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Multiple cellular roles of *Neurospora crassa plc-1*, *splA2*, and *cpe-1* in regulation of cytosolic free calcium, carotenoid accumulation, stress responses, and acquisition of thermotolerance[§]

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Phospholipase C1 (PLC1), secretory phospholipase A2 (sPLA2) and Ca^{2+}/H^+ exchanger proteins regulate calcium signaling and homeostasis in eukaryotes. In this study, we investigate functions for phospholipase C1 (*plc-1*), sPLA2 (*splA2*) and a Ca^{2+}/H^+ exchanger (*cpe-1*) in the filamentous fungus *Neurospora crassa*. The *Δplc-1*, *ΔsplA2*, and *Δcpe-1* mutants exhibited a growth defect on medium supplemented with the divalent ionophore A23187, suggesting that these genes might play a role in regulation of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) in *N. crassa*. The strains lacking *plc-1*, *splA2*, and *cpe-1* possessed higher carotenoid content than wild type at 8°C, 22°C, and 30°C, and showed increased ultraviolet (UV)-survival under conditions that induced carotenoid accumulation. Moreover, *Δplc-1*, *ΔsplA2*, and *Δcpe-1* mutants showed reduced survival rate under hydrogen peroxide-induced oxidative stress and induced thermotolerance after exposure to heat shock temperatures. Thus, this study revealed multiple cellular roles for *plc-1*, *splA2*, and *cpe-1* genes in regulation of $[Ca^{2+}]_c$, carotenoid accumulation, survival under stress conditions, and acquisition of thermotolerance induced by heat shock.

Keywords: calcium signaling, Ca^{2+}/H^+ exchanger, phospholipase C-1, secretory phospholipase A2, *Neurospora crassa*

Introduction

Cell signaling requires messengers including calcium ion (Ca^{2+}) whose concentration varies with space, time and amplitude (Berridge *et al*., 1998; Clapham, 2007). Binding of Ca^{2+} to target proteins changes their conformation, charge, and thereby, governs protein functions (Clapham, 2007). Thus, Ca^{2+} has evolved as a universal messenger that plays a versatile role in intracellular signaling in all cell types and tissues (Berridge *et al*., 1998; Kazmierczak *et al*., 2013). Ca^{2+}-signaling is typically triggered by a transient increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$). The resting $[Ca^{2+}]_c$ is ~100 nM; however, when Ca^{2+}-signaling is triggered, $[Ca^{2+}]_c$ rises transiently upto 1 μM or more (Chin and Means, 2000; Bootman *et al*., 2001).

In the filamentous fungus *Neurospora crassa*, low $[Ca^{2+}]_c$ is maintained using active transport mechanisms across the plasma membrane and buffering Ca^{2+} in organelles (Bowman *et al*., 2011). In *N. crassa*, Ca^{2+}-signaling is known to be involved in regulating a variety of processes such as Ca^{2+} stress tolerance, the circadian clock, growth, hyphal tip branching, ion transport, sexual development and UV survival (Deka *et al.*, 2011; Deka and Tamuli, 2013; Tamuli *et al*., 2011, 2013; Kumar and Tamuli, 2014). The predicted Ca^{2+}-signaling machinery of *N. crassa* is complex and contains many different proteins including four novel phospholipase C-δ subtype (PLC-δ) proteins (Galagan *et al*., 2003; Borkovich *et al*., 2004). The PLC protein cleaves phosphotidylinositol -4, 5-bisphosphate (PIP2) into second messengers, namely, inositol 1, 4, 5-trisphosphate (IP_3) and diacylglycerol (DAG) (Chae *et al*., 2007). In animal cells, IP_3 diffuses through the cytosol and binds to the IP_3 receptors, inducing Ca^{2+} release from vacuoles, whereas DAG activates protein kinase C (PKC), triggering a range of cellular activities (Cornelius and Nakashima, 1987; Chae *et al*., 2007). Interestingly, no recognizable IP_3 receptors has been identified in *N. crassa* and both of its PKC proteins lack a C2 domain with Ca^{2+} binding sites (Galagan *et al*., 2003; Borkovich *et al*., 2004).

