp | TGGACGGGAGGATAAGATCTAAACCTGCC | This study |
| 41 | Duplex NCA-2_1 | tggctctgctacggcctgcatttcca | This study |
| 42 | Duplex NCA-2_1C | ACCGAGACAGATGGCGCGAGCTAAACGCT | This study |
| 43 | Duplex NCA-2_2 | gatctgctgcctgagctgcgtggttcg | This study |
| 44 | Duplex NCA-2_2C | CTCAGACGGAGCGCTCGACTGCCCAGC | This study |
| 45 | Duplex NCA-2_1 Mut | tggctgctgctCAATATAAtcgattttca | This study |
| 46 | Duplex NCA-2_1 Mut C | ACCGAGACAGATTTATAGCTAAACGCT | This study |
| 47 | Duplex NCA-2_2 Mut | gatctgctgcgtCTACTAtgacggttcg | This study |
Table 3 List of elements binds to the promoter of *hsp80*

| Sl No. | Name of the upstream element                                              | Sequence (5'→3')     |
|--------|---------------------------------------------------------------------------|----------------------|
| 1      | Ribosomal RNA processing element                                          | atctttTTTCTact      |
| 2      | pH responsive regulators                                                  | caccGCCAagtgctata    |
| 3      | Yeast heat shock factors                                                  | gattctgccgtTTCAagaagcctcttgattg |
| 4      | Pheromone response elements                                               | tcttgaAACAggc        |
| 5      | Calcineurin-responsive zinc finger 1 binding factor                       | gtGGCTg              |
| 6      | Yeast stress response elements                                            | GgacccaGGGGccgg       |
| 7      | Repressor of hypoxic genes                                                | cccaTCGTtcagc        |
| 8      | Regulator of Drug Sensitivity                                             | ttgCGGCcgttg          |

**Figures**
Figure 1

Transcriptional studies of the hsp6, cnb-1, and crz-1 genes under the heat shock condition. (A) Relative expression of hsp60 in the wild type and cnb-1RIP strains (599 A, 600 A, and 602 A) under the heat shock conditions. Fold changes were calculated using 2-ΔΔCT method (Livak and Schmittgen 2001) by taking control (without heat shock) as calibrator and β-tubulin as endogenous control. The standard deviation was calculated from the data of three individual experiments each with three triplicates (n = 9). (B) Relative expression of hsp80 was performed in the cnb-1RIP strains (599 A, 600 A and 602 A) in comparison to the wild type under heat shock conditions. Fold changes were calculated using 2-ΔΔCT method (Livak and Schmittgen 2001) by taking control (without heat shock) as calibrator and β-tubulin as endogenous control. The standard deviation was calculated from the data of three individual experiments each with three triplicates (n = 9) with P values <0.05 (*), <0.01 (**), <0.001 (***) and otherwise non-significant (ns) as measured by one-way ANOVA test. (C) Relative expression studies for cnb-1, hsp60, hsp80, and crz-1 was performed in the wild type strain under heat shock conditions in VG liquid media supplemented with or without FK506 as indicated. Fold changes were calculated using 2-ΔΔCT method (Livak and Schmittgen 2001) by taking control wild type (without heat shock) as calibrator and β-tubulin as endogenous control. The standard deviation was calculated from the data of three individual experiments each with three triplicates (n = 9) with P values <0.05 (*), <0.01 (**), <0.001 (***) and otherwise non-significant (ns) as measured by one-way ANOVA test.
Figure 2

Transcriptional analysis of hsp in the wild type, Δtrm-9, Δnca-2 and their respective double mutants. (A) Relative expression of hsp60 across trm-9, nca-2 and their double mutants being subjected to thermal stress. Fold changes were calculated using 2-ΔΔCT method (Livak and Schmittgen 2001) by taking as wild type (with heat shock) control as calibrator and β-tubulin as endogenous control. The standard deviation was calculated from the data of three individual experiments each with three triplicates (n = 9). The Standard deviation was calculated from the data of three individual experiments each with three triplicates (n = 9) with P values <0.05 (*), <0.01 (**), <0.001 (***) and otherwise non-significant (ns) as measured by one-way ANOVA test. (B) Relative expression of hsp80 across trm-9, nca-2 and their double mutants being subjected to thermal stress. Fold changes were calculated using 2-ΔΔCT method (Livak and Schmittgen 2001) by taking as wild type (with heat shock) control as calibrator and β-tubulin as endogenous control. The standard deviation was calculated from the data of three individual experiments each with three triplicates (n = 9). The Standard deviation was calculated from the data of three individual experiments each with three triplicates (n = 9) with P values <0.05 (*), <0.01 (**), <0.001 (***) and otherwise non-significant (ns) as measured by one-way ANOVA test. (C) Relative expression of trm-9 and nca-2 in the wild type strains being subjected to thermal stress. Fold changes were calculated using 2-ΔΔCT method (Livak and Schmittgen 2001) by taking as wild type (without heat shock) control as calibrator and β-tubulin as endogenous control. The standard deviation was calculated from the data of three individual experiments each with three triplicates (n = 9). The Standard deviation was calculated
from the data of three individual experiments each with three triplicates (n = 9) with P values <0.05 (*), <0.01 (**), <0.001 (***) and otherwise non-significant (ns) as measured by one-way ANOVA test.

