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Neurospora crassa homologue of Neuronal Calcium Sensor-1 has a role in growth, calcium stress tolerance, and ultraviolet survival

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Abstract NCU04379 gene encodes a conserved Ca^{2+} and/or calmodulin binding protein that possesses a consensus signal for N-terminal myristoylation and four EF-hands, characteristics of Neuronal Calcium Sensor-1proteins. The NCU04379.2 knockout mutant shows slow growth rate, increased sensitivity to calcium and ultraviolet (UV) irradiation, and a wild-type fragment carrying NCU04379 gene complements the mutant. Therefore, NCU04379 gene has a role in growth, calcium stress tolerance, and UV survival. Crosses homozygous for ΔNCU04379.2 mutant strains were fully fertile; however, we found evidence for involvement of Ca^{2+}/calmodulin-dependent protein kinase encoding genes NCU02283 and NCU09123 in sexual development.

Keywords Calcium signaling genes · Calcium sensitivity · Ca^{2+}/calmodulin-dependent protein kinase · DNA damage · Neuronal Calcium Sensor-1 · Sexual development · UV survival

Introduction

The filamentous fungus *Neurospora crassa* possesses complex calcium (Ca^{2+})-signaling system that appears to be significantly different from plant and animal cells, especially in relation to second messenger systems responsible for Ca^{2+}-release from internal stores (Galagan et al. 2003). The genome analysis of Neurospora has revealed three Ca^{2+} channel proteins, nine Ca^{2+}/cation-ATPases, six recognizable Ca^{2+}/H^{+} exchangers, two novel putative Ca^{2+}/Na^{+} exchangers, four novel phospholipase C-δ subtype (PLC-δ) proteins, 23 Ca^{2+} and/or calmodulin (CaM) binding proteins, and one CaM (Galagan et al. 2003; Borkovich et al. 2004). One of the 23 Ca^{2+} and/or CaM binding proteins, the product of NCU04379 gene, shows significant sequence homology to a protein called Frequenin (Frq) in Drosophila (Pongs et al. 1993), Frq1 in yeast (Hendricks et al. 1999), and Neuronal Calcium Sensor-1 (NCS-1) in mammalian cells (McFerran et al. 1998; Tamuli et al. 2011). These proteins belong to the Neuronal Calcium Sensor (NCS) branch of the CaM superfamily, characterized by a consensus signal for N-terminal myristoylation and four EF-hand Ca^{2+}-binding sites (Johnson et al. 1994; Ames et al. 1996; Strahl et al. 2007; Tamuli et al. 2011). Members of the NCS protein superfamily mediate the effects of the cytosolic Ca^{2+} (Ikura 1996).

Cytosolic Ca^{2+} plays a central role as an intracellular signal, however, high concentrations of Ca^{2+} are toxic to the cell, and therefore, cytosolic free Ca^{2+} ($[Ca^{2+}]_c$) is effectively regulated in Neurospora, Arabidopsis, and human (Cornelius and Nakashima 1987; Sanders et al. 2002; Berridge et al. 1998). The $[Ca^{2+}]_c$ is regulated by specialized proteins, for example, $[Ca^{2+}]_c$ is removed by the ATP-dependent pumps located in the rat plasma membrane (Ambudkar and Baum 1988; Ambudkar et al. 1989; Haghighat and Al-Hashimi 1999). In *N. crassa*, excess and hazardous amounts of $[Ca^{2+}]_c$ are sequestered through vacuolar uptake (Cornelius and Nakashima 1987), although, the mechanism of the vacuolar uptake is still unknown.

One of the versatile Ca^{2+}-signaling proteins, the Ca^{2+}-modulated protein CaM plays an important role in

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mediating the effects of $[Ca^{2+}]_c$ and is believed to be crucial in modulating DNA repair, DNA synthesis, and cell proliferation in both CHO and human cell lines (Chafouleas et al. 1984; Pavelic 1987; Chard 1987; Mirzayans et al. 1995). The process of DNA repair has been extensively studied in *N. crassa*, where three genes, *upr-1* (*ncrev3*), *mus-42* (*ncrev1*), and *mus-26* (*ncrev7*) proved to be induced by DNA damage and function in the mutagenic translesion DNA synthesis (TLS) pathway (Sakai et al. 2002; 2003). However, it is still not known whether and how CaM or any other Ca^{2+}-signaling protein plays a role in DNA damage repair process in *N. crassa*.

Thus far, detailed knowledge about the genes involved in Ca^{2+}-mediated signal response pathway is lacking for *N. crassa* and very few Ca^{2+}-signaling genes have been characterized. To understand the cellular roles of additional Ca^{2+}-signaling genes, we have studied 18 Ca^{2+}-signaling mutants. We report in this paper that a homologue of NCS-1 encoding gene NCU04379 has a role in growth, Ca^{2+} stress tolerance, and UV survival. Moreover, crosses homozygous for ΔNCU04379.2 mutant strains were fully fertile, however, we found evidence for involvement of NCU02283 and NCU09123 genes, encoding a Ca^{2+}/CaM-dependent protein kinase type I and a CAMK-1, respectively, in sexual development.