PLC proteins are involved in diverse cell functions in different organisms. In the budding yeast *Saccharomyces cerevisiae*, Plc1p, a phosphatidylinositol-specific phospholipase C (PI-PLC) shows sequence homology to the mammalian PI-PLC-δ isoforms and is involved in nutritional and stress-related responses (Flick and Thorner, 1993). In *S. cerevisiae*, Plc1p is necessary for growth at nonpermissive temperatures (above 35°C), survival under hyperosmotic stress, and utilization of galactose, raffinose, or glycerol as a carbon source at permissive temperatures (23 to 30°C) (Flick and Thorner, 1993). In the grey mold fungus *Botrytis cinerea*, a necrotrophic plant pathogen, PLC1 homologue *BcPLC1* functions in growth, conidiation, germination and virulence (Schumacher *et al*., 2008). In *Magnaporthe oryzae*, the rice blast fungus, the *MoPLC1* gene encodes a fungal PI-PLC-δ isoform that regulates intracellular Ca^{2+} fluxes essential for fungal development, appressorium formation and pathogenicity (Rho *et al*., 2009). Moreover, two other PLC isozymes *MoPLC2* and *MoPLC3* exhibit, respectively, 58% and 49% identity to the *PLC1* gene of *N. crassa* (NCU06245), and play distinct roles in *M. oryzae* including regulating development and appres-

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sorium-mediated penetration (Choi *et al.*, 2011). In *Alternaria alternata*, the citrus fungal pathogen, the *PLC1* homologue plays an important role in vegetative growth, conidial formation, Ca^{2+} homeostasis, and virulence (Tsai and Chung, 2014). In the encapsulated yeast and human pathogen *Cryptococcus neoformans*, *CnPlc1*, a homologue of the mammalian PI-PLC-δ, supplies the IP3 substrate required for the catalytic activity of the major IP3 kinase, Arg1, which is essential for cellular homeostasis and virulence (Lev *et al.*, 2013).

In contrast to these other fungi, little is known about the *N. crassa* homologues of PLC homologs. The NCU06245 gene encodes a homologue of PLC-δ that is highly divergent among the natural isolates of *N. crassa*. The NCU06245 gene exhibits high incidence of polymorphisms resulting in a coding sequence changes for amino acid positions 200–250 unique to *Neurospora* (Gavric *et al.*, 2007).

The *N. crassa* Ca^{2+}-signaling machinery also includes 23 members of Ca^{2+} and/or CaM binding protein families (Galagan *et al.*, 2003; Borkovich *et al.*, 2004; Tamuli *et al.*, 2013). One member of this group shows sequence similarity to secretory phospholipase A2 (sPLA2). sPLA2, comprises a diverse family of low-molecular weight secretory enzymes possessing the ability to hydrolyze the ester bond at the *sn-2* position of glycerophospholipids in a Ca^{2+} dependent manner, thereby, liberating fatty acids and lysophospholipids (Murakami and Kudo, 2002). Various sPLA2 proteins play roles in numerous biological processes such as atherosclerosis, host defense, inflammation, and regulation of eicosanoid synthesis (Murakami and Kudo, 2002; Boilard *et al.*, 2010). A novel secretory protein, p15, isolated from *Helicosporium sp.* HN1 has been shown to induce outgrowth of neurites and neuronal differentiation from rat pheochromocytoma PC12 cells (Wakatsuki *et al.*, 1999; 2001). Another sPLA2 homologue Scp15 isolated from *Streptomyces coelicolor* displays similar neurite inducing activity in PC12 cells (Nakashima *et al.*, 2003). Fungi possesses a heterogeneous group of PLA_2 proteins with a variety of structural domains that could play an important role in nutrient acquisition and interaction with the host (Köhler *et al.*, 2006). In the symbiotic fungus *Tuber borchii*, the phospholipase A TbSP1 is strongly up-regulated and activated via autoproteolysis that may enhance establishment of symbiosis and mycorrhiza formation in response to nutrient starvation (Soragni *et al.*, 2001; Cavazzini *et al.*, 2013). In the filamentous ascomycete *Aspergillus oryzae*, two distinct $sPLA_2s$, sPlaA and sPlaB exhibit distinct physiological properties such as pH optimum for enzyme activity, Ca^{2+} requirement, substrate preferences, expression profile, and cellular localization (Nakahama *et al.*, 2010). In mice, sPLA2 regulates phagocytosis and contributes to the innate immune response against *Candida albicans* (Balestrieri *et al.*, 2009). Furthermore, in humans, a group of sPLA2 enzymes

