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The *Neurospora crassa cmd*, *trm-9*, and *nca-2* Genes Play a Role in Growth, Development, and Survival in Stress conditions

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Abstract The calmodulin protein antagonists, trifluoperazine (TFP) and chlorpromazine (CPZ) inhibit the growth, carotenoids accumulation and sexual development of *Neurospora crassa*. In addition, *N. crassa* strains lacking *trm-9*, a cation-ATPase, showed defect in growth. Moreover, strains lacking both *trm-9* and another Ca^{2+}-ATPase *nca-2*, exhibited a severe growth defect, an increased sensitivity to $CaCl_2$, and a reduction in acquisition of thermotolerance induced by heat shock temperature. Therefore, the *cmd*, *trm-9*, and *nca-2* play a role in growth, survival in calcium stress and induced heat shock temperature in *N. crassa*.

Keywords Calcium signaling; Ca^{2+}/cation ATPases; Calmodulin; *Neurospora crassa*; *nca-2*; thermotolerance

Introduction

Calcium (Ca^{2+}) signaling is involved in regulating numerous processes in eukaryotes ranging from fungi to mammals. The Ca^{2+}-signaling process is initiated primarily due to transient raise in concentration of cytosolic free Ca^{2+} ($[Ca^{2+}]_c$), which is recognized by Ca^{2+} sensor proteins. One of the versatile and evolutionary conserved Ca^{2+}-sensor proteins is calmodulin (CaM), which binds Ca^{2+} with high affinity and specificity. CaM plays an important role in modulating DNA repair, DNA synthesis, cell proliferation, cyclic nucleotide and glycogen metabolism, secretion, motility and Ca^{2+} transport (Means and Dedman 1980; Smallwood et al., 2009). CaM also plays an important role for the regulation of stress response pathways in pathogenic fungi *Candida albicans* and *Cryptococcus neoformans* (Kraus and Heitman, 2003). In the budding yeast *Saccharomyces cerevisiae*, CaM is required for mitotic progression and acquisition of induced thermotolerance (Iida et al., 1995). Similar to the *S. cerevisiae*, in the filamentous fungi *Aspergillus nidulans*, CaM is critical for the progression through the G2/M transition (Kahl and Means 2003).

The filamentous fungus *Neurospora crassa* has a unique calcium (Ca^{2+}) signaling machinery, CaM is encoded by NCU04120 that appears to be an essential gene for viability (Galagan et al., 2003; Borkovich et al., 2004; Tamuli et al., 2013). In *N. crassa*, unlike the vertebrate counterparts, only one CaM gene has been identified (Capelli et al., 1993; Cox et al., 1982; Perez et al., 1981; Galagan et al., 2003). In vertebrates, CaM protein is encoded by multiple genes, for example, six genes have been detected in zebra fish, three genes in human and rat, two genes in frog and two genes in chicken (Luan et al., 2007). Coding sequence of the CaM encoding gene NCU04120 contains six exons and five introns, and CaM possesses conserved EF-hand domains (Tamuli et al., 2013). In *N. crassa* CaM antagonists, trifluoperazine (TFP) and chlorpromazine (CPZ) caused shortening of period length of the conidiation rhythm and light induced phase shifting (Sadakane and Nakashima 1996; Suzuki et al., 1996). In addition, possible role of CaM in activation of chitin synthase enzyme in *N. crassa* was studied by examining the effects of TFP on protoplast regeneration (Suresh and Subramanyam, 1997).

One of the targets of CaM is the Ca^{2+}-ATPase, a Ca^{2+} pump that help in fine tuning of Ca^{2+} homeostasis in cells by pumping Ca^{2+} out of cells. Ca^{2+}-ATPases hydrolyze ATP to catalyze active Ca^{2+}-efflux across biological membranes, and maintain a steep Ca^{2+} gradient across the plasma membrane (Hao et al.,

1994). CaM stimulates plasma membrane Ca^{2+}-ATPase (PMCA) activity by binding to an autoinhibitory domain of PMCA. The CaM-binding domain is located near the C-terminus of PMCA (Osborn et al., 2004; Giacomello et al., 2013). Besides interacting with (Ca^{2+}+Mg^{2+}) -activated ATPase in isolated cardiac sarcoplasmic reticulum and RBC membrane, CaM also interacts with ciliary dynein ATPase of *Tetrahymena* (Blum et al., 1980; Kirchberger and Antonetz, 1982; Lopes et al., 1990). In plants, Ca^{2+}-activated CaM regulates different Ca^{2+}-ATPases (Peerseen et al., 1997; Harper et al., 1998; Hong et al., 1999; Chung et al., 2000; Malmström et al., 1997, 2000). In *N. crassa*, nine ATPases have been identified and they possess conserved cation transporter/ATPase domain in the proteins (Galagan et al., 2003; Borkovich et al., 2004; Tamuli et al., 2013). *N. crassa* ATPases are found distributed in different branches during a phylogenetic analysis, NCA1 in ERCA, NCA2 and NCA3 in PMCA, PMR1 in PMR1, and PH-7 in ENA branch (Haro et al., 1991; Benito et al., 2000). Lack of NCA-2 results in slow growth, Ca^{2+} sensitivity, female sterility, and accumulation of more Ca^{2+} than the wild-type; indicating that it functions in the plasma membrane to pump Ca^{2+} out of the cell (Bowman et al., 2011). NCA-2 is more similar to the PMC-type proteins of animal cell than the Pmc1p in *S. cerevisiae* that resides in the vacuole (Bowman et al., 2011). In addition, one of the cation-ATPases *trm-9*, which is encoded by the gene NCU04898, shows sequence homology to *spf1* gene of *S. cerevisiae.* SPF1 family ATPases genes are conserved from yeast to human; however, the functions of these ATPases remain unclear. SPF1 is not essential for cell viability and its substrate specificity is unknown and loss of SPF1 may perturb homeostasis of ions that affects modification and sorting of proteins in the secretory pathway of yeast (Cronin et al., 2000; Suzuki, 2001).

To investigate the cellular role of CaM in *N. crassa*, we used CaM antagonists, TFP and CPZ. Moreover, we studied two other genes *trm-9* and *nca-2* using their knockout mutants. We found that the *cmd*, *trm-9*, and *nca-2* genes play a role in growth, Ca^{2+} sensitivity, and in acquisition of thermotolerance induced by heat shock temperature in *N. crassa*.

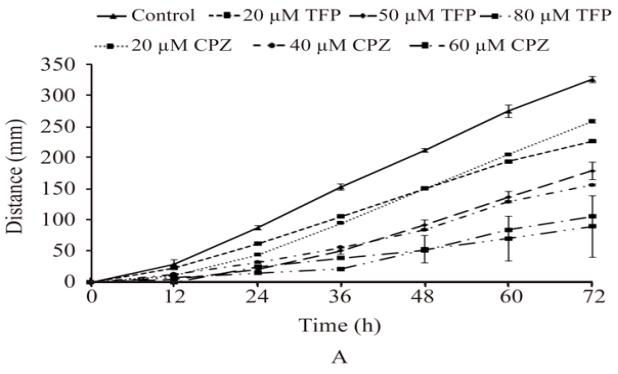
Materials and Methods

Sequence analysis

BLAST (Altschul et al., 1990) analysis was performed using software tools available from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the proteins were selected based on *E* value, % identities and gapes as described previously (Tamuli et al., 2011). The Conserved Domain Database (CCD) (Marchler-Bauer et al., 2009) database was used to identify conserved domains in the protein. The homologue protein sequences were aligned with ClustalX 1.83 (Thompson et al., 1997) and visualized using GeneDoc (Nicholas et al., 1997). Phylogenetic trees were constructed from the aligned sequences using the minimum-evolution method (Rzhetsky and Nei, 1992), 500 bootstrap replications as test of phylogeny (Felsenstein 1985) and the software MEGA5 (Tamura et al., 2011). Promoter region was analyzed by selecting ~2 kb sequences from upstream of Transcription Start Site and transferred to MatInspector in Genomatix software (http://www.genomatix. de/cgi-bin//matinspector_prof/mat_fam) to predict transcription factor binding sites (Quandt et al., 1995).