Materials and methods

Strains, growth, and crosses

The wild-type and Ca^{2+}-signaling mutant strains (Table 1) were obtained from the Fungal Genetics Stock Center (FGSC), University of Missouri, Kansas City, MO 64110 (McCluskey 2003). The Ca^{2+}-signaling mutants were generated using a high-throughput gene knockout procedure, developed by the *Neurospora* genome project (http://www.dartmouth.edu/~neurosporagenome/proj_overview.html; Colot et al. 2006).

Growth, crossing, and maintenance of Neurospora strains were essentially as described by Davis and De Serres (1970). Growth was initially measured by placing either conidia or a plug of agar containing mycelium in the center of a petri dish and colony diameter was measured every 2–3 h to obtain linear rates of diameter increase over a period of 28 h. Strains that show lower growth rate on

Table 1 *N. crassa* strains used in this study

S. no.	FGSC no. (*a*/*A*) blank;or strain no.	NCU no. or strain type	Type of protein	Source
1.	11169/11170	02814.2	Ca^{2+} and/or CaM binding protein	FGSC
2.	11246/11247	06650.2	Ca^{2+} and/or CaM binding protein	FGSC
3.	11403/11404	04379.2	Ca^{2+} and/or CaM binding protein	FGSC
4.	11405/11406	05255.2	Ca^{2+} and/or CaM binding protein	FGSC
5.	11537/11536	06177.2	Ca^{2+} and/or CaM binding protein	FGSC
6.	11541/11542	06948.2	Ca^{2+} and/or CaM binding protein	FGSC
7.	12448/12449	02283.2	Ca^{2+} and/or CaM binding protein	FGSC
8.	12548/12547	09123.2	Ca^{2+} and/or CaM binding protein	FGSC
9.	15898/15899	01564.2	Ca^{2+} and/or CaM binding protein	FGSC
10.	11248/11249	07075.2	Ca^{2+}/H^{+} exchanger	FGSC
11.	11407/11408	06366.2	Ca^{2+}/H^{+} exchanger	FGSC
12.	11685/11686	00916.2	Ca^{2+}/H^{+} exchanger	FGSC
13.	12376/12375	00795.2	Ca^{2+}/H^{+} exchanger	FGSC
14.	11255/11256	08147.2	Ca^{2+}-ATPase	FGSC
15.	11410/11409	07966.2	Cation-ATPase	FGSC
16.	11530/11529	02826.2	Ca^{2+}/Na^{+} exchanger	FGSC
17.	13036/13037	05154.2	Ca^{2+}-ATPase	FGSC
18.	11271/11272	09655.2	Phospholipase C	FGSC
19.	988/987	Wild-type		FGSC
20.	i-94-120a	*erg-3*	Sterol $\Delta^{14,15}$-reductase	Laboratory stock (Prakash et al. 1999)
21.	34-97-3a	*upr-1*	NCREV3	Laboratory stock (Tamuli et al. 2006)

petri dish were further analyzed by using standard race tube assay (Ryan et al. 1943; Ryan 1950). Growth rates were calculated as cm h^{-1} in both cases.

Assay for calcium and UV sensitivity

Vogel's glucose medium (Davis and De Serres 1970) was supplemented with various concentrations of $CaCl_2$, NaCl and sucrose as indicated. The 0 M $CaCl_2$ control plates were prepared using Vogel's Medium N (Vogel 1964) without $CaCl_2 \cdot 2H_2O$. Growth rate on these plates were determined as described above.

UV sensitivity was essentially as described by Kato and Inoue (2006). Briefly, conidia were grown in flasks containing Vogel's glucose medium at 30°C for 5 days, harvested and assayed for UV sensitivity. For UV dose dependency of the survival of *N. crassa*, conidia were irradiated at various doses of UV and aliquots were sampled and plated after appropriate dilution. The plates were grown at 30°C for 3 days in dark and number of colonies on each plate was counted.

PCR amplification, cloning, and transformation

PCRs were performed using custom oligonucleotide primers (Metabion GmbH, Germany), Phusion High-Fidelity PCR Kit (Finnzymes, Finland) and the manufacturer's protocol. A 3,972 bp of NCU04379 fragment from the wild-type was PCR amplified by using the primers NCU04379-5F 5′ GCTCGAAAGTTTAGTCCTGG 3′and NCU04379-3R 5′ CCCAGTAACGTCTCTTTTGC 3′ (http://www.dartmouth.edu/~neurosporagenome/knockouts_completed.html) and cloned into the *SmaI* site of pBARGEM7-1 (FGSC 19; Pall and Brunelli 1993) that resulted in pRD-1. This construct was transformed into the ΔNCU04379.2 recipient as described by Bhat et al. (2004). Transformants were selected on plate containing basta (200 μg/ml), and initial heterokaryotic transformants were crossed with the opposite mating type of the ΔNCU04379.2 mutant strain to isolate homokaryotic strains.