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

Sl. No.	FGSC no. (a/A) or strain no.	NCU no. or strain type	Type of protein	Source
1	11248/11249	7075.2	Ca^{2+}/H^{+} exchanger	FGSC
2	12376/12375	795.2	Ca^{2+}/H^{+} exchanger	FGSC
3	11530/11529	2826.2	Ca^{2+}/H^{+} exchanger	FGSC
4	11407/11408	6366.2	Ca^{2+}/H^{+} exchanger	FGSC
5	12468/NA	8490.2	Ca^{2+}/H^{+} exchanger	FGSC
6	NA/11253	7605.2	Ca^{2+} permeable channel	FGSC
7	11707/11708	6703.2	Ca^{2+} permeable channel	FGSC
8	13287/NA	3305.2	Ca^{2+} ATPase	FGSC
9	NA/13071	4736.2	Ca^{2+} ATPase	FGSC
10	13036/13037	5154.2	Ca^{2+} ATPase	FGSC
11	NA/13040	4898.2	Ca^{2+} ATPase	FGSC
12	11410/11409	7966.2	Cation- ATPase	FGSC
13	12022/NA	1266.2	Phospholipase C	FGSC
14	11411/NA	6245.2	Phospholipase C	FGSC
15	11271/11271	9655.2	Phospholipase C	FGSC
16	12023/NA	2175.2	Phospholipase C	FGSC
17	11405/11406	5225.2	Ca^{2+} and/or CaM binding protein	FGSC
18	13049/NA	2115.2	Ca^{2+} and/or CaM binding protein	FGSC
19	15890/NA	2738.2	Ca^{2+} and/or CaM binding protein	FGSC
20	11541/11542	6948.2	Ca^{2+} and/or CaM binding protein	FGSC
21	11403/11404	4379.2	Ca^{2+} and/or CaM binding protein	FGSC
22	11531/NA	3750.2	Ca^{2+} and/or CaM binding protein	FGSC
23	12448/12449	2283.2	Ca^{2+} and/or CaM binding protein	FGSC
24	12548/12547	9123.2	Ca^{2+} and/or CaM binding protein	FGSC
25	11169/11170	2814.2	Ca^{2+} and/or CaM binding protein	FGSC
26	11545/NA	9212.2	Ca^{2+} and/or CaM binding protein	FGSC
27	11246/11247	6650.2	Ca^{2+} and/or CaM binding protein	FGSC
28	11537/11536	6177.2	Ca^{2+} and/or CaM binding protein	FGSC
29	988/987	Wild type		FGSC

possibly plays a novel anti-inflammatory role in immune complex-mediated arthritis (Boilard *et al*., 2010). However, little is known about the sole sPLA2 protein identified in *N. crassa*.

The high $[Ca^{2+}]_c$ is reduced to the resting level by another group of Ca^{2+}-signaling proteins, Ca^{2+} exchangers that pump Ca^{2+} out of the cell and transport Ca^{2+} into the intracellular Ca^{2+} storage organelles with the simultaneous exchange of positive ions across membranes (Zelter *et al*., 2004; Tamuli *et al*., 2013). The *N. crassa* Ca^{2+}-signaling machinery possesses six novel Ca^{2+}/H^+ exchangers and two putative Ca^{2+}/Na^+ exchangers, and all of these exchangers contain conserved Ca^{2+} exchanger domains (Galagan *et al*., 2003; Borkovich *et al*., 2004; Zelter *et al*., 2004). One of the *N. crassa* Ca^{2+}/H^+ exchangers called CAX is homologous to Vcx1p from *S. cerevisiae*. The *Δcax* mutant accumulates very little Ca^{2+} in the dense vacuolar fraction, and therefore, CAX could be involved in maintaining intracellular Ca^{2+} levels in *N. crassa* (Bowman *et al*., 2011). Phylogenetic analysis has revealed that one of the *N. crassa* Ca^{2+}/H^+ exchangers, encoded by NCU06366, is significantly different from homologues found in *S. cerevisiae* and *M. grisea* (Zelter *et al*., 2004). However, detailed knowledge about the NCU06366-encoded Ca^{2+}/H^+ exchanger has remained largely unknown.

In this work, we describe the cellular roles of *plc-1*, s*plA2*, and NCU06366 in regulation of $[Ca^{2+}]_c$, carotenoid accumulation, survival under stress conditions, and acquisition of thermotolerance.

Materials and Methods

Strains, media, and growth conditions

N. crassa wild type strains 74-OR23-1 A (FGSC 987) and OR8-1 a (FGSC 988), Ca^{2+} signaling mutants ΔNCU06245.2 a (FGSC 11411), ΔNCU06650.2 A (FGSC11247), ΔNCU-06650.2 a (FGSC11247), ΔNCU06366.2 A (FGSC 11408), ΔNCU06366.2 a (FGSC 11407), and other strains (Table 1) were obtained from the Fungal Genetics Stock Center (FGSC; University of Missouri, Kansas city, MO 64110) (McCluskey, 2010). We verified knockout mutants using the gene-specific primers along with the common reverse *hph* primer in polymerase chain reactions (Supplementary data Fig. S1). Media and procedures for growth and maintenance of *N. crassa* strains were essentially as described (Davis and De-Serres, 1970). For vegetative growth, strains were routinely cultured on 1X Vogel's (Vogel, 1964) glucose medium (VGM) with 1.5% D-glucose as a carbon source and 1.5% Bacto Grade Agar (SRL).