Strains, growth, crosses maintenance

N. crassa wild-type strains 74-OR23-1 *mat A* (FGSC 987), 74-OR8-1 *mat a* (FGSC 988), Ca^{2+} signaling mutant strain ΔNCU04898.2::*hph mat A* (FGSC 1304 0), and ΔNCU04736.2::*hph mat* A (FGSC 13071) were generated by the *Neurospora* genome project and obtained from the Fungal Genetics Stock Center (FGSC; University of Missouri, Kansas city, MO 64110) (Colot et al., 2006; McCluskey 2010). The ΔNCU04898.2::*hph* ΔNCU04736.2::*hph* double mutant was generated by crossing the individual single mutant strain, and presence of the knockout alleles



A

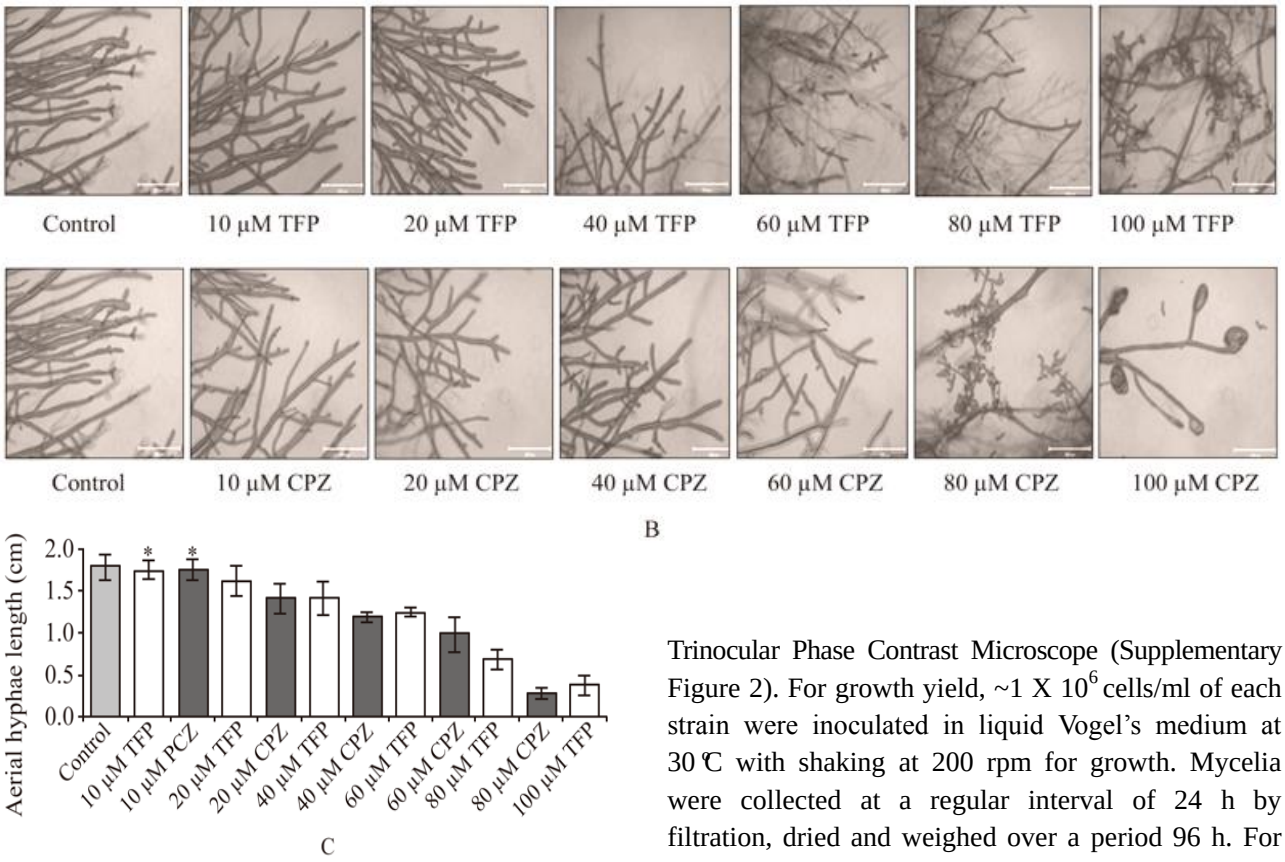


Figure 1 Effect of trifluoperazine (TFP) and chlorpromazine (CPZ) on growth of *N. crassa*. (A) Effect of TFP and CPZ at various concentrations on apical growth. (B) Abnormal hyphal morphology with increasing concentrations of TFP and CPZ. (C) Aerial hyphae length of cultures grown for 72 h in various concentrations of TFP and CPZ. Error bars indicate the standard errors calculated from the data for three independent experiments. Statistically significant values are indicated by asterisks, *P < 0.05

were verified using polymerase chain reactions (PCR) of the progeny strains (Supplementary Figure 1).

Growth, crossing, and maintenance of *Neurospora* strains were essentially as described by Davis and De Serres (1970). The apical growth was analyzed by using standard race tube assay and calculated as cm h^{-1} (Ryan et al., 1943, 1950). For aerial hyphae, ~1 X 10^6 cells/ml of each strain was grown in liquid Vogel's sucrose media (VSM) and incubated at 30°C for 48 h in dark followed by 24 h light illumination at room temperature and height of aerial hyphae was measured (Deka and Tamuli, 2013). Conidial count was done after 72 hours of growth; a sample of each strain was withdrawn and harvested using sterile water followed by conidial counting using a haemocytometer under a Trinocular Phase Contrast Microscope (Supplementary Figure 2). For growth yield, ~1 X 10^6 cells/ml of each strain were inoculated in liquid Vogel's medium at 30 ℃ with shaking at 200 rpm for growth. Mycelia were collected at a regular interval of 24 h by filtration, dried and weighed over a period 96 h. For analysis of hyphal morphology, strains were grown for 12 h on a thin layer of Vogel's agar on glass slide, and observed under microscope at 20X magnification. In addition, statistical significance was performed according to variance analysis (ANOVA, $P < 0.05$).

