RESEARCH NOTE

Neurospora crassa ncs-1, *mid-1* and *nca-2* double-mutant phenotypes suggest diverse interaction among three Ca^{2+}-regulating gene products

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Introduction

The calcium (Ca^{2+}) signalling system in the filamentous fungus *Neurospora crassa* is unique and significantly different from that in plants and animals, mainly with regard to the second messenger systems involved in Ca^{2+}-release from internal stores (Galagan *et al.* 2003). The complex Ca^{2+} signalling system in *N. crassa* contains 48 Ca^{2+} signalling proteins including a Ca^{2+} and/or CaM binding protein called neuronal calcium sensor-1 (NCS-1), a Ca^{2+}-permeable channel MID-1 and a PMCA-type Ca^{2+}-ATPase NCA-2 (Zelter *et al.* 2004; Tamuli *et al.* 2013). Here, we show that *ncs-1*, *mid-1* and *nca-2* interactions affect growth, carotenoid accumulation, Ca^{2+} stress tolerance, ultraviolet (UV) survival and circadian-regulated conidiation in *N. crassa*.

The *N. crassa* homologue of NCS-1 has a role in growth, Ca^{2+} stress tolerance and UV survival (Deka *et al.* 2011; Tamuli *et al.* 2011). MID-1 is necessary for Ca^{2+}-homeostasis in *N. crassa* (Lew *et al.* 2008). Mid-1 in *Gibberella zeae* has a role in growth, development and ascospore discharge (Cavinder *et al.* 2011). NCA-2 is responsible for adaptation to stress conditions and functions to pump Ca^{2+} out of the cell in *N. crassa* (Benito *et al.* 2000; Bowman *et al.* 2011).

In *Schizosaccharomyces pombe*, Ncs1p, the homologue of NCS-1, promotes Ca^{2+}-induced closure of Yam8p Ca^{2+} channel, a homologue of the MID1 (Hamasaki-Katagiri and Ames 2010). The Ca^{2+}-sensitive phenotype of the *ncs1* deletion mutant is rescued by a *yam8* deletion, indicating that Ncs1p negatively regulates Yam8p in *S. pombe* (Hamasaki-Katagiri and Ames 2010). However, no information is available regarding the interaction between homologues of Ncs1p and Yam8p in *Neurospora*. In addition, previous studies had indicated NCS-1 and NCA-2 roles in controlling Ca^{2+} levels in *N. crassa*; however, relationship between these two proteins remained unknown. Here, we study *ncs-1*, *mid-1* and *nca-2* mutations, suggesting their proteins interact to regulate various cell functions.

Materials and methods

Strains, growth, assay for Ca^{2+} and UV sensitivity

The *N. crassa* wild-type strains 74-OR23-1 *A* (FGSC 987) and OR8-1 *a* (FGSC 988), *ras-1^{bd}A* (FGSC 1858), *ras-1^{bd}a* (FGSC 1859); and the Ca^{2+}-signalling knockout mutants, Δ*NCU04379.2::hph A* (FGSC 11404), Δ*NCU04379.2::hph a* (FGSC 11403), Δ*NCU06703.2::hph A* (FGSC 11708), Δ*NCU06703.2::hph a* (FGSC 11707), Δ*NCU04736.2::hph A* (FGSC 13071) were obtained from the Fungal Genetics Stock Center (FGSC, Kansas, USA) (McCluskey 2003). The *NCU04379*, *NCU06703* and *NCU04736* genes encode the *N. crassa* homologue of NCS-1 (Deka *et al.* 2011), MID-1 (Lew *et al.* 2008) and NCA-2 (Bowman *et al.* 2009), respectively. The Δ*ncs-1*Δ*nca-2*, Δ*ncs-1*Δ*mid-1* and Δ*mid-1*Δ*nca-2* double mutants were generated by crossing the single mutant strains of opposite mating types and mutant alleles were confirmed by the PCR analysis of f_1 progeny (figure 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/). A minimum of two strains were generated for each of the double mutants. Growth, crossing and maintenance of *Neurospora* strains were essentially as described by Davis and De Serres (1970). Ca^{2+} and UV sensitivity assays were performed as previously described (Deka *et al.* 2011).