Sequence analysis

BLAST (Altschul et al. 1990) analysis was performed using software tools available from NCBI (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi), the Conserved Domain Database (CCD; Marchler-Bauer and Bryant 2004; Marchler-Bauer et al. 2009) was used to identify conserved domains in the protein. Protein sequences were aligned with ClustalX 1.83 (Thompson et al. 1997) and transferred to GeneDoc for visualization (Nicholas et al. 1997). Phylogenetic trees were constructed from these alignments using the minimum-evolution method (Rzhetsky and Nei 1992), bootstrap replications as test of phylogeny (Felsenstein 1985) and the software MEGA4 (Tamura et al. 2007).

Results

The ΔNCU04379.2 mutant grows slowly and shows hypersensitivity to $CaCl_2$ stress

We have studied growth rate for 18 Ca^{2+}-signaling mutants (Table 1, entries 1–18), of which the ΔNCU04379.2 mutant (Supplementary Fig. 1) grows consistently slower than the wild-type strain (Fig. 1). The average growth rates were 0.357 and 0.264 cm h^{-1}, for the wild-type and ΔNCU04379.2 mutant strains, respectively ($n = 3$). The slow growth phenotype of the ΔNCU04379.2 mutant prompted us to investigate the ergosterol profile in the mutant, since ergosterol is absent in the *erg-3* mutant that grows slowly (Prakash et al. 1999). Sterol from the ΔNCU04379.2 mutant has UV absorption maxima at 272, 282, and 293 nm thereby indicating presence of ergosterol (Supplementary Fig. 2). To test the effects of Ca^{2+} on growth, we supplemented Vogel's glucose agar media with various amount of $CaCl_2$. We found that the ΔNCU04379.2 mutant is hypersensitive to $CaCl_2$ stress. The ΔNCU04379.2 mutant showed severe growth defect on media supplemented with 0.3 M and 0.4 M $CaCl_2$ (Supplementary Fig. 3), and growth rate of the ΔNCU04379.2 mutant was lower than the wild-type (Fig. 2a). In order to test the effect of Ca^{2+} deprivation, the wild-type and ΔNCU04379.2 mutant strains were grown on Vogel's glucose agar media with various amount of EGTA that has high affinity and selectivity for free Ca^{2+} (Tsien 1980). The decrease of EGTA, and the corresponding increase of Ca^{2+} levels, revealed the higher Ca^{2+}-sensitivity of the ΔNCU04379.2

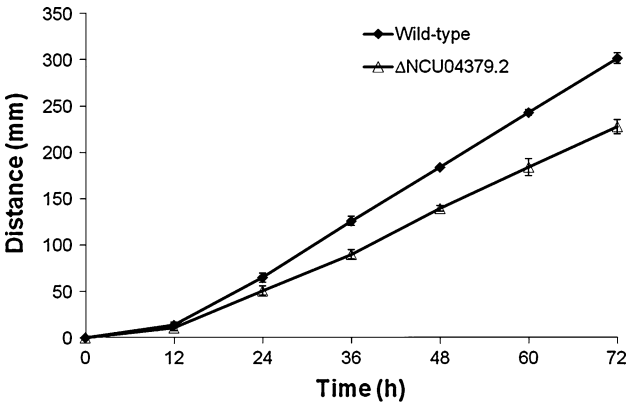


Fig. 1 The ΔNCU04379.2 mutant grows slowly. Rates of apical growth of the wild-type and ΔNCU04379.2 knockout mutant strains were measured using race tubes. *Error bars* indicate the standard errors calculated from the data for three independent experiments

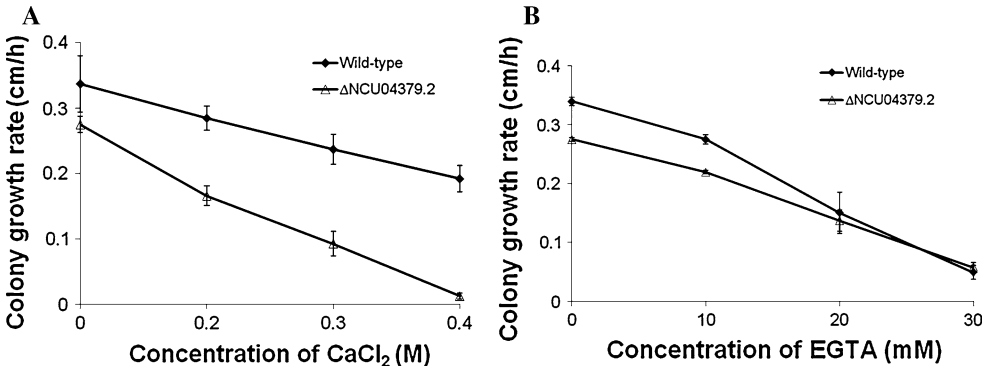


Fig. 2 The ΔNCU04379.2 mutant is sensitive to $CaCl_2$. Growth rate of the wild-type and the ΔNCU04379.2 mutant on Vogel's glucose medium supplemented with different concentrations of $CaCl_2$ (**a**) and EGTA (**b**). *Error bars* indicate the standard errors calculated from the data for three independent experiments

mutant relative to the wild-type (Fig. 2b). To determine whether the Ca^{2+} sensitivity phenotype of the ΔNCU04379.2 mutant is specific to Ca^{2+} stress or due to a mere osmotic effect, we have studied the growth characteristics of the ΔNCU04379.2 mutant on media supplemented with NaCl and sucrose. However, the ΔNCU04379.2 mutant is not sensitive to either NaCl or sucrose stress (data not shown). These results suggest that NCU04379 plays a role in growth and Ca^{2+} stress tolerance.