Sequence analysis

The sequences of *N. crassa* NCU06245, NCU06650 and NCU06366 proteins were downloaded from Broad Institute Neurospora database version 7 (http://www.broadinstitute.org/annotation/genome/neurospora/MultiHome.html) and used as queries to search against the non-redundant protein sequence databases at the NCBI using BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi; Altschul *et al*., 1990) with the default parameters. Proteins were selected based on % identities, % similarities and *E* values. Multiple sequence alignments of the selected proteins were built using CLUSTALX 1.83 (Thompson *et al*., 1997) and alignments visualized using the GeneDoc program (Nicholas and Nicholas, 1997). These alignments were used for constructing phylogenetic trees using the minimum-evolution method (Rzhetsky and Nei, 1992), 500 bootstrap replication as test of phylogeny (Felsenstein, 1985) and the software MEGA5 (Tamura *et al*., 2011).

Calcium ionophore assay

To test for elevated intracellular Ca^{2+} sensitivity in the mutants, an agar block was inoculated onto one corner of petri dishes and 10 µl each of a 9.5 mM stock solution of A23187 (test) or ethanol (control) was spotted onto the other corner on the VGM media as described previously (Lew *et al*., 2008). Plates were incubated at 30°C for 2–3 days and colonies were observed under a Trinocular inverted microscope (Axio Vert.A1 FL, Carl Zeiss). Hyphal images were captured with an AxioCam ICc3 CCD camera. The petri dishes were also photographed after 38 h of growth using a digital camera (Nikon Coolpix P500).

Carotenoid analysis

For carotenoid analysis, sterile petri dishes containing liquid VGM supplemented with 0.2% tween 80 were inoculated with conidia at a concentration of ~1×10^6 conidia/ml (Zalokar, 1954). Cultures were initially incubated for 48 h in the dark at 30°C and further incubated at the indicated temperature either with or without an exposure to white light for 24 h (illuminated with two fluorescent bulbs, Philips TL-D 18W/54 lamp, 18W, 6500 K, 1015 lumens). Mycelia from these cultures were collected, lyophilized and pulverized into fine powder with mortar and pestle. Subsequently, acetone and hexane were used in consecutive steps for extraction of total carotenoids from 25 mg (dry weight) of the powdered sample. Total carotenoid content was determined by measuring the absorbance value at 470 nm and using the formula:

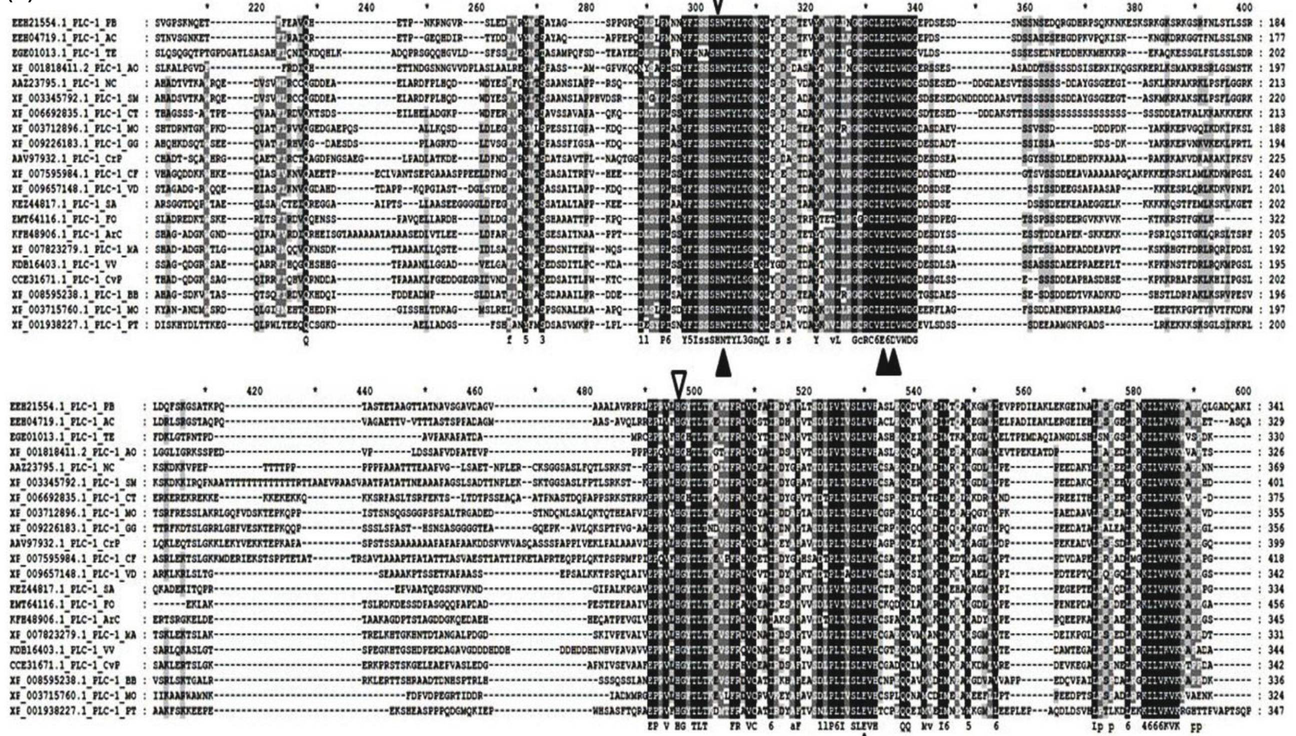
Total carotenoid content (µg/g) = [Total absorbance × Total volume of extract (1 ml) × 10^4] / [Absorption coefficient (2500) × sample weight (g)] as described previously (Rodriguez-Amaya and Kimura, 2004).