Figure 3

NCA-2 has a role in the calcineurin mediated Ca2+ stress response. (A) Relative expression of nca-2 in the cnb-1RIP mutants under high concentrations of Ca2+. Fold changes were calculated using 2-ΔΔCT method (Livak and Schmittgen 2001) by taking wild type (with 200 mM CaCl2) control as calibrator and β-tubulin as endogenous control. The standard deviation was calculated from the data of three individual experiments each with three triplicates (n = 9) with P values <0.05 (*), <0.01 (**), <0.001 (***) and otherwise non-significant (ns) as measured by one-way ANOVA test. (B) Relative expression of cnb-1, nca-2, and crz-1 in the wild type under high concentrations of Ca2+ in VG liquid media supplemented with or without FK506 as indicated. Fold changes were calculated using 2-ΔΔCT method (Livak and Schmittgen 2001) by taking wild type control as calibrator and β-tubulin as endogenous control. The standard VG media contains 0.68 mM CaCl2. The standard deviation was calculated from the data of three individual experiments each with three triplicates (n = 9) with P values <0.05 (*), <0.01 (**), <0.001 (***) and otherwise non-significant (ns) as measured by one-way ANOVA test.
Figure 4

Chromatin immunoprecipitation for the binding of CRZ-1 to the promoter of hsp80. (A) Schematic representation of the primer pairs used for hsp80 ChIP analysis. Five pairs of primers were designed to perform PCR for the hsp80 promoter region using the DNA template obtained in the ChIP are located between the 5' UTR of the hsp80 gene and the hsp80 ORF. The primers 3F and 4R to amplify 174 bp fragment (I), 4F and 3R to amplify 231 bp fragment (II), 2F and 5R to amplify 173 bp fragment (III), Chip 5F and 2R to amplify 331 bp fragment (IV), 1F and 1R were used to PCR amplify fragment (V) of 545 bp size as indicated in the diagram. The ATG start codon is indicted using an arrow. (B) PCR analysis of the ChIP assay to identify the CRZ-1 binding region in the hsp80 promoter sequence. The PCR products were resolved using a 1.2% agarose gel and 100 bp ladder (New England Biolabs, USA) as marker. (C) The PCR amplification for each of the five fragments (I to V) is indicated in the gel. The analysis of the PCR revealed a positive product for the fragment I and IV of 174 bp and 331 bp, respectively, and the band intensity was further enhanced when the 559 strain was grown in the presence of heat shock stress condition. The antibody control (Ab-) indicates the control PCR for the sample, where only beads were
used for immunoprecipitation. The wild type genomic DNA has used a positive PCR control for amplification of all the five fragments.

Figure 5

Electrophoretic mobility shift assay to identify the CRZ-1 binding nucleotide sequence in the promoter of hsp80. (A) Schematic representation for the position of the PCR primers in the hsp80 promoter to map the CRZ-1 binding sequence in the fragments I and IV identified using the ChIP analysis. The primers, indicated using arrows, were used for PCR amplification of four different DNA probes (I), (IV), (VI), and (VII). Also, the positions of the duplex DNA probes in the CRZ-1 binding regions located in the hsp80 promoter fragments of 174 bp and 161 bp size are show. The positions of the two duplex DNA probe 1 and 2 of 30 bp each are indicated below the hsp80 promoter. The position of mutated probes 3 and 4 are the same as that of 1 and 2. The primers to obtain each of the probes are indicated in the parentheses for the respective probe. The size (in bp) of the PCR products and the ATG start codon, shown using an arrow, are indicated. (B) CRZ-1 binding to the specific DNA probes in the promoter of hsp80. The 100 bp DNA ladder (New England Biolabs, USA) was used as DNA marker (lane 1), DNA probes only controls (lanes 2, 4, 6, and 8), and the reaction mixture containing both the DNA probe and the CRZ-1::5xGly::V5::GFP protein (lanes 3, 5, 7, and 9) were resolved in a 5% non-denaturing polyacrylamide gel and stained with SYBR® Green. The DNA probe I (lanes 2 and 3), IV (lanes 4 and 5), VI (lanes 6 and 7), and VII (lanes 8 and 9) were used either alone or with the protein as described above. The gel shifts were observed for the probes I of 174 bp (lane 3) and VI of 161 bp (lane 7), which fall within the probe IV of 331 bp that has also shown a shift (lane 5). Therefore, CRZ-1::5xGly::V5::GFP protein has two binding
regions in the hsp80 promoter. (C) Identification of the CRZ-1 binding sequence in the hsp80 promoter region. The DNA probes 1 and 2 were used alone as controls (lanes 1 and 3, respectively) or together with the CRZ-1::5xGly::V5::GFP protein (lanes 2 and 4, respectively). Shift was observed when probes 1 and 2 were used together with the protein (lanes 2 and 4), showing that CRZ-1::5xGly::V5::GFP binds to probes 1 and 2 that contain the predicted CRZ-1 binding sequence sites 5’-CCTTCACA-3’ and 5’-AGCGGAGC-3’.