The ΔNCU04379.2 mutant is sensitive to UV

The sensitivity of the ΔNCU04379.2 mutant to Ca^{2+} stress prompted us to test if ultraviolet (UV) stress has any effect on this strain. Previous work had also indicated that CaM and its binding proteins are crucial in modulating DNA repair (Chard 1987; Mirzayans et al. 1995). We assayed the UV-sensitivity of the ΔNCU04379.2 mutant qualitatively and quantitatively. The ΔNCU04379.2 mutant shows an increased sensitivity to UV irradiation as revealed by the spot test (Supplementary Fig. 4). Dose–response curves constructed following exposure of the wild-type, and the ΔNCU04379.2 mutant to UV irradiations also suggested increased sensitivity of the ΔNCU4379.2 mutant (Fig. 3). However, UV sensitivity of the ΔNCU4379.2 mutant is less than the *upr-1* mutant constructed in the laboratory (Fig. 3; Tamuli et al. 2006). These data indicate that NCU04379 plays a role in UV-survival.

Complementation of the ΔNCU04379.2 mutant

The plasmid vector pRD-1 was transformed into the ΔNCU04379.2 mutant and initial transformants were crossed with the opposite mating type strain of the ΔNCU04379.2 mutant to obtain homokaryotic strains. We have tested three homokaryotic transformants and they complement growth, $CaCl_2$, EGTA, and UV phenotypes of the ΔNCU04379.2 mutant (Supplementary Fig. 5). We have also obtained additional homokaryotic transformants

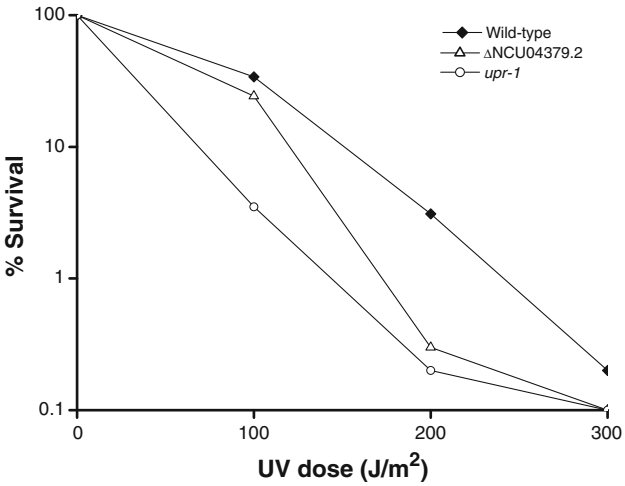


Fig. 3 The ΔNCU04379.2 mutant is sensitive to UV. Dose–response curves of the wild-type, the ΔNCU04379.2 mutant, and the *upr-1* mutant on exposure to UV irradiations. The *upr-1* null mutant was constructed in the laboratory using repeat-induced point mutation (Tamuli et al. 2006), and used to compare the relative UV sensitivity. *Each point* represents the mean of at least three independent experiments

and all of them complement the ΔNCU04379.2 mutant phenotypes (data not shown). Therefore, we conclude that NCU04379 gene has a role in growth, Ca^{2+} stress tolerance, and UV survival.

Sequence analysis of the product of NCU04379 gene

NCU04379 gene is predicted to encode a Ca^{2+} and/or CaM binding protein of 190 amino acid residues (GenBank accession number EAA28220.1) that shows sequence similarity to the *Aspergillus fumigatus*, *Magnaporthe grisea*, *Saccharomyces cerevisiae*, and *Homo sapiens* NCS-1 (also known as Frq) homologues (91, 92, 59, and 66% identity; 95, 97, 79, and 82% similarity; *e*-values 9e-96, 8e-96, 4e-64, and 2e-68, respectively). NCU04379 gene encodes a protein that also possesses a consensus signal for N-terminal myristoylation and four EF-hand Ca^{2+}-binding