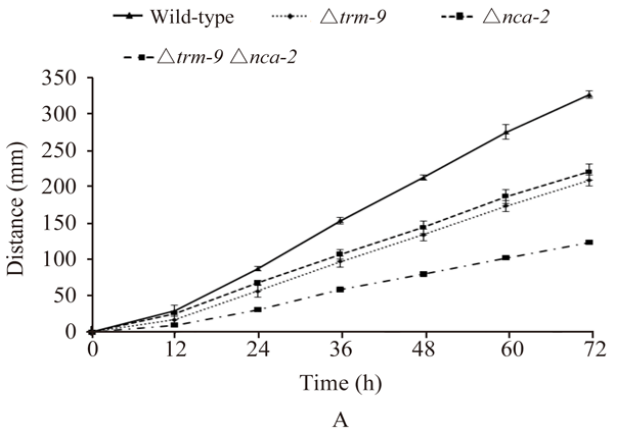
Assay for calcium sensitivity and thermotolerance

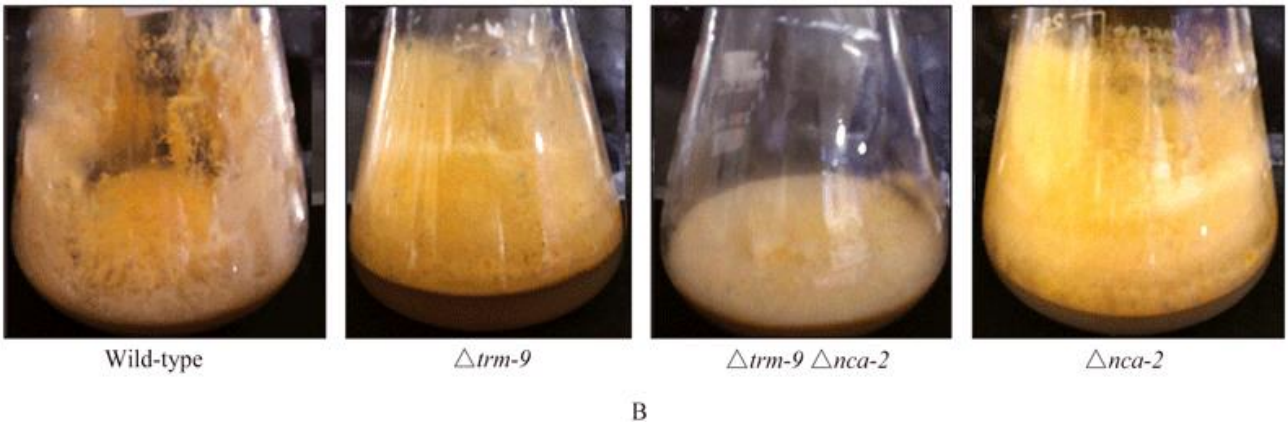
Assay for calcium sensitivity was done essentially as described previously (Deka et al., 2011). Briefly, conidia was placed in the centre of petri dishes containing Vogel's glucose (1.5%) media supplemented with 0.0 M, 0.2 M, 0.3 M, 0.4 M $CaCl_2$ incubated at 30 ℃ and colony diameter was measured every 3 h over a period of 24 h and growth rates were calculated as cm h^{-1}. For measuring thermotolerance, three days-old conidia were inoculated into liquid Vogel's Medium at a concentration of ~1 X 10^6 cells/ml and germinated for 2 h with shaking at 200 rpm at 30°C. These germlings were exposed to different heat treatment condition in two sets one set was held at 30°C for uninduced condition and the other set at 44°C for induced condition for 30 min, then one set of each were given a lethal heat shock at 52°C for 20 min. (Yang Qi and Borkovich, 1999; Kumar and Tamuli, 2014). After that these conidia were spread on sorbose agar (0.05 % fructose, 0.05 % glucose, 2% sorbose, 2 %

Bacto agar) plate and incubated at 30°C for 2 days. Percent survival was obtained by dividing the number of viable colonies on plates subjected to heat treatment by the number of viable colonies on plates held at 30°C (control) and multiplying by 100.

Carotenoid accumulation

To measure carotenoid accumulation, ~1 X 10^6 cells/ml of each *N. crassa* strains were inoculated into Vogel's sucrose (2%) medium supplemented with 0.2% Tween 80 used as a wetting agent to prevent conidiation (Zalokar, 1954) and kept for growth at 30°C for two days in dark, and at room temperature for one day under light. After that mycelia were filtered, lyophilized and powdered. Carotenoids were extracted from 50 mg lyophilized powder by using acetone and hexane. Total carotenoids content were calculated by measuring the maximum absorbance value at 470 nm and using formula: Total carotenoid content (µg/g) = [Total absorbance x Total volume of extract (ml) x 10^4]/ [Absorbance coefficient (2500) x Sample weight (g)] (Rodriguez-Amaya and Kimura 2004).





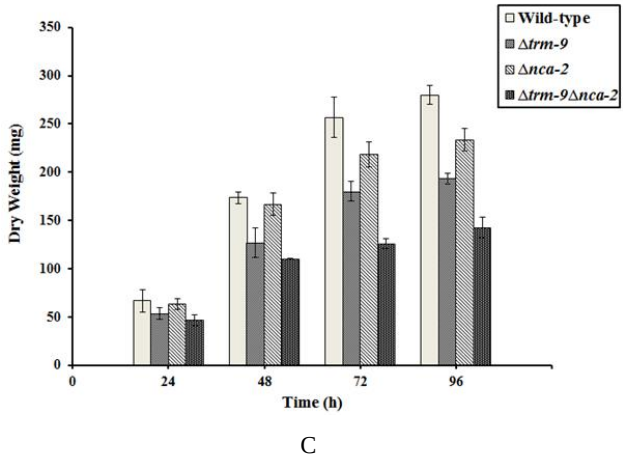


Figure 2 Growth phenotypes. (A) Rate of apical growth of the wild-type, Δ*trm-9*, Δ*nca-2*, and Δ*trm-9*Δ*nca-2* strains were measured using race tubes. Growth rate of Δ*trm-9*Δ*nca-2* double mutant strain was lesser as compared to parental single mutants and wild-type strain. (B) Colony morphology of wild-type, Δ*trm-9*, Δ*nca-2*, and Δ*trm-9*Δ*nca-2* double mutant strains. The Δ*trm-9*Δ*nca-2* double mutant strain showed matty-like growth and reduced pigmentation. (C) Dry weight of Δ*trm-9*, Δ*nca-2*, Δ*trm-9*Δ*nca-2* and wild-type strains. Dry weight yield of Δ*trm-9*Δ*nca-2* double mutant strain was less than parental single mutants and the wild-type

Results and Discussion

NCU04120, NCU04898, and NCU04736 genes encode CaM, TRM-9, and NCA-2, respectively, and contain conserved domains

The *N. crassa* calmodulin gene (*cmd*) NCU04120 encodes a highly conserved Ca^{2+} signaling protein CaM that possesses four conserved EF hand motifs (Supplementary Figure 3A). Similarly, the NCU04898 encodes a Ca^{2+}/cation ATPases annotated as TRM-9 (http://www.broadinstitute.org/annotation/genome/neur

ospora/MultiHome.html). The TRM-9 possesses one conserved E_1-E_2 ATPases domain, and one halo-acid dehalogenase like hydrolase domain (Supplementary Figure 3B). In addition, phylogenetic analysis revealed that CaM and TRM-9