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Carotenoid accumulation

To test for carotenoid accumulation, ~1 × 10^6 wild type and mutant strains spores were inoculated into flasks containing 25 mL of Vogel's liquid medium supplemented with 0.2% Tween 80 as a wetting agent to avoid conidiation (Zalokar 1954). The cultures were incubated initially in continuous darkness at 30°C for 48 h and then exposed to cool-white light at 22°C for 24 h (illuminated with two fluorescent bulbs (Philips Lifemax Tubelight TL-D 18W/54, Philips, Kolkata, India), 18 W, 6500 K, 1015 lumens). Mycelia were then filtered, lyophilized and powdered with mortar and pestle. Acetone and hexane were used in consecutive extractions of total carotenoid from 50 mg of lyophilized sample. Total carotenoid content of the wild type and mutant strains were calculated by measuring the maximum absorbance value at 470 nm and using the formula: total carotenoid content (μg/g) = [total absorbance × total volume of extract (1 mL) × 10^4]/[absorption coefficient (2500) × sample weight (g)] (Rodriguez-Amaya and Kimura 2004).

Circadian-regulated conidiation

The *N. crassa* strains were inoculated at one end of the race tube containing 1 × Vogel's salts, 0.1% D-glucose, 0.17% L-arginine, 50 ng/mL biotin and 1.5% bacto agar. The cultures were incubated at 25°C in constant light for 24 h and the growth fronts were marked. The cultures were then shifted to constant darkness at 25°C and the growth fronts were marked under a red safe light at regular interval of 24 h for five days. Period lengths were calculated by multiplying distance between conidial bands by the inverse of slope (http://www.fgsc.net/teaching/circad.htm).

Results and discussion

Effect of Δncs-1 Δmid-1 and Δnca-2 mutations in colony morphology, growth rate, aerial hyphae and carotenoid accumulation

We studied morphology and growth rates of the *ncs-1*, *mid-1* and *nca-2* knockout mutants. We found that only the Δ*ncs-1*Δ*nca-2* double mutant showed novel colony morphology with a characteristic swollen sponge-like colony (figure 2a in electronic supplementary material). The average growth rates followed the order wild type > Δ*nca-2* > Δ*ncs-1* > Δ*ncs-1*Δ*nca-2* > Δ*mid-1*Δ*nca-2* ≅ Δ*mid-1* > Δ*ncs-1*Δ*mid-1* (figure 1; table 1 in electronic supplementary material). Growth rates for both Δ*ncs-1*Δ*nca-2* and Δ*ncs-1*Δ*mid-1* double mutants were lower than the parental single mutants. However, the Δ*mid-1*Δ*nca-2* double mutant showed growth rate similar to that of the Δ*mid-1* mutant. In addition, the Δ*ncs-1* and Δ*nca-2* mutants showed shorter aerial hyphae and this phenotype was aggravated in the Δ*ncs-1*Δ*nca-2* double mutant (figure 2b in electronic supplementary material). Interestingly, aerial hyphae growth in the Δ*mid-1* mutant was comparable to the wild type; however, the Δ*ncs-1*Δ*mid-1* and Δ*mid-1*Δ*nca-2* double mutants showed shorter aerial hyphae like the Δ*ncs-1* and Δ*nca-2* mutants, respectively (figure 2b in electronic supplementary material).

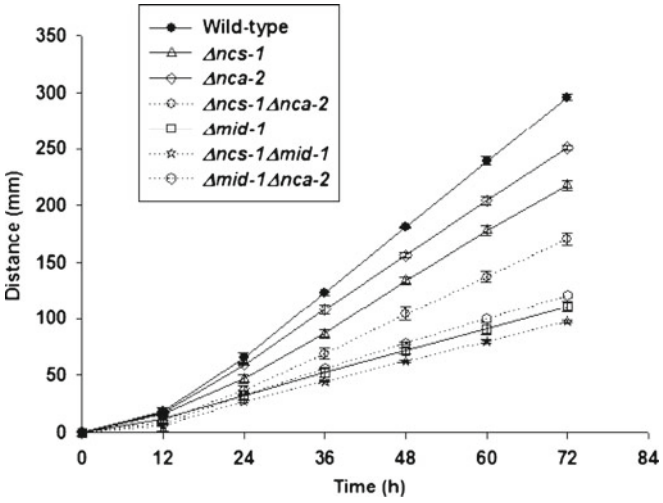

Figure 1. Growth rates. Rates of apical growth of the wild type and mutant strains in race tubes are plotted against the indicated time interval. Error bars show the standard errors calculated from the data for three independent experiments.