UV sensitivity assay

Conidia were grown in flasks containing VGM (without tween 80 supplement) agar medium at 30°C in the dark for 48 h and then at the indicated temperature with or without illumination as described for the carotenoid analysis above. For quantitative analysis, conidia were plated on FGS (0.05% fructose, 0.05% glucose, 0.05% sorbose plus 2% agar) medium and irradiated with different doses of UV in a UVC 500 cross linker (Hoefer, UK). The plates were incubated at 30°C in the dark for 48 h and number of colonies on each plate counted.

Oxidative stress resistance and thermotolerance studies

For hydrogen peroxide induced oxidative stress assay, conidia of *N. crassa* strains were inoculated at a concentration of



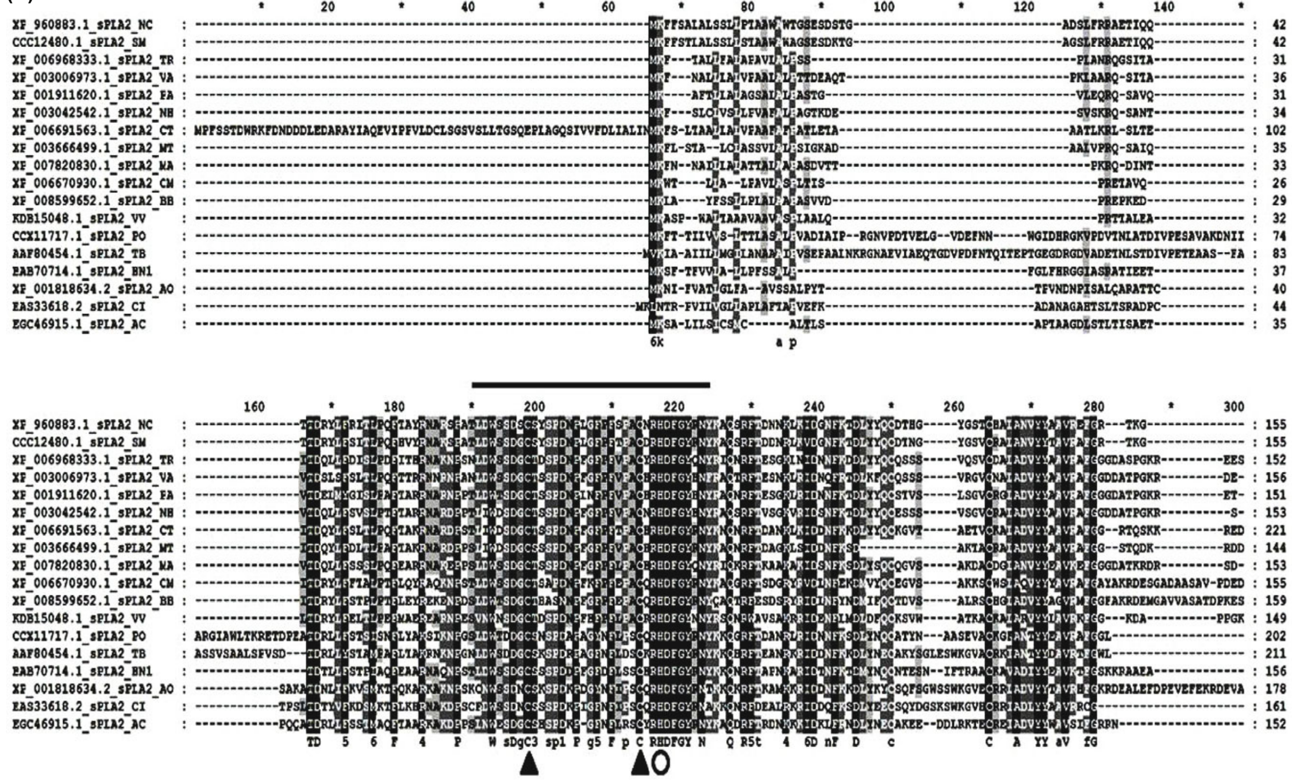


Fig. 1. Continued

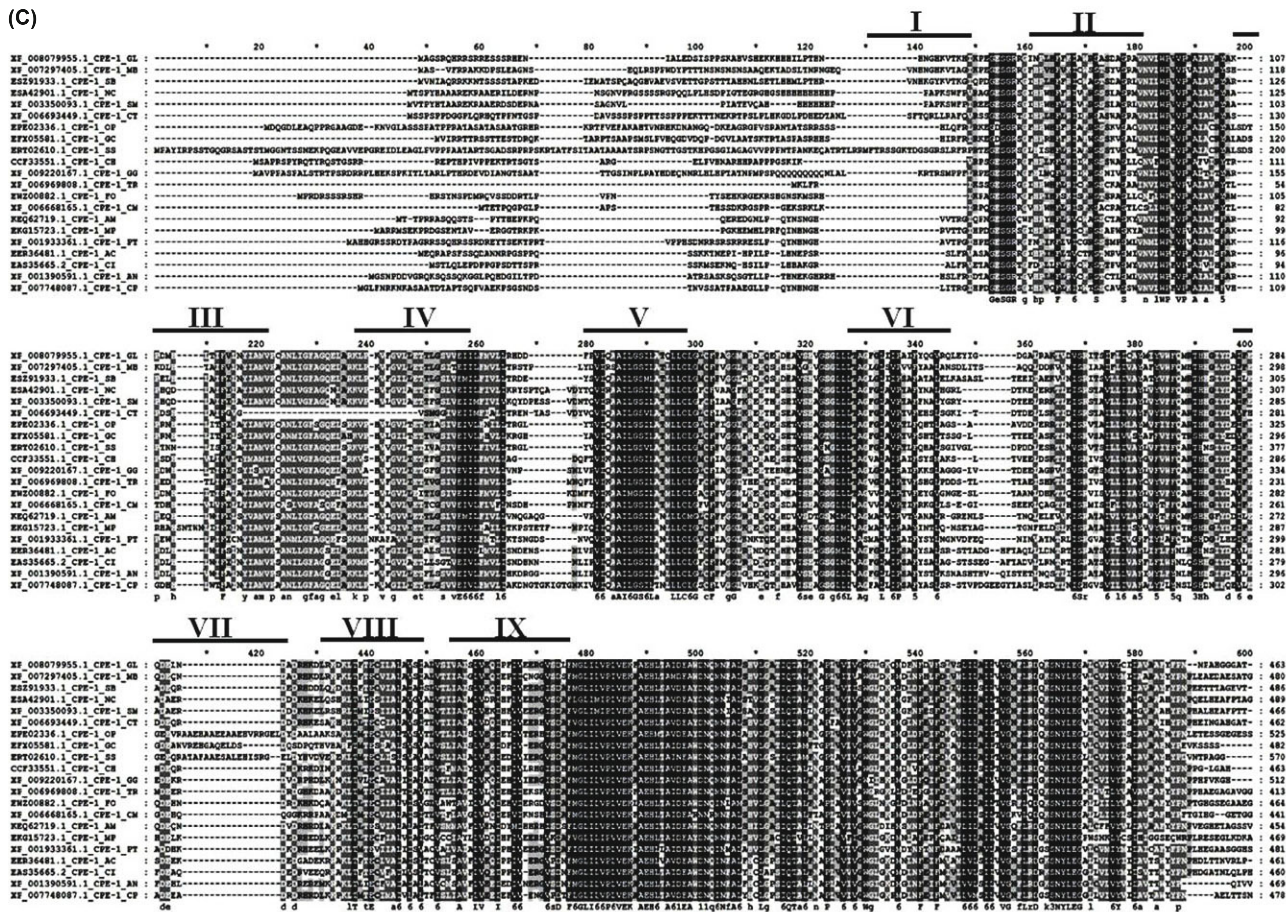


Fig. 1. Sequence analysis of *plc-1*, *splA2*, and *cpe-1* homologues. (A) Sequence alignment of PLC-1 homologues. The positions of the hydrogen binding residues are indicated above the sequence (inverted triangles) and the Ca^{2+} binding residues are indicated below the sequence (filled triangles) (Chung *et al.*, 2006). (B) Sequence alignment of sPLA2 homologues. The predicted secretion signal peptide is underlined in red. The highly conserved portion of sPLA2 (positions 122-153) is underlined in black. The positions of the catalytic histidine residue (open circle) and disulphide bonded cysteine residues (filled triangles) are indicated below the sequences (Soragni *et al.*, 2001). (C) Sequence alignment of CPE-1 homologues. The sequence corresponds to transmembrane segments (I to IX) as predicted with TMHMM program are shown underlined (http://www.cbs.dtu.dk/services/TMHMM/) (Zelter *et al.*, 2004; Guttery *et al.*, 2013). The sequences used in the analyses are AC, *A. capsulatus*; ArC, *Acremonium chrysogenum*; AM, *Aureobasidium melanogenum*; AN, *Aspergillus niger*; AO, *A. oryzae*; BB, *Beauveria bassiana*; CF, *Colletotrichum fioriniae*; CH, *C. higginsianum*; CI, *Coccidioides immitis*; CM, *Cordyceps militaris*; CP, *Cladophialophora psammophila*; CrP, *C. parasitica*; CvP, *Claviceps