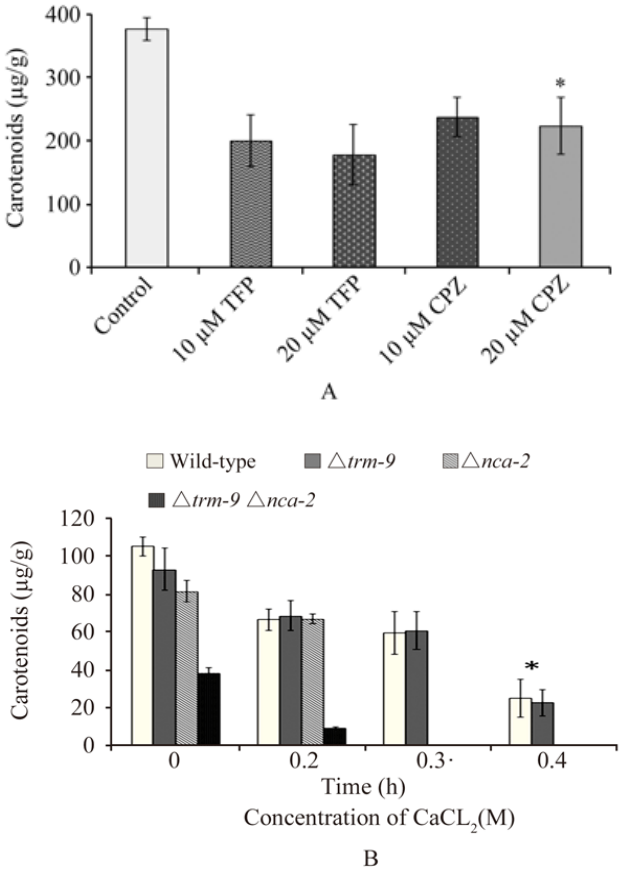


Figure 3 Analysis of carotenoids content. (A) Carotenoid content of wild-type strain in the presence of CaM antagonist TFP and CPZ. Standard errors calculated from the data for three independent experiments are shown using error bars. TFP act as negative regulator for carotenoid accumulation whereas CPZ act as positive regulator during carotenoid accumulation. (B) Carotenoids accumulation of wild-type, Δ*trm-9*, Δ*nca-2*, and Δ*trm-9*Δ*nca-2* double mutant strains during Ca^{2+}stress. Carotenoids extracted from these strains grown in Vogel's liquid medium without $CaCl_2$, supplemented with various concentrations of $CaCl_2$. Carotenoids were extracted and estimated in μg carotenoids per g of dry weight. Error bars show the standard errors calculated from the data for three independent experiments. Statistically significant values are indicated by asterisks, *P < 0.05

proteins are clustered with homologues from related (Supplementary Figure 4). Promoter analysis revealed important putative regulatory elements involved in transcription of *cmd* and *trm-9* gene by using MatInspector software (Supplementary Figure 5). The NCU04736 gene was previously shown to encode *nca-2* (Bowman et al., 2009; Bowman et al., 2011).

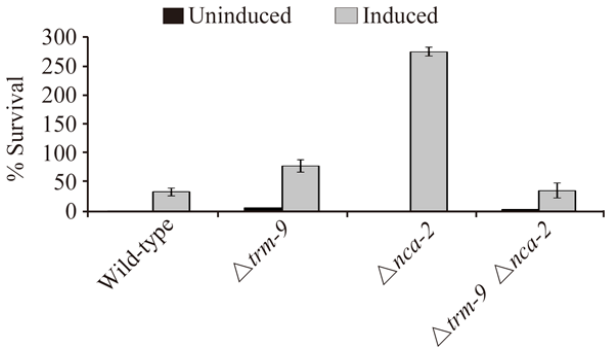


Figure 4 Thermotolerance measurement of wild-type, Δ*trm-9*, Δ*nca-2*, and Δ*trm-9*Δ*nca-2* double mutant strains in induced (44℃) and uninduced (30℃) conditions. Each data point represents the mean of three independent experiments

The *cmd*, *trm-9*, and *nca-2* genes are involved in growth

We used CaM antagonists, trifluoperazine (TFP) and chlorpromazine (CPZ) to study the effect of CaM inhibition on growth of wild-type strain of *N. crassa*. We found that both TFP and CPZ inhibit growth, a hyphal branching, and development of aerial hyphae in *N. crassa* (Figure 1). Moreover, addition of TFP (10, 20, 40, 60, 80, and 100 μM) or CPZ (20, 40, 60, 80, 100 μM) in the synthetic crossing medium (SCM) causes a defect in perithecia formation in *N. crassa* and results in a sterile phenotype (data not shown). In addition, the *cmd* transcript level was found to be decreased in the presence of TFP and CPZ as revealed by the real time PCR analysis (Supplementary Figure 6). These results suggest that CaM play a role in vegetative growth, hyphal development, and sexual development in *N. crassa*.

The Δ*trm-9* mutant displayed a slow growth phenotype (Figure 2A). However, the slow growth phenotype of the Δ*trm-9* mutant was not due to a defect in the ergosterol profile (Supplementary Figure 7) and the growth of the Δ*trm-9* mutant was not affected by addition of various amounts of $CaCl_2$, sucrose and NaCl in the medium indicating that the Δ*trm-9* mutant is insensitive to these stress conditions (data not shown). The growth defect was more severe in the Δ*trm-9*Δ*nca-2* double mutant that showed distinct

colony morphology with matty-like colony growth (Figure 2B). In addition, the dry weight of the strains followed the order wild-type>Δ*nca-2*> Δ*trm-9*> Δ*trm-9*Δ*nca-2* (Figure 2C). The Δ*trm-9*Δ*nca-2* double mutant also showed sensitivity to $CaCl_2$, reduced aerial hyphae development and ultraviolet (UV) survival (Supplementary Figure 8). Therefore, these results suggested that lack of both *trm-9* and *nca-2* result in impaired growth, hyphae development and conidial development in *N. crassa*.

Carotenoids accumulation

We also analyzed carotenoid accumulation in *N. crassa* in the presence of TFP and CPZ to investigate the role of CaM in carotenoid accumulation. The carotenoid profile of the wild-type strain in presence of the inhibitors followed the order wild-type > 10 μM CPZ > 20 μM CPZ >10 μM TFP > 20 μM TFP (Figure 3A). Therefore, these results indicate the CaM protein might modulate carotenoid accumulation in *N. crassa*. The difference of carotenoids accumulation in presence of TFP and CPZ might be due to the difference of mechanism of inhibition mediated by TFP and CPZ. Furthermore, carotenoids accumulation in the Δ*trm-9*Δ*nca-2* double mutant was lower than either of the parental single mutant strains, and reduced further on medium supplemented with high concentrations of $CaCl_2$ (Figure 3B). In addition, Δ*nca-2* mutant was unable to grow on medium supplemented with 0.3 M $CaCl_2$ or more (supplementray Figure 8A) and consequently, no carotenoids was accumulated; however, accumulation of carotenoids in the Δ*trm-9* was similar to the wild-type (Figure 3B). Therefore, these results suggest that *nca-2* plays a role in carotenoids biosynthesis.

Lack of both nca-2 and trm-9 affect in acquisition of induced thermotolerance

We studied the ability of the Δ*trm-9*Δ*nca-2* double mutant in acquisition of induced thermotolerance. The Δ*trm-9*Δ*nca-2* double mutants showed decreased survival in induced thermotolerance as compared to parental single mutants. The survival in induced heat shock temperature followed the order Δ*nca-2*> Δ*trm-9*>Δ*trm-9*Δ*nca-2*>wild-type (Figure 4). Therefore, lack of both *nca-2* and *trm-9* had a negative effect in acquisition of induced thermotolerance.