We also performed quantitative analysis of carotenoid accumulation in the mutants to test the effect of these mutations in carotenoid synthesis. The carotenoid profile followed the order Δ*mid-1*Δ*nca-2* > Δ*ncs-1*Δ*mid-1* > Δ*mid-1* > Δ*ncs-1* > Δ*nca-2* > wild type ≅ Δ*ncs-1*Δ*nca-2* (figure 2). Thus, except the Δ*ncs-1*Δ*nca-2* double mutant, the other mutants produced more carotenoid than the wild type which suggests that the wild-type gene products negatively affect carotenoid accumulation. Accumulation of the xanthophyll

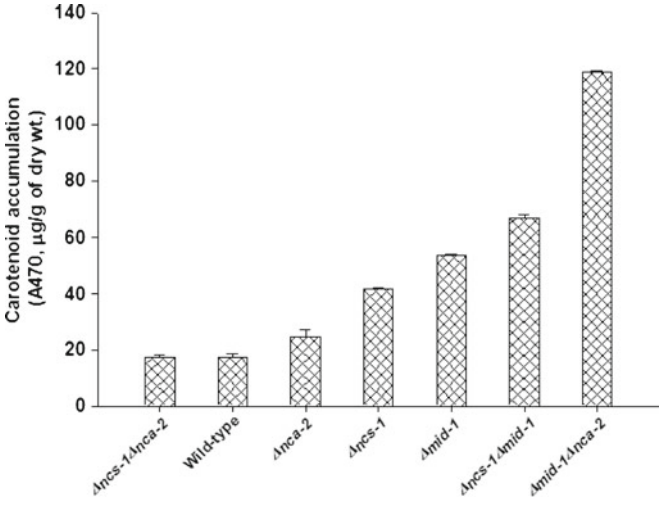

Figure 2. Total carotenoid contents. Carotenoid extracted from the indicated *N. crassa* strains are expressed as μg carotenoid per gram of dry weight. Error bars show the standard errors calculated from the data for three independent experiments.

neurosporaxanthin and variable amounts of carotenoid precursors results in characteristic orange pigmentation of conidia and mycelia in *N. crassa* (Avalos *et al.* 2013). Our studies indicate that Ca^{2+} signalling pathway could be involved in regulating carotenoid accumulation in *N. crassa*.

The Δncs-1Δnca-2 double mutant was hypersensitive to Ca^{2+} and UV stress, and the Δmid-1 and Δnca-2 mutants showed increased sensitivity to UV

The Δ*ncs-1* and Δ*nca-2* mutants were sensitive to Ca^{2+} stress (Bowman *et al.* 2011; Deka *et al.* 2011). In addition, growth of the Δ*mid-1* mutant was inhibited at low extracellular or elevated intracellular Ca^{2+} (Lew *et al.* 2008). To test if Ca^{2+} stress tolerance is influenced by the interactions of *ncs-1*, *mid-1* and *nca-2*, we studied sensitivity of the mutants to various concentrations of $CaCl_2$. The Ca^{2+} stress tolerance followed the order Δ*mid-1* > wild type > Δ*ncs-1*Δ*mid-1* > Δ*nca-2* > Δ*ncs-1* > Δ*mid-1*Δ*nca-2* > Δ*ncs-1*Δ*nca-2* (figure 3a, table 2 in electronic supplementary material).