purpurea*; CT, *Chaetomium thermophilum*; FO, *Fusarium oxysporum*; GC, *Grosmannia clavigera*; GG, *Gaeumannomyces graminis*; HN1, *Helicosporium* sp.; GL, *Glarea lozoyensis*; MA, *Metarhizium anisopliae*; MB, *Marssonina brunnea*; MO, *M. oryzae*; MP, *Macrophomina phaseolina*; MT, *Myceliophthora thermophila*; NC, *N. crassa*; NH, *Nectria haematococca*; OP, *Ophiostoma piceae*; PA, *Podospora anserine*, PB, *Paracoccidioides brasiliensis*; PO, *Pyronema omphalodes*; PT, *Pyrenophoratritici-repentis*; SA, *Scedosporium apiospermum*; SB, *Sclerotinia borealis*; SM, *Sordaria macrospora*; SS, *Sporothrix schenckii*; TB, *T. borchii*; TE, *Trichophyton equinum*; TR, *Trichoderma reesei*; VA, *Verticillium alfalfae*; VD, *Verticillium dahliae*; VV, *Villosiclava virens*. Conserved amino acids are indicated in black (100%), dark grey (>80%) and light grey (>60%) in the alignments.

~1×10^6 conidia/ ml into liquid VGM and germinated with shaking at 200 rpm for 2 h in the dark at 30°C. Germlings were supplemented with hydrogen peroxide (test) or without (control) at a final concentration of 10 mM and further germinated for 1 h at 30°C. Germlings were plated on FGS medium and incubated at 30°C for 24 h. Percent survival was scored by dividing the number of viable colonies from plates exposed to H_2O_2 (test) by the number of colonies from plates not exposed to H_2O_2 (control) and multiplying by 100.

Similarly, for thermotolerance assays, 2 h germlings obtained as mentioned above were held at 30°C (control), 30°C (uninduced thermotolerance), and 44°C (induced thermotolerance). Both 30°C (uninduced) and 44°C (induced) germlings were then given a 52°C lethal heat shock for 20 min. Germlings were finally plated on FGS media followed by incubation at 30°C for 24 h (Yang and Borkovich, 1999). Percent survival was calculated by dividing the number of viable colonies from plates subjected to heat-treatment (induced or uninduced) by the number of colonies on plates held at 30°C (control) and multiplying by 100.

Statistical analysis

Statistical analysis was carried out using the data obtained from at least three independent replicates by one-way ANOVA (Microsoft Excel).