Conclusions

CaM and its target proteins mediate diverse cellular functions. The CaM antagonists TFP and CPZ affect growth, aerial hyphae development, carotenoids accumulation and sexual development in *N. crassa*. In addition, Δ*trm-9* mutant has a slow growth phenotype and less dry weight content. Moreover, the Δ*trm-9*Δ*nca-2* double mutant showed a severe growth defect, less carotenoid accumulation, reduced conidial count, an increased sensitivity to $CaCl_2$, and reduced viability in acquisition of thermotolerance induced by heat shock temperature. Thus, in this study, we have shown that *cmd*, *trm-9*, and *nca-2* genes play an important role in growth, pigmentation, and stress-tolerance in *N. crassa*.

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Supplementary Materials

Supplementary Figure 1: PCR analysis for double mutant confirmation. The Δ*trm-9*Δ*nca-2* double mutants were verified by using the forward primers NCU04898-5F 5' GGTTAGTGAGCTTTGAGTCG 3' and NCU04736 -5F 5' TACACTGGTAATGGACCACG 3' specific for upstream of the open reading frame of genes *trm-9* and *nca-2*, respectively, and with the common reverse primer 5HPHR 5' ATCCACTTAACGTTACTGAAATC 3' that is specific for the *hph* cassette used to generate the knockout mutants (Colot et al., 2006; Deka et al., 2011). Amplification of PCR products of size ~1.2 and ~1.018 kb indicate the presence *trm-9* and *nca-2* knockout alleles respectively. The wild-type was used as negative controls for the knockout alleles (indicated in the parenthesis) using the allele specific primer pairs. PCR products were visualized in a 0.8% agarose gel with 1 kb DNA ladder.

Supplementary Figure 2: Conidial cell count of wild-type, Δ*trm-9*, Δ*nca-2*, and Δ*trm-9*Δ*nca-2* double mutant strains grown in Vogel's glucose medium. Conidia count of wild-type and mutant strains are plotted with relative counting with respect to wild-type. Error bars indicate the standard errors calculated from the data for three independent experiments. Conidial cell count of Δ*trm-9*Δ*nca-2* double mutant strain was less than the parental single knockout mutant strains and wild-type.

Supplementary Figure 3: Sequence alignment of *cmd* and *trm9* homologues. (A) Sequence alignment of the *Neurospora crassa* CaM homologues. (B) Sequence alignment of the *trm-9* homologues. Conserved amino acids are indicated in black (100%), dark gray (>80%) and light gray (>60%). The homologue sequences used for the sequence analysis are from *Ajellomyces capsulatus* (AC), *Ajellomyces dermatitidis SLH14081* (AD), *Aspergillus fumigatus* (AF), *Aspergillus nidulans* (AN), *Botryotinia fuckeliana B05.10* (BF), *Candida albicans* (CA), *Coccidioides immitis* (CI), *Cordyceps militaris CM01* (CM), *Coccidioides posadasii* (CP), *Dichotomomy cescejpii* (DC), *Esox lucius* (EL), *Grosmannia clavigera kw1407* (GC), *Glomerella graminicola* (GG),*Gibberella zeae PH-1* (GZ), *Homo sapiens* (HS), *Komagataella pastoris* (KP), *Magnaporthe grisea* (MG), *Neurospora crassa* (NC), *Neurospora tetrasperma* (NT), *Procambarus clarkii* (PC), *Phytophthora infestans T30-4* (PI), *Rhodomonas sp. CCMP768* (RS), *Saccharomyces cerevisiae* (SC), *Schistosoma mansoni* (SM), *Spathaspora passalidarum* (SP), *Trichoderma reesei* (TR), and *Talaromyces stipitatus ATCC 10500* (TS).

Supplementary Figure 4: Phylogenetic analysis of the (A) CaM and (B) TRM-9 proteins. Protein sequences are described using GenBank accession numbers, phylum is indicated at major clades, and bar indicates scale of genetic distances. The homologue sequences used for the sequence analysis are from *Ajellomyces capsulatus* (AC), *Ajellomyces dermatitidis SLH14081* (AD), *Aspergillus fumigatus* (AF), *Arthroderma gypseum* (AG), *Aspergillus kawachii* (AK), *Aspergillus nidulans* (AN), *Aspergillus oryzae* (AO), *Botryotinia fuckeliana B05.10* (BF), *Candida albicans* (CA), *Coccidioides immitis* (CI), *Cordyceps militaris CM01* (CM), *Coccidioides posadasii* (CP), *Dichotomomy cescejpii* (DC), *Drosophila melanogaster* (DM), *Exophiala dermatitidis* (ED), *Esox lucius* (EL), *Eurotium rubrum* (ER), *Emericella unguis* (EU), *Grosmannia clavigera kw1407* (GC), *Glomerella graminicola* (GG), *Gibberella zeae PH-1* (GZ), *Homo sapiens* (HS), *Komagataella pastoris* (KP), *Metarhizium anisopliae* (MA), *Magnaporthe grisea* (MG), *Neurospora crassa* (NC), *Neurospora tetrasperma* (NT), *Ogataeapara polymorpha* (OP), *Paracoccidioides brasiliensis* (PB), *Procambarus clarkii* (PC), *Puccinia graminis f. sp. Tritici* (PG), *Phytophthora infestans T30-4* (PI), *Penicillium rolfsii* (PR), *Pyrenophora tritici-repentis* (PT), *Rhodomonas sp. CCMP768* (RS), *Saccharomyces cerevisiae* (SC), *Saccharomyces cerevisiae RM11-1a* (SC RM11), *Saccharomyces cerevisiae* x *Saccharomyces kudriavzevii VIN7* (SCSK), *Sordaria macrospora* (SM), *Spathaspora passalidarum* (SP), *Scheffersomyce sstipitis* (SS), *Trichophyton equinum* (TE), *Trichoderma reesei* (TR), *Talaromyces stipitatus ATCC 10500* (TS), and *Verticillium albo-atrum* (VA).

Supplementary Figure 5: Promoter analysis of (A) *cmd* and (B) *trm-9* gene of *N. crassa*. Gray boxes showed the important regulatory sequences of gene and transcription start site (TSS) are indicated by using arrows.

Supplementary Figure 6: Expression studies of *cmd* gene in the presence of inhibitors. Fold change in expression was calculated by $2^{-\Delta\Delta Ct}$ method, using wild-type and β-tubulin as calibrator and endogenous control respectively. Standard errors calculated from the data for two independent experiments are shown using error bars.

Supplementary Figure 7: Ergosterol is present in the Δ*trm-9* mutant and Δ*trm-9*Δ*nca-2* double mutant strains. Profile of the sterols extracted from the wild-type, Δ*nca-2*, Δ*trm-9*, Δ*trm-9*Δ*nca-2*, and *erg-3* mutant strains were analysed by UV spectrophotometer.

Supplementary Figure 8: (A) Calcium sensitivity, (B) development of aerial hyphae, and (C) UV survival. (A) Ca^{2+}sensitivity analysis of wild-type, Δ*trm-9*, Δ*nca-2*, and Δ*trm-9*Δ*nca-2* double mutant strains. Colony diameter (cm h^{-1}) were measured at regular intervals and plotted against various concentrations of $CaCl_2$. Standard errors calculated from the data for three independent experiments are shown using error bars. (B) Aerial hyphae development of the wild-type, Δ*trm-9*, Δ*nca-2*, and Δ*trm-9*Δ*nca-2* double mutant strains on VSM agar media in test tube. The aerial hyphae growth of Δ*trm-9*Δ*nca-2* double mutant strain was less as compared to parental single mutants and wild-type strain. (C) UV survival. Spot-test analysis of wild-type, Δ*trm-9*, Δ*nca-2*, and Δ*trm-9*Δ*nca-2* double mutant and *upr-1* mutant strain grown on VG agar at 30°C for 48 h in dark then illuminated for 24 h. UV survival assay was done essentially as described previously (Deka et al., 2011).