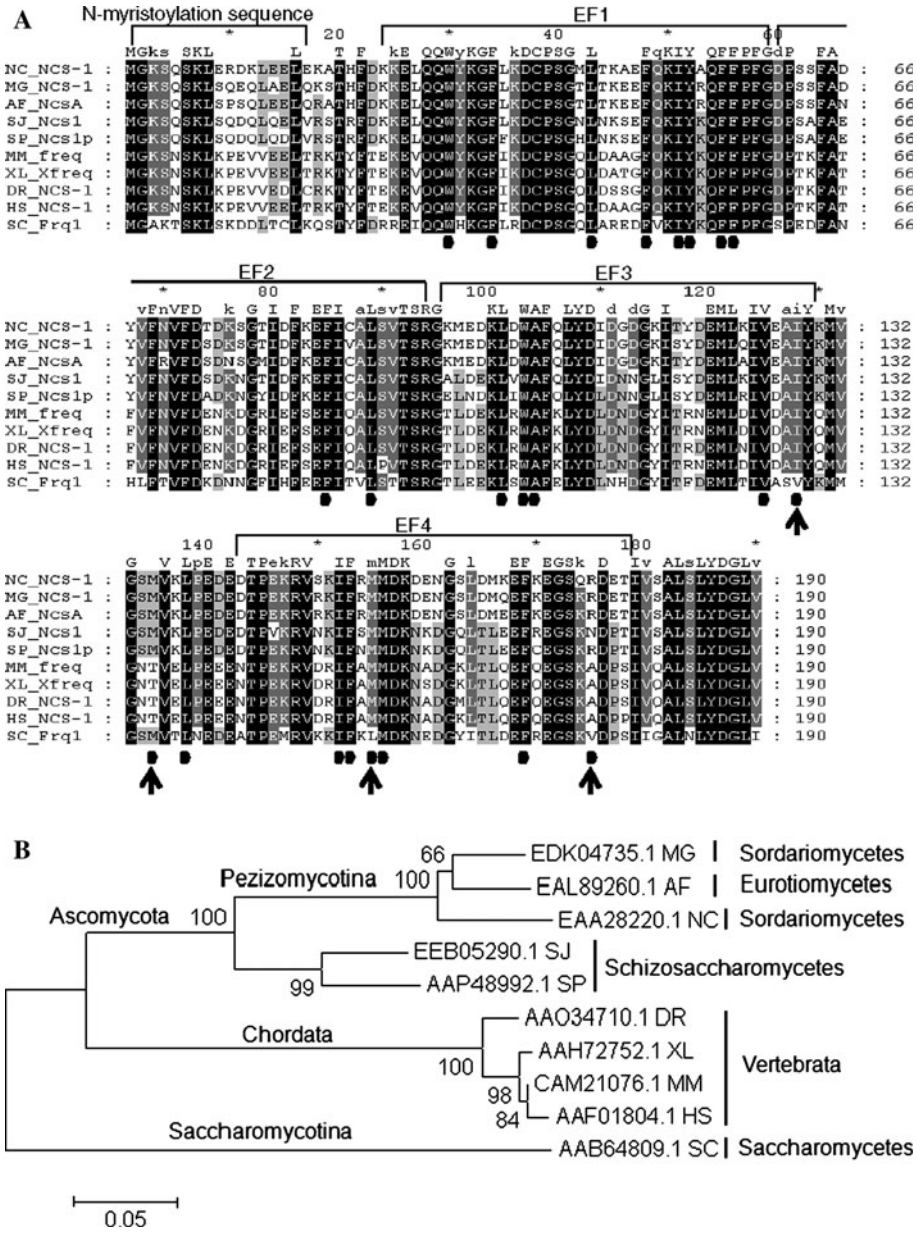


Fig. 4 Alignment and phylogenetic analysis of NCS-1 homologues. **a** Sequence alignment of NCS-1 homologues. The positions of the N-terminal myristoylation sequence and the four EF-hands (EF1, EF2, EF3, and EF4) along with the consensus sequence of the alignment are shown above the sequences. *Solid circles* below the Frq1 sequence indicate the hydrophobic residues that constitute the binding interface with the target peptide of the Frq1 (Strahl et al. 2007), and the *arrow heads* indicate residues that are found altered in other sequences in the alignment. AF, *Aspergillus fumigatus*; DR, *Danio rerio*; HS, *Homo sapiens*; MG, *Magnaporthe grisea*; MM, *Mus musculus*; NC, *Neurospora crassa*; SC, *Saccharomyces cerevisiae*; SJ, *Schizosaccharomyces japonicus*; SP, *Schizosaccharomyces pombe*; XL, *Xenopus laevis*. Conserved amino acids are indicate in *black* (100%), *dark gray* (>80%) and *light gray* (>60%). **b** Phylogenetic analysis of NCS-1 proteins using the minimum-evolution method, 500 Bootstrap replications (bootstrap values are indicated in the point at nodes) as test of phylogeny, and the software MEGA4

sites like the NCS-1 homologues in *A. fumigatus*, *Danio rerio*, *H. sapiens*, *M. grisea*, *Mus musculus*, *S. cerevisiae*, *Schizosaccharomyces japonicus*, *S. pombe*, and *Xenopus laevis* (Fig. 4a). A phylogenetic analysis with a subset of NCS-1 homologues from various organisms revealed that NCU04379 product clustered with the Pezizomycotina clade (Fig. 4b). These results indicate that NCU04379 gene encodes a homologue of NCS-1 in *N. crassa*.

Crosses homozygous for ΔNCU04379.2 mutant strains are fully fertile

To investigate the role of NCU04379 gene in sexual development, we did crosses homozygous for ΔNCU04379.2 mutant strains and all such crosses were fully fertile. We have extended this to another 17 Ca^{2+}-signaling mutants, of which 15 were fully fertile in homozygous crosses (Supplementary Table 1, entries 1–16). However, crosses homozygous for ΔNCU02283.2 mutant strains produce normal looking perithecia, only a few asci were recovered from all perithecia from one petri dish, and display a barren phenotype (produce very few ascospores; Supplementary Table 1, entry 17; Singh et al. 2009). Additionally, crosses homozygous for ΔNCU09123.2 mutant strains display an intermediate phenotype between ΔNCU02283.2 and wild-type (produce a few hundred ascospores; Supplementary Table 1, entry 18). However, both ΔNCU02283.2 and ΔNCU09123.2 mutants mate successfully with wild-type

and produce thousands of ascospores (data not shown). To exclude the possibility that a direct contact between mutant and wild-type allows complementation of the mutant, we performed crosses using ΔNCU02283.2 and ΔNCU09123.2 mutant strains both as male and female parents, however, crosses were fully fertile. These results indicate that both NCU02283 and NCU09123 play a role in sexual development in a recessive manner.