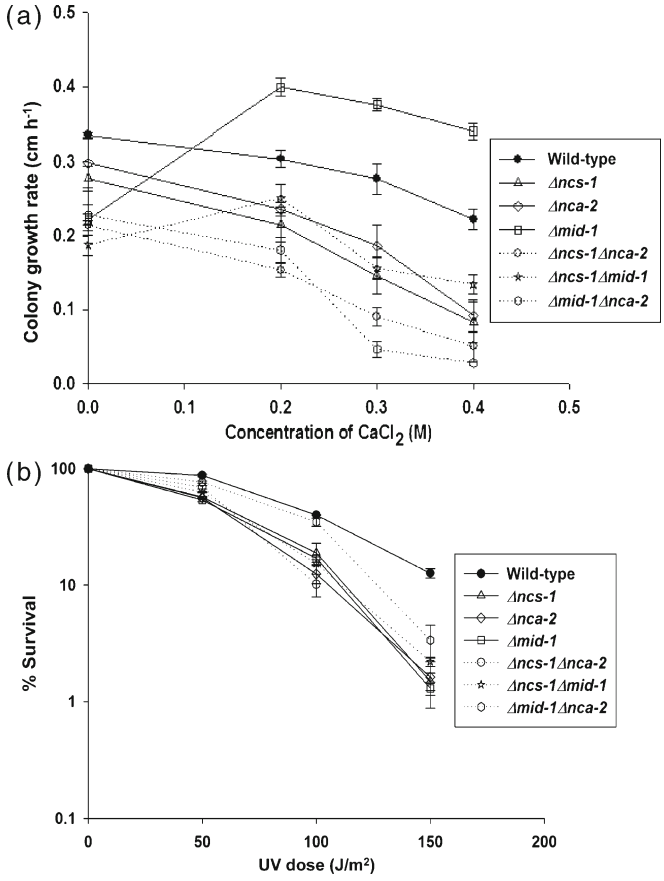

Figure 3. Analysis of Ca^{2+} and UV sensitivity. (a) Ca^{2+} sensitivity assay. Colony diameter (cm h^{-1}) of the *N. crassa* were measured at regular intervals and plotted against various concentrations of $CaCl_2$. Standard errors calculated from the data for three independent experiments are shown using error bars. (b) Dose-response curves of the *N. crassa* wild type and the mutant strains on exposure to UV irradiations. Each data point represents the mean of at least three independent experiments.

Thus, Δ*nca-2* mutation had a synergistic effect on Ca^{2+} sensitivity phenotype of the Δ*mid-1* and Δ*ncs-1* mutants. The growth of Δ*mid-1* mutant was stimulated ~83.8% at 0.2 M $CaCl_2$ concentrations. Interestingly, stimulation of the growth rate of the Δ*mid-1* mutant at 0.2 M $CaCl_2$ was suppressed in the Δ*ncs-1* and Δ*nca-2* backgrounds, growth stimulation was only ~34% in the Δ*ncs-1*Δ*mid-1* double mutant and fully disappeared in the Δ*mid-1*Δ*nca-2* double mutant. The Ca^{2+}-permeable channels regulate passive inflow of Ca^{2+} into the cell, whereas the Ca^{2+}-ATPases reduce the cytosolic free Ca^{2+} ($[Ca^{2+}]_c$) by active pumping of Ca^{2+} into internal stores or efflux of Ca^{2+} from the cell (Møller *et al.* 1996; Zelter *et al.* 2004). This could explain the epistasis of the *nca-2* over *mid-1* for Ca^{2+} sensitivity. It is possible that NCS-1 is also involved in decreasing $[Ca^{2+}]_c$ by stimulating Ca^{2+}-efflux. The Ca^{2+}-sensitivity phenotype was aggravated in the Δ*ncs-1*Δ*nca-2* double mutant, indicating that negative interaction of *ncs-1* and *nca-2* plays a critical role in reducing hazardous amount of $[Ca^{2+}]_c$. However, Ca^{2+} sensitivity phenotype of the Δ*ncs-1* mutant was not fully rescued by the Δ*mid-1* mutant, indicating that the relationship between *ncs-1* and *mid-1* genes are not the same in *N. crassa* as their homologues in *S. pombe* (Hamasaki-Katagiri and Ames 2010; figure 3a).

The null mutant of *N. crassa* homologue of *ncs-1* was sensitive to UV (Deka *et al.* 2011). We tested the sensitivity of the double mutants in exposure to UV irradiation (figure 3b). Both Δ*mid-1* and Δ*nca-2* mutants showed an increased sensitivity to UV; however, to our surprise, the Δ*mid-1*Δ*nca-2* double mutant was more tolerant to UV than either of the single mutants (figure 3b). The Δ*ncs-1*Δ*nca-2* double mutant showed severe sensitivity to UV; however, UV sensitivity of the Δ*ncs-1*Δ*mid-1* double mutant was comparable to the single mutants (figure 3b). These results indicated involvement of MID-1 and NCA-2 in UV induced DNA damage repair. Besides, interactions of *mid-1* with *nca-2* and *ncs-1* with *nca-2* affect the UV survival in a negative and positive manner, respectively.

