Abstract/Synopsis

The calcium (Ca^{2+}) signaling system in the filamentous fungus *Neurospora crassa* is unique and this system lacks receptors for second messengers such as inositol-1,4,5-trisphosphate ($InsP_3$), ryanodine and cyclic ADP ribose (cADPR) that are responsible for Ca^{2+} release from internal stores in other organisms. $InsP_3$ is synthesized by phospholipase C-δ subtype (PLC-δ) proteins. These proteins are identified in Neurospora as well as in other filamentous fungi; however, signaling via $InsP_3$ receptor is still unclear since no recognizable $InsP_3$ receptors have been identified in Neurospora. In addition, ADP-ribosyl cyclase protein that synthesizes second messengers such as cADP ribose or NAADP and ryanodine receptor, which are the key components of Ca^{2+} release mechanisms in animal cells, are not known in Neurospora and other fungi. Furthermore, no homologues of either sphingosine kinase, which synthesizes the second-messenger sphingosine-1-phosphate, or the sarcoplasmic reticulum Ca^{2+} release channel, SCaMPER, which is a possible target of sphingolipids, could be identified in *N. crassa*. These notable differences suggest that filamentous fungi might possess novel intracellular Ca^{2+} release mechanism that remains to be identified that might provide novel antifungal targets for drug discovery. In filamentous fungi including *N. crassa* where growth pattern and development are more complex than lower fungi, there is evidence for the involvement of Ca^{2+} in numerous physiological processes including cell cycle, circadian rhythms, cytoskeletal organization, hyphal branching, hyphal orientation, hyphal tip growth, infection structure differentiation, sporulation and spore germination. However, detailed knowledge about the main proteins involved in Ca^{2+}-mediated signal response pathway is still lacking for *N. crassa* or, indeed, any other filamentous fungus. Therefore, I have decided to work on three broad objectives such as

(i) to screen *N. crassa* Ca^{2+} signaling knockout mutants to identify the Ca^{2+} signaling genes involved in various cell functions,

(ii) to understand the molecular mechanism of selected Ca^{2+}signaling gene by identifying the critical amino acid residues through site-directed mutagenesis approach, and

(iii) to study the genetic interactions of selected Ca^{2+} signaling genes to determine their epistatic relationship.

Major conclusions of the study

In this work, I have studied the cellular roles of Ca_{2+} signaling genes in *Neurospora crassa* using knockout mutant strains. I have shown that NCU04379 gene is involved in growth, calcium (Ca^{2+}) stress tolerance and ultraviolet (UV) survival. This is the first report demonstrating the involvement of Ca^{2+} signaling gene in UV-induced DNA damage and repair process in *N. crassa*. The sequence analysis has revealed that NCU04379 encodes a protein of 190 amino acid residues that shows sequence similarity to the homologues of Neuronal Calcium Sensor-1 (NCS-1). Moreover, the NCU04379 encoded protein is found to be highly conserved from fungi to mammals and contain consensus sequence for N-terminal myristoylation (NMT), and four EF hand domains (EF1, EF2, EF3, and EF4) for Ca^{2+} binding. Therefore, NCU04379 encodes a homologue of NCS-1 in *N. crassa*.

I performed site-directed mutational analysis to identify critical residues of NCS-1 homologue in *N. crassa*. The glycine to alanine mutation in the N-myristoylation site (G2A) impaired NCS-1 function for UV survival, indicating that N-myristoylation is essential for NCS- 1 function in UV induced DNA damage and repair process. The arginine to alanine mutation (R175A) in the hydrophobic pocket of NCS-1 resulted in an increased UV sensitivity in the *ncs-1*^{R175A} mutant than the Δ*ncs-1* mutant, suggesting that arginine 175 plays a critical role UV survival and this could explain the molecular basis of *N. crassa* homologue of NCS-1 for its novel involvement in UV-induced DNA damage repair process. The glutamate to glutamine mutation in the Ca^{2+} binding site (E120Q) of the EF hand domain 3, completely abolished NCS-1 functions in growth, Ca_{2+}stress tolerance, and UV survival; these results suggested that Ca^{2+} binding is essential for functions of NCS-1 in *N. crassa*. Thus, site-directed mutational analysis identified three critical amino residues in the NCS-1 homologue of *N. crassa*. In addition, Δ*ncs-1*Δ*nca-2*, Δ*ncs-1*Δ*mid-1*, and Δ*mid-1*Δ*nca-2* double mutants were generated to study genetic interaction among *ncs-1*, *mid-1*, and *nca-2* genes in *N. crassa*. The *mid-1* and *nca-2* genes encode a Ca^{2+}-permeable channel and a Ca^{2+}-ATPase, respectively.

Studies on the double mutants revealed that these genes synthetically regulate growth, aerial hyphae development, carotenoid accumulation, Ca^{2+} stress tolerance, sensitivity to the respiratory by-product CO_2, and UV survival. In addition to the previously identified *ncs-1* gene, *mid-1*and *nca-2* genes also play a role in UV-induced DNA damage repair

as revealed by the UV sensitivity analysis. Therefore, a complex genetic interaction of *ncs-1*, *mid-1*, and *nca-2* genes regulate multiple cell functions in *N. crassa*. Moreover, expression profile of *ncs-1*, *mid-1* and *nca-2* in response to Ca^{2+} stress also supported that synthetic interaction of these genes play a role in Ca^{2+} stress tolerance.