The shift was not observed for probes 3 and 4 containing mutations in the predicted CRZ-1 binding sites (lanes 6 and 8). In lanes 9 and 10, 5xGly::V5::GFP protein with probes 1 and 2 was used as a negative control, and no shift was observed.

Figure 6

Chromatin immunoprecipitation for the binding of CRZ-1 to the promoter of nca-2. (A) Schematic representation of the primer pairs and their positions for nca-2 ChIP analysis. The primers designed to perform PCR for the nca-2 promoter region using the DNA template obtained in the ChIP are located between the 5’ UTR and ORF of the nca-2 gene. The primers Chip NCA-2 1F and Chip NCA-2 1R were used to PCR amplify a fragment of 400 bp size as indicated in the diagram. (B) The PCR products were resolved using a 1.2% agarose gel to identify the CRZ-1 binding region in the nca-2 promoter sequence.
The PCR amplification for the fragment is indicated in the gel. The analysis of the PCR revealed a positive product for the fragment of 400 bp in size, and the band intensity was further enhanced when the 559 strain was grown in the presence of 0.2 M CaCl2 stress condition. The antibody control (Ab-) indicates the control PCR for the sample, where only beads were used for immunoprecipitation. The 100 bp DNA ladder (New England Biolabs, USA) was used as marker.

Figure 7

Electrophoretic mobility shift assay to identify the CRZ-1 binding nucleotide sequence in the promoter of nca-2 (A) Schematic representation for the position of the PCR primers to map the CRZ-1 binding sequence in the nca-2 promoter shown using a bar. The primers, indicated using arrows, used for PCR amplification of three different DNA probes (I), (II), and (III) of indicated size (bp) were for the EMSA. The relative position of the TATA box with respect to the primer position is shown. Also, the positions of the two duplex DNA probe A and B of 30 bp each are indicated below the nca-2 promoter. The position of mutated probes C and D is the same as that of A and B. The primers to obtain each of the probes are indicated in the parentheses for the respective probe. (B) CRZ-1 binding to the specific DNA probe in the promoter of nca-2. The 100 bp DNA ladder (New England Biolabs, USA) used as marker (lane 1), DNA probes only controls (lanes 2, 4, and 6), and the reaction mixture containing both the DNA probe and the CRZ-1::5xGly::V5::GFP protein (lanes 3, 5, and 7) were resolved in a 5% non-denaturing polyacrylamide gel and stained with SYBR® Green. The DNA probe I (lanes 2 and 3), II (lanes 4 and 5), and III (lanes 6 and 7) were used either alone or with the protein as described above. The gel shifts were observed for probes I of 400 bp (lane 3) and II of 220 bp (lane 5), which further maps the CRZ-1::5xGly::V5::GFP protein binding region in the nca-2 promoter. (C) Identification of the CRZ-1 binding sequence in the nca-2 promoter
region. The DNA probes A, B, C, and D were used alone as controls (lanes 1, 3, 5, and 7, respectively) or together with the CRZ-1::5xGly::V5::GFP protein (lanes 2, 4, 6, and 8, respectively). A shift was observed when probe A was used together with the protein (lane 2), showing that CRZ-1::5xGly::V5::GFP binds to probe A that contains the predicted CRZ-1 binding sequence site 5'-ACCGCGCC-3'. The shift was not observed either for the probe B having the sequence 5'-TGCGCAGC-3' or probe C containing mutations in the predicted CRZ-1 binding site as mentioned in probe A (lanes 4 and 6). No binding was observed in lane 8 containing probe D, which is the mutated version of probe B. In the lane 9, 5xGly::V5::GFP protein with probe A and was used as a negative control, and no shift was observed. Similarly, in lane 10, 5xGly::V5::GFP protein with probe B was used, and there was no shift.

Figure 8

Model of calcineurin-CRZ-1 signaling pathway in the regulation of Hsp80 and NCA-2 in N. crassa. The model depicts an insight into the regulation of Hsp80 and NCA-2 via calcineurin-CRZ-1 pathway in response to abiotic stress condition. The heat shock and Ca2+ stress conditions in the cell result in the increase of cytosolic [Ca2+]c, which activates Ca2+ sensors, including calmodulin (CaM) that follows activation of calcineurin and CRZ-1. Activated CRZ-1 then nuclear localized and binds the promoter of its target genes, including hsp80 and nca-2, and upregulates their expression to cope the heat shock and Ca2+ stress responses in N. crassa. Supplementation of FK506, an inhibitor calcineurin, prevents
calcineurin activation thereby downregulates the expressions of crz-1, hsp80, and nca-2 ensuing decreased viability of the cells in response to stress conditions. Also, another probable mechanism for regulation of hsp80 which indicates there could be a possible interaction of Hsp80 with CNA, which can further drive the calcineurin-CRZ-1 signaling pathway in response to stress conditions, is shown in the model.

**Supplementary Files**

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- SupplementaryTables16012021.docx