NCU02283 gene is predicted to encode a Ca^{2+}/CaM-dependent protein kinase type I, Ca^{2+}/CaMKI (accession number XP_959927.2), shares 90% identity and 94% similarity in amino acid sequence with a putative CaM kinase, CgCMK, of *Colletotrichum gloeosporioides* (Kim et al. 1998). Both CgCMK and the *N. crassa* Ca^{2+}/CaMKI possess 11 conserved kinase domains and one putative CaM-binding domain, however, of nine putative phosphorylation sites of CgCMK, only five are conserved in the *N. crassa* Ca^{2+}/CaMKI (Fig. 5a). NCU09123 gene encodes another Ca^{2+}/CaMK, CAMK-1(accession number XP_958895.2), which is highly similar to other eukaryotic Ca^{2+}/CaM-dependent kinases, specifically at the kinase domain (Yang et al. 2001; Valle-Aviles et al. 2007). The Ca^{2+}/CaMKI and CAMK-1 proteins from *N. crassa* form a clade with other Sordariomycetes in a phylogenetic analysis with a subset of homologues from fungi (Fig. 5b, c).

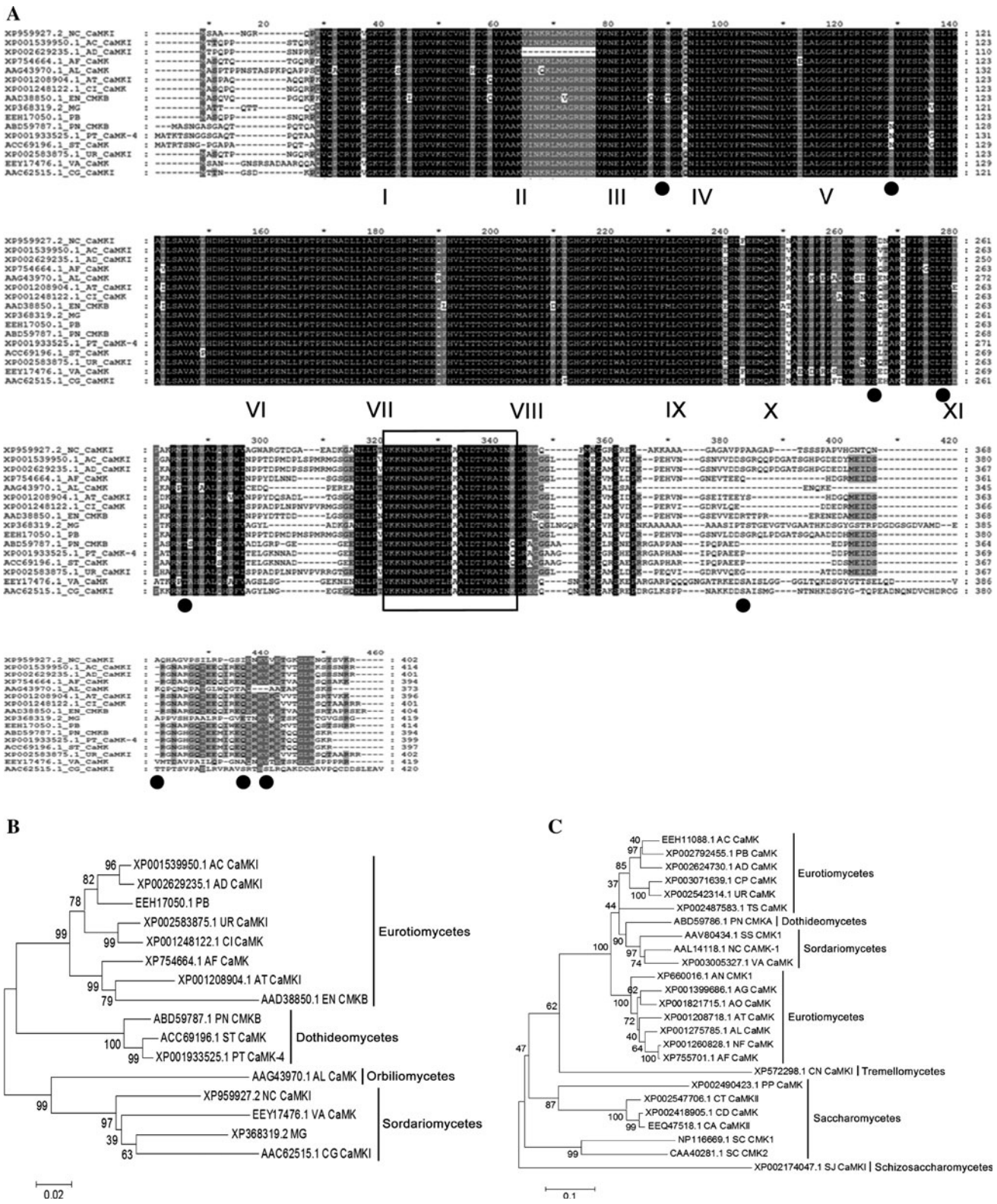
Fig. 5 Alignment and phylogenetic analysis of calcium/calmodulin-dependent protein kinases (Ca^{2+}/CaMKs). **a** Alignment of Ca^{2+}/CaMKI sequences. The sequences in the box represent the putative CaM-binding domain, and *solid circles* below the CG sequence indicate potential autophosporylation sites containing the $^{R}_{K}XX^{T}_{S}$ consensus phosphorylation site for CaMKs (Kim et al. 1998). AC, *Ajellomyces capsulatus*; AD, *Ajellomyces dermatitidis*; AL, *Arthrobotrys dactyloides*; AF, *Aspergillus fumigatus*; AT, *Aspergillus terreus*; CG, *Colletotrichum gloeosporioides*; CI, *Coccidioides immitis*; EN, *Emericella nidulans*; MG, *Magnaporthe grisea*; NC, *Neurospora crassa*; PB, *Paracoccidioides brasiliensis*; PN, *Phaeosphaeria nodorum*; PT, *Pyrenophora tritici-repentis*; ST, *Setosphaeria turcica*; UR, *Uncinocarpus reesii*; VA, *Verticillium albo-atrum*. Conserved amino acids are indicated in *black* (100%), *dark gray* (>80%) and *light gray* (>60%). **b** Phylogenetic analysis of Ca^{2+}/CaMKI proteins using the minimum-evolution method, 500 Bootstrap replications (bootstrap values are indicated in the point at nodes) as test of phylogeny, and the software MEGA4. **c** Phylogenetic analysis of CAMK-1 proteins using the minimum-evolution method, 500 Bootstrap replications (bootstrap values are indicated in the point at nodes), as test of phylogeny and the software MEGA4. The sequences used are AC, *Ajellomyces capsulatus*; AD, *Ajellomyces dermatitidis*; AF, *Aspergillus fumigates*; AG, *Aspergillus niger*; AL, *Aspergillus clavatus*; AN, *Aspergillus nidulans*; AO, *Aspergillus oryzae*; AT, *Aspergillus terreus*; CA, *Candida albicans*; CD, *Candida dubliniensis*; CN, *Cryptococcus neoformans*; CP, *Coccidioides posadasii*; CT, *Candida tropicalis*; NC, *Neurospora crassa*; NF, *Neosartorya fischeri*; PB, *Paracoccidioides brasiliensis*; PN, *Phaeosphaeria nodorum*; PP, *Pichia pastoris*; SC, *Saccharomyces cerevisiae*; SJ, *Schizosaccharomyces japonicus*; SS, *Sporothrix schenckii*; TS, *Talaromyces stipitatus*; UR, *Uncinocarpus reesii*; VA, *Verticillium albo-atrum*