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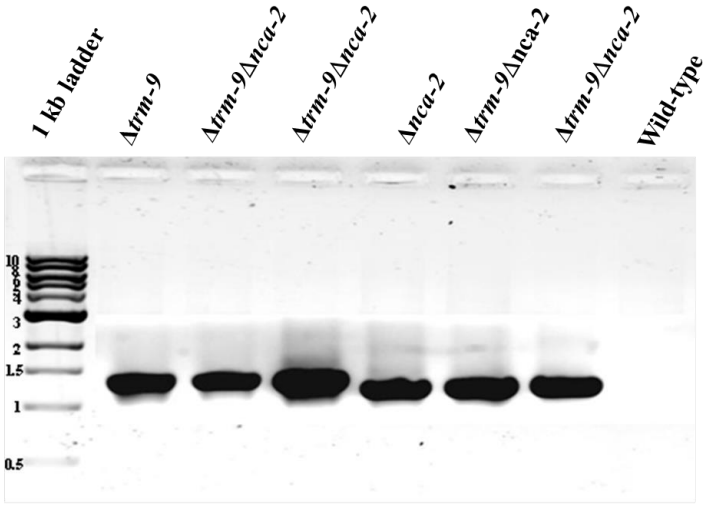
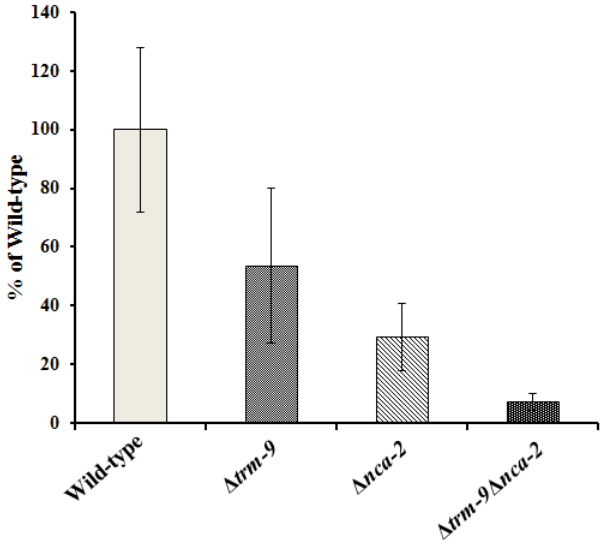
Supplementary Figure 1

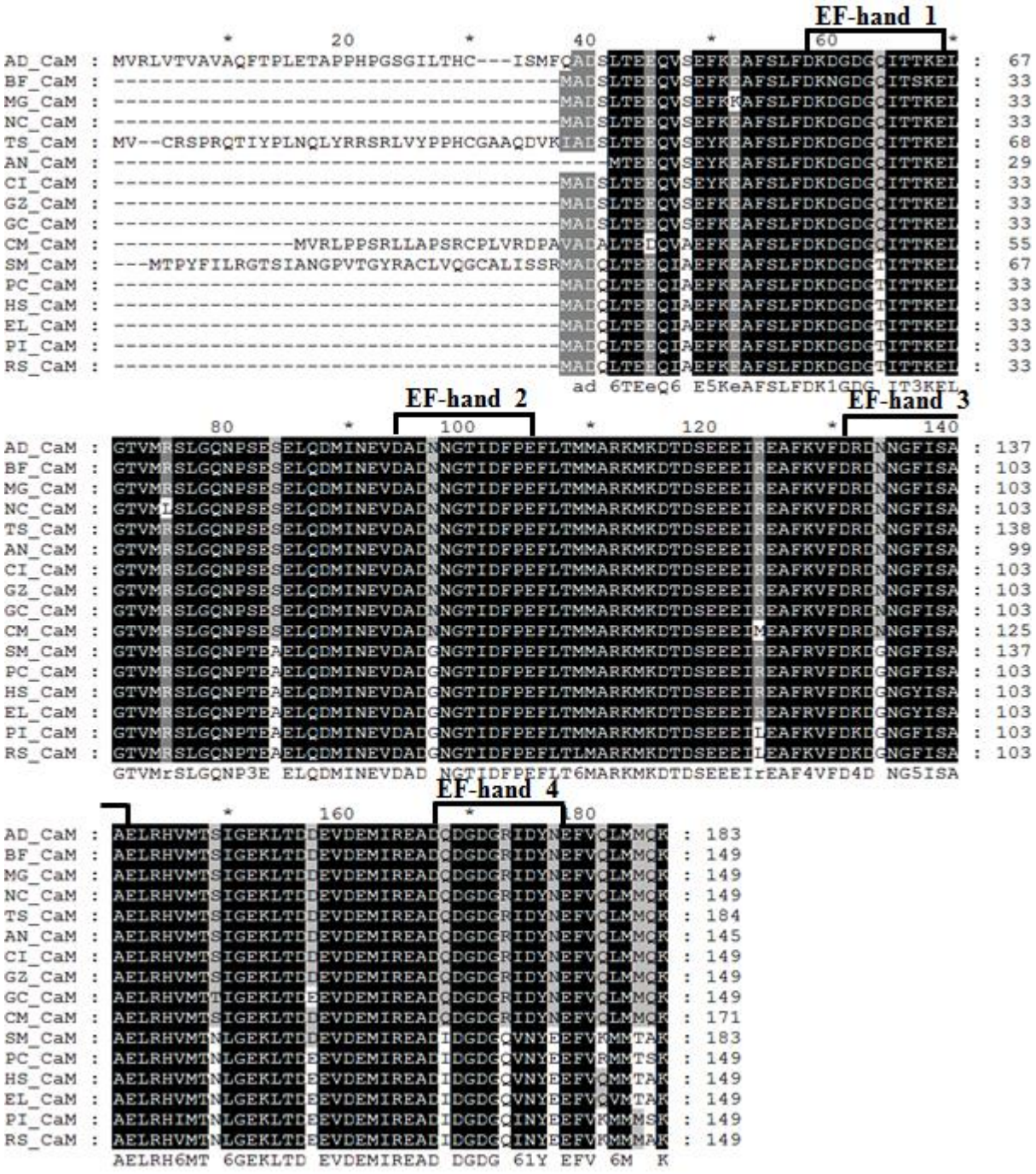


Supplementary Figure 2



Supplementary Figure 3

A.