Results and Discussion

Sequence analysis of NCU06245, NCU06650 and NCU06366

N. crassa NCU06245, NCU06650, and NCU06366 encode three distinct types of Ca^{2+}signaling proteins. The NCU-06245 gene is predicted to encode a 711 amino acid residues phospholipase C-1 (PLC-1) with similarity to PLC homologues in *Ajellomyces capsulatus*, *Colletotrichum higginsianum*, and *MoPLC2* and *MoPLC3* of *M. oryzae* (43, 52, 51, and 43% identities; Fig. 1A). Sequence alignment of NCU06245 homologues revealed important Ca^{2+} and H^+ binding residues similar to PLC homologues from *Cryphonectria parasitica*, *M. grisea*, and *Arabidopsis thaliana* (Chung *et al.*, 2006). The NCU06650 gene is predicted to encode a conserved hypothetical secretory phospholipase A2 (sPLA2) of 186 amino acid residues that shares sequence similarity with *A. capsulatus*, *A. oryzae*, *T. borchii*, and *Verticillium alfalfa* sPLA2 proteins (55, 41, 50, and 57% identities; Fig. 1B). The protein encoded by the NCU06650 gene also possesses conserved signal peptide residues (positions 1–30) and a highly conserved region (positions 122–153) that possesses catalytic histidine residues and disulphide-bonded cysteine residues similar to the sPLA2 homologue of *T. borchii* (Soragni *et al.*, 2001). The NCU06366 gene encodes a Ca^{2+}/H^+ exchanger (calcium proton exchanger-1; CPE-1) of 505 amino acid residues sharing sequence similarity with *A. capsulatus*, *A. niger*, *C. higginsianum* and *M. oryzae* homologues of CPE (50, 52, 59, and 68% identities; Fig. 1C). The NCU06366 gene product contains nine transmembrane helices as predicted by the TMHMM program (http://www. cbs.dtu.dk/services/TMHMM/; Zelter *et al.*, 2004; Guttery *et al.*, 2013). These results indicate that NCU06245, NCU06650 and NCU06366 correspond to *N. crassa* homologues of the *plc-1*, *splA2*, and *cpe-1*genes, respectively. In addition, the PLC-1, sPLA2, and CPE-1 proteins from *N. crassa* were found clustered within the Sordariomycetes clade during phylogenetic analysis with a subset of homologues from other fungi (Supplementary data Fig. 2).