Discussion

Calcium plays an important role in intracellular signaling system in eukaryotes including fungi (Gadd 1994; Shaw and Hoch 2001; Sanders et al. 2002). To understand the cellular roles of the Ca^{2+}-signaling genes in *N. crassa*, we have studied 18 Ca^{2+}-signaling mutants. The ΔNCU04379.2 mutant grows slowly, shows increased sensitivity to Ca^{2+} and UV irradiation. In addition, we found evidence for involvement for NCU02283 and NCU09123 genes in sexual development. Reverse transcription-PCR (RT-PCR) did not show expression of the NCU04379, NCU02283, and NCU09123 gene products in the corresponding mutants (data not shown). We did not notice any phenotype for the remaining 15 Ca^{2+}-signaling mutants in growth, Ca^{2+} stress tolerance, and sexual development. NCU04379, NCU02283, and NCU09123 genes encode, respectively, a homologue of NCS-1, a Ca^{2+}/CaMKI, and a CAMK-1 in *N. crassa*.

In *S. cerevisiae*, Frq1, the NCS-1 ortholog, is essential for cell growth and viability (Hendricks et al. 1999). In *M. grisea*, null-mutants for a neuronal calcium sensor-1/frequenin like gene, *Mg-NCS-1*, showed normal growth, however, high concentrations of Ca^{2+} and acidic conditions suppressed the growth (Saitoh et al. 2003). In *S. pombe*, *ncs1Δ*, deletion mutant of NCS-1 homolog, showed starvation independent sexual development and Ca^{2+}-sensitivity (Hamasaki-Katagiri et al. 2004). In *A. fumigatus*, the *NCS-1* homologue NcsA is involved in sterol distribution in the tip and polar establishment, the *ΔncsA* mutant was more resistant to $CaCl_2$ and sensitive to EGTA (Mota Júnior et al. 2008). We found that knockout mutant of *N. crassa* homologue of NCS-1 displays both slow growth and Ca^{2+}-sensitivity phenotypes (Figs. 1, 2). Additionally, UV-sensitivity of the ΔNCU04379.2 mutant strain uncovers a novel function of NCS-1 homologue in *N. crassa* (Fig. 3). This indicates involvement of NCU04379 gene product in UV-induced DNA damage repair. UV light absorption may cause DNA damage primarily through formation of cyclobutane pyrimidine dimers (CPD; Lippke et al. 1981) and pyrimidine (6–4) pyrimidone photoproducts (6–4PP; Mitchell and Nairn 1989) leading to induction of DNA repair mechanisms or apoptosis (Lo et al. 2005). In human cells, the CaM protein antagonists fully or partially block double-stranded DNA repair and CaM overexpression activates H2AX mediated DNA repair after irradiation (Wang et al. 2000; Herman et al. 2002; Smallwood et al. 2009).