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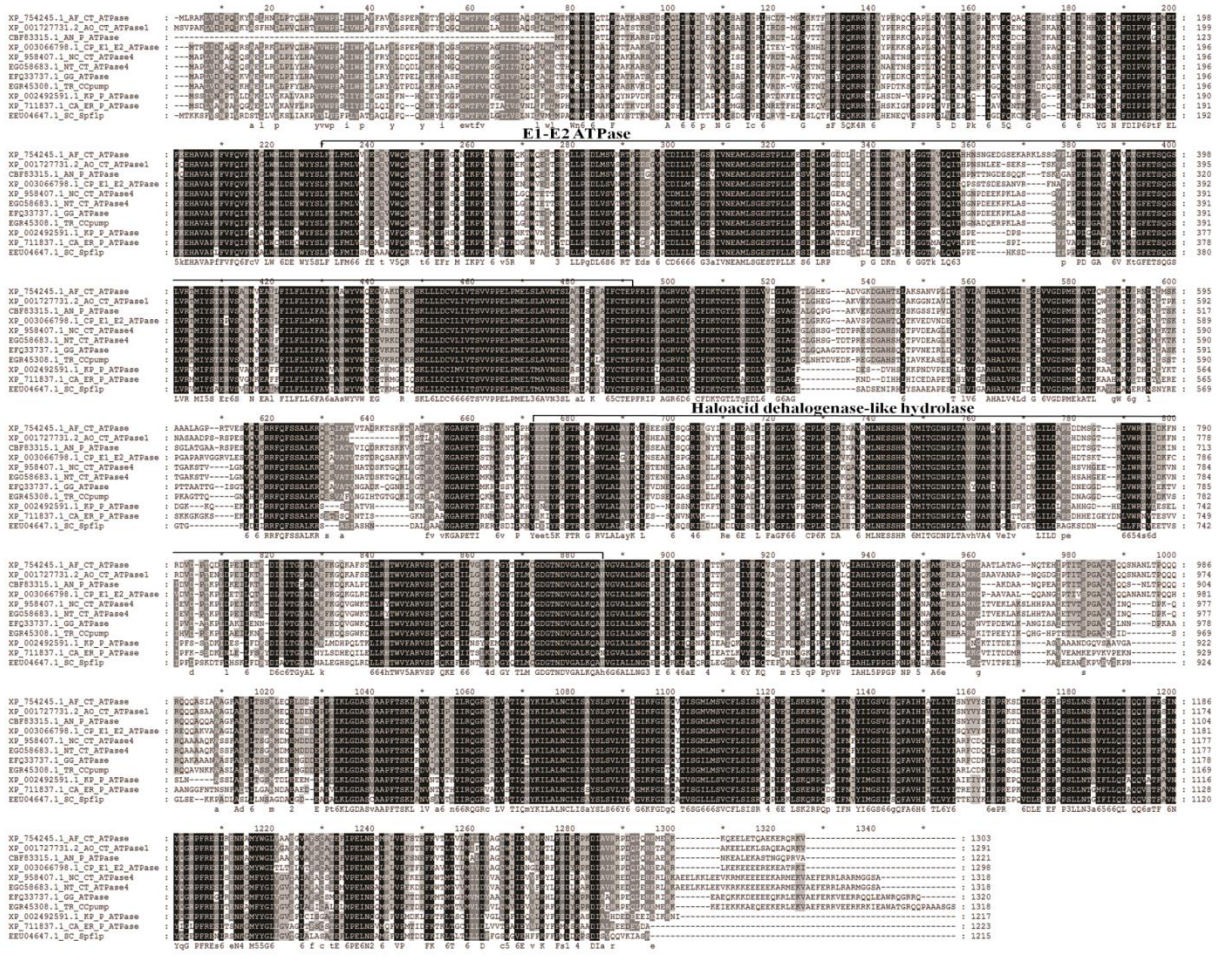
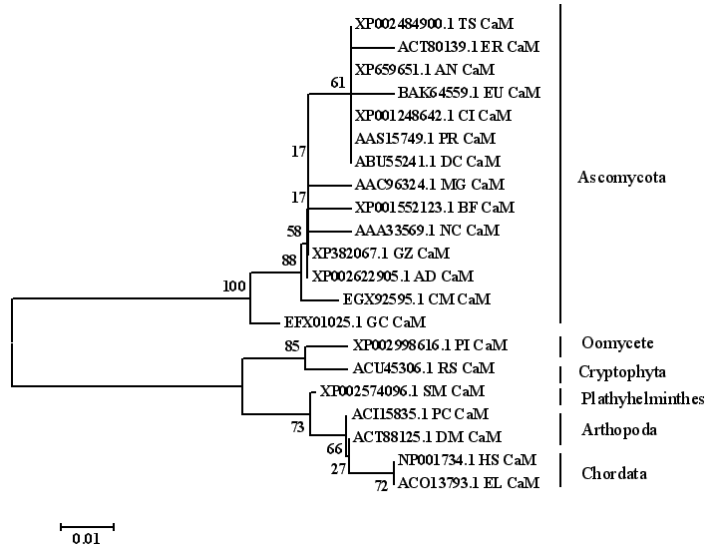


Supplementary Figure 4

A.



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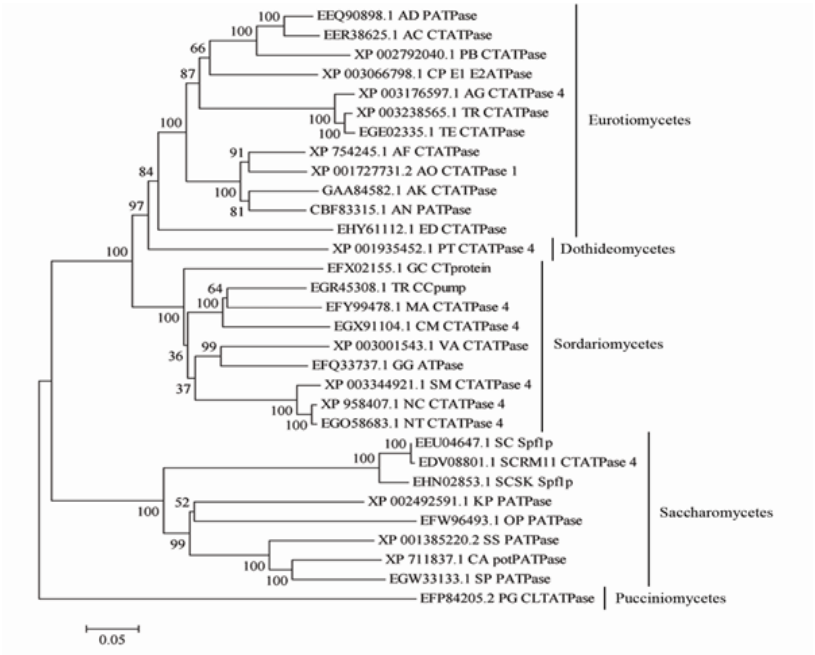
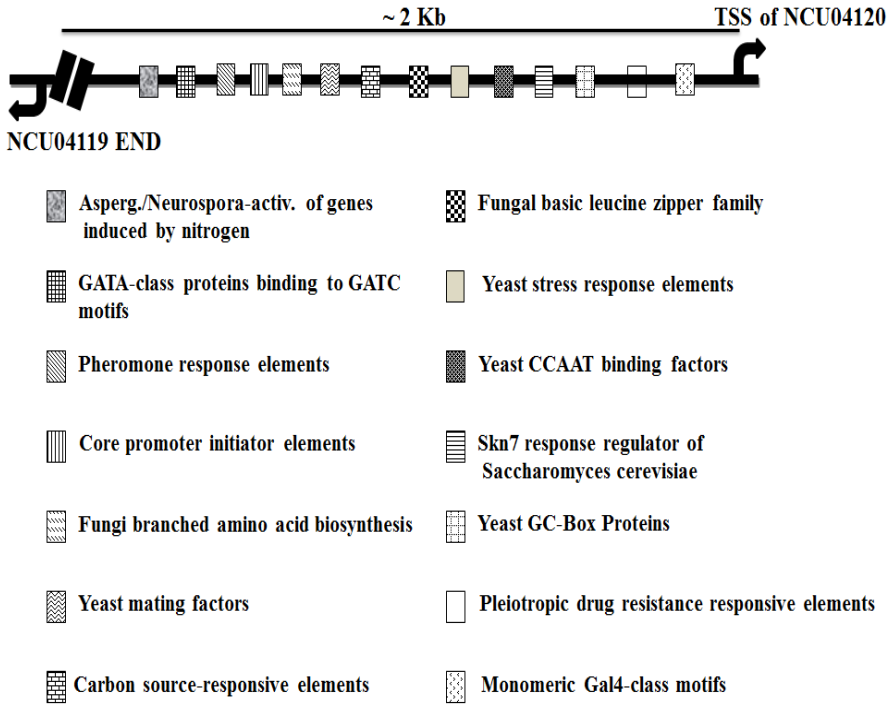


Supplementary Figure 5

A.