The Δncs-1Δnca-2, Δncs-1Δmid-1 and Δmid-1Δnca-2 double mutant showed lesser sensitivity to respiratory byproduct CO_2 and produced conidial bands

The analysis of the knockout mutants for the genes *ncs-1*, *mid-1* and *nca-2* indicated their involvement in stress tolerance. Therefore, we also tested if any of the mutants could tolerate the respiratory byproduct CO_2 and produce conidial bands race tube. The conidiation of the wild type in a race tube is suppressed due to accumulation of CO_2. However, conidiation persists despite the CO_2 accumulation in a race tube in the *band* (*bd*) mutant *ras-1*^{bd} that carries a T79I point mutation in *ras-1*; therefore, the *ras-1*^{bd} mutant is used in circadian rhythm studies (Sargent and Kaltenborn 1972; Belden *et al* 2007). We found that both Δ*ncs-1*Δ*mid-1* and Δ*mid-1*Δ*nca-2* double mutants produce

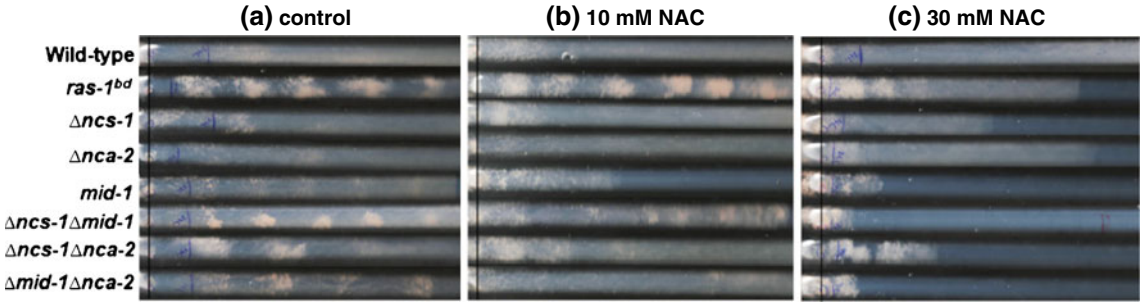

Figure 4. Circadian-regulated conidiation. (a) The Δ*ncs-1*Δ*mid-1* and Δ*mid-1*Δ*nca-2* double mutants produce distinct conidial bands at regular intervals in race tubes. In the Δ*ncs-1*Δ*nca-2* double mutants, bands were distinct till 72 h. (b) Rhythmic conidiation patterns of the Δ*ncs-1*Δ*mid-1* and Δ*mid-1*Δ*nca-2* double mutants were suppressed in the medium supplemented with 10 mM NAC. (c) Growth defects of the *N. crassa* strains in the medium supplemented with 30 mM NAC.

distinct conidial bands with an increased period length of 25.41 ± 0.48 h and 23.96 ± 0.58 h, respectively, and the *ras-1*^{bd} control showed a period length of 22.48 ± 0.72 (figure 4a). The Δ*ncs-1*Δ*nca-2* double mutant showed a less robust effect and produced distinct conidial bands up to 72 h (figure 4a). To test if the conidial band were produced due to the increased level of reactive oxygen species (ROS), we supplemented race tube medium with antioxidant N-acetyl-L-cysteine (NAC) to reduce ROS. Conidial bands were suppressed in the double mutants on addition of 10 mM NAC; however, NAC at 30 mM caused growth arrest, indicating a toxic effect at high concentration (figures 4b, c). In summary, Δ*ncs-1*Δ*nca-2*, Δ*ncs-1*Δ*mid*-1 and Δ*mid-1*Δ*nca-2* double mutants showed lesser sensitivity than the single mutants and the wild-type strains to the respiratory byproduct CO_2 and produced conidial bands, which was possibly due to an increased ROS level in these mutants. Thus, our finding that Δ*ncs-1*Δ*nca-2*, Δ*ncs-1*Δ*mid-1* and Δ*mid-1*Δ*nca-2* double mutants produced distinct conidial bands under the conditions normally unfavourable for conidiation in race tube, indicate that wild-type gene products synthetically act as negative regulators of circadian-regulated conidiation. We also tested if the Δ*ncs-1* mutant could show any synthetic effect in the knockout background of another Ca^{2+}-signalling gene NCU02814 (*prd-4*). The Δ*ncs-1*Δ*prd-4* double mutant did not show any synthetic effect on growth, Ca^{2+} and UV stress tolerance and period length; therefore, ruling out the hypothesis that similar genetic interactions exist between any of the Ca^{2+} signalling gene pair (data not shown). Thus, we showed that genetic interactions of *ncs-1*, *mid-1* and *nca-2* regulate multiple cellular processes in *N. crassa*.

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