In addition, growth rate of the ΔNCU04379.2 and other Ca^{2+}-signaling mutants (Table 1) were comparable with the wild-type on plates containing hydrogen peroxide



(3 mM) and phytosphingosine (PHS; 5 μg/ml), therefore, we did not find evidence for involvement of these genes in H_2O_2 or PHS mediated cell death in *N. crassa* (Castro et al. 2008; data not shown). The ΔNCU04379.2 mutant phenotypes were complemented upon pRD-1 transformation (Supplementary Fig. 5). Therefore, we conclude that NCU04379 gene has a role in growth, Ca^{2+} stress tolerance, and UV survival in *N. crassa*.

The predicted protein product of NCU04379 gene consists of 190 amino acid residues, which possesses a consensus signal for N-terminal myristoylation and four EF-hand Ca^{2+}-binding sites, and shows high sequence similarity to NCS-1 proteins (Fig. 4). *N. crassa* homologue of NCS-1 has 58% identity with *S. cerevisiae* Frq1. Recent NMR derived structure of Frq1 had identified hydrophobic residues in the groove that constitute the binding interface with the target peptide (Strahl et al. 2007). These hydrophobic residues are also conserved in *N. crassa* homologue of NCS-1 except for alterations in three residues of which V175R may be the most significant alteration (Fig. 4a). Moreover, both Cys residues in *N. crassa* homologue of NCS-1 appear to be buried in the interior (Fig. 4a). *S. cerevisiae* Frq1 also has two Cys residues, however, one is near its N-terminus and the other buried in the interior (Ames et al. 2000). It will be interesting to investigate the effects of these alterations.

Crosses homozygous for ΔNCU04379.2 mutant strains are fully fertile. In addition, our preliminary work on ΔNCU02283.2 and ΔNCU09123.2 mutants indicate that their normal gene functions are essential for fertility. NCU02283 gene is predicted to encode a Ca^{2+}/CaMKI and its homologue in *C. gloeosporioides*, CgCMK, might be involved in germination and appressorium induction (Kim et al. 1998). NCU09123 gene encodes a CAMK-1 that phosphorylates the *N. crassa* circadian clock protein FREQUENCY (FRQ; Yang et al. 2001). In *Sporothrix schenckii*, *sscmk1*, a CAMK-1 homologue might be regulating dimorphism (Valle-Aviles et al. 2007). Ca^{2+}-signaling proteins also play an important role in development in higher organisms. In the late stages of embryogenesis *X. laevis*, CaMKIx, a Ca^{2+}/CaM-dependent protein kinase I is activated upon phosphorylation that can phosphorylate various proteins including synapsin I, histones, and myelin basic protein (Kinoshita et al. 2004). In human, activation of Ca^{2+} channels leads to Ca^{2+} influx that is the pivotal step in initiation of acrosome reaction during fertilization (Ma and Shi 1999). *N. crassa* undergoes a complex sexual developmental process to form protoperithecia when subjected to nitrogen starvation, light and low temperature (Perkins and Barry 1977; Nelson and Metzenberg 1992; Read 1994; Nelson 1996; Coppin et al. 1997). Specialized receptive hyphae called trichogynes are extended from the protoperithecia and fuses with the fertilizing cell of the opposite mating type. After fertilization, protoperithecia develop into perithecia where multiple asci, each containing eight ordered ascospores, are formed (Raju 1992; Kim and Borkovich 2006). Apart from few *s*exual *dev*elopment (*sdv*) and pheromone related genes, little is known about sexual developmental process in *N. crassa* (Johnson 1978; Nelson and Metzenberg 1992; Kim and Nelson 2005; Kim and Borkovich 2006; Iyer et al. 2009). It is possible that the NCU02283 and NCU09123 encoded Ca^{2+}/CaMKs phosphorylate proteins necessary for the sexual development. Our preliminary data indicate that wild-type fragments carrying NCU02283 and NCU09123 genes complement the respective mutant strains (Kumar and Tamuli, unpublished).

Thus, in this study we have shown that a *N. crassa* homologue of NCS-1 encoding gene NCU04379 has a role in growth, Ca^{2+} stress tolerance, and UV survival. Additionally, we found evidence for involvement of Ca^{2+}/CaMKs encoding genes NCU02283 and NCU09123 in sexual development. Future work will enable us to determine the molecular mechanisms of their actions.

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