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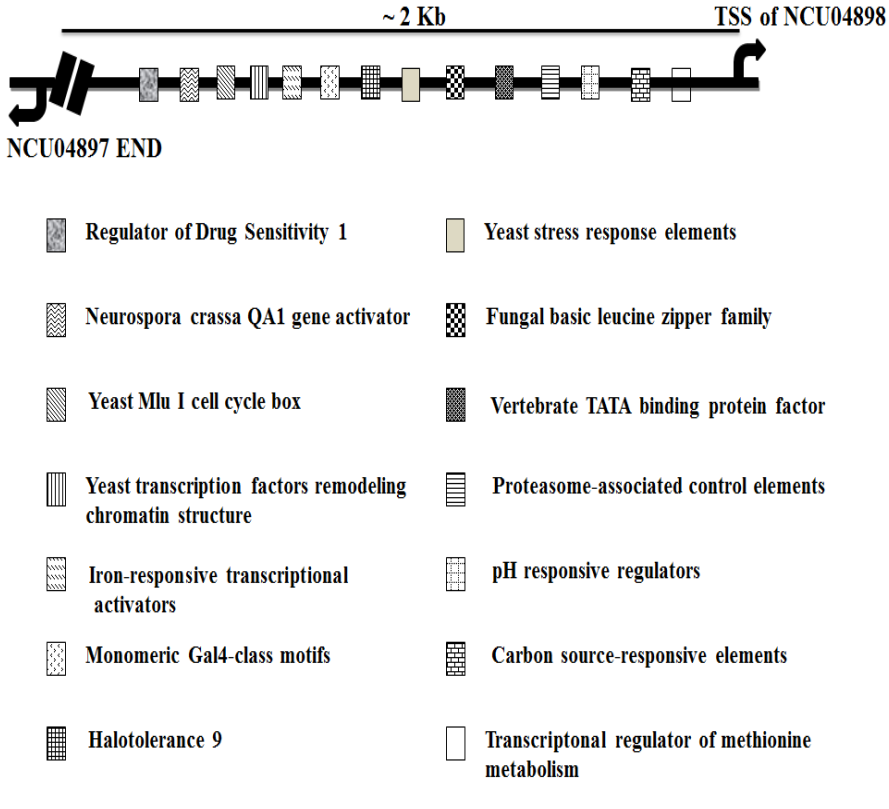
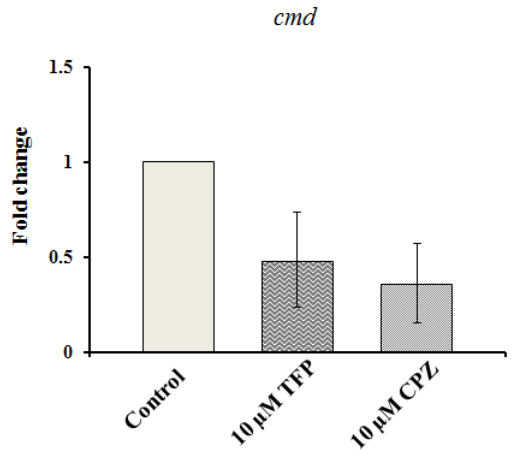
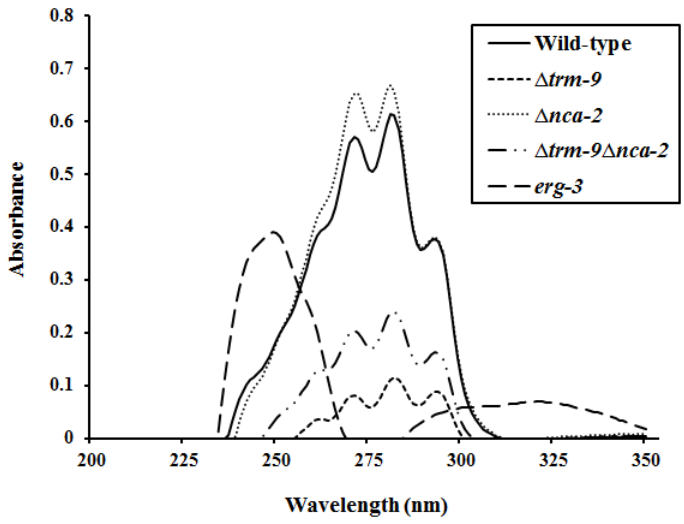


Supplementary Figure 6

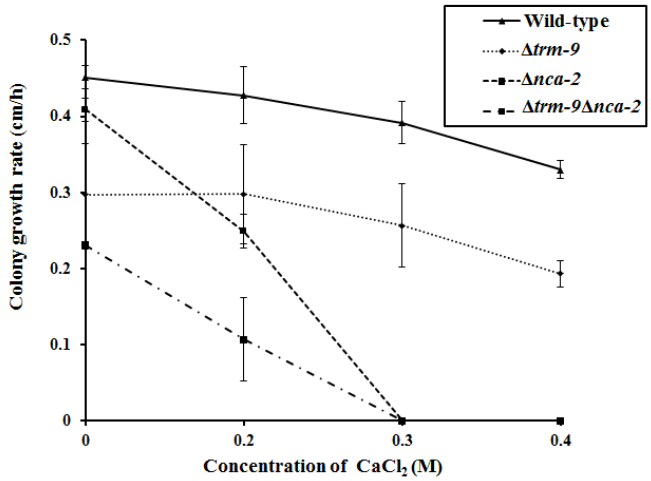


Supplementary Figure 7



Supplementary Figure 8

A.



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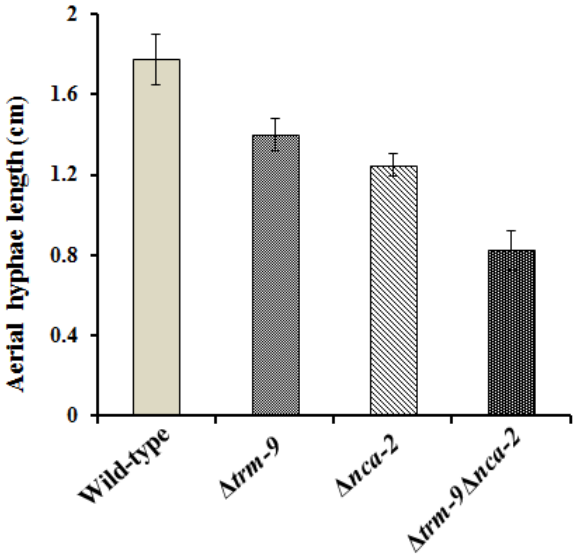

C.