Δplc-1, *ΔsplA2* and *Δcpe-1* mutants show growth defects in response to increased intracellular free Ca^{2+}

We found that growth rates of the *Δplc-1*, *ΔsplA2*, and *Δcpe-1* mutants on VGM were similar to the wild-type (Supplementary data Table S1). However, the *Δplc-1*, *ΔsplA2*, and *Δcpe-1* mutants exhibited morphological defects when cultured on VGM containing the divalent ionophore A23187 (Fig. 2). The A23187 ionophore causes an increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$; Nelson *et al.*, 2004). In the ionophore zone, the *Δplc-1* mutant exhibited reduced branching with characteristic spherical sac-like structures at the apex of the growing hyphae. Similar sac like structures and more compact hyphal branching was observed in the *ΔsplA2* mutant. In case of the *Δcpe-1*mutant, apical hyper branching of hyphae was observed. These results indicate that the *plc-1*, *splA2*, and *cpe-1* genes may play a role in the regulation of $[Ca^{2+}]_c$ in *N. crassa*. We also studied growth of 25 additional knockout mutants lacking predicted Ca^{2+} signaling on medium containing the A23187 ionophore, but, these mutants were similar to wild-type (Supplementary data Table S2).

To test whether the hyphal growth defect of *Δplc-1*, *ΔsplA2*, and *Δcpe-1* mutants upon ionophore treatment was due to dissipation of membrane potential, we used the membrane potential sensitive fluorescent dye DiBAC that fluoresces when membranes are depolarized (Alcántara-Sánchez *et al.*, 2004). Germlings of *Δplc-1*, *ΔsplA2*, and *Δcpe-1*mutants

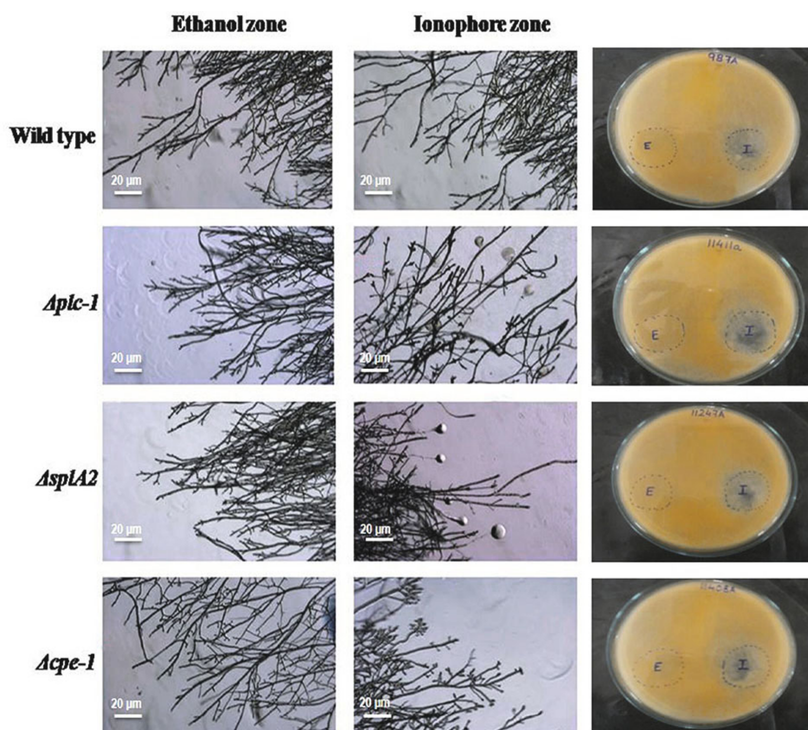


Fig. 2. Calcium ionophore assay. *N. crassa* wild type and the three mutant strains were grown in petri dishes containing the ionophore A23187 zone (test) and the ethanol zone (control). Images were taken when hyphae of the strains touched both the zones (38 h of growth at 30°C). Ten images were captured for each strain and a representative image is shown.

Table 2. **Carotenoid content of *N. crassa* strains at three different temperatures.**

Strains	Carotenoids (μg/g dry wt.)[a]					
	8°C		22°C		30°C	
	Light	Dark	Light	Dark	Light	Dark
Wild type	151 ± 20.1	21 ± 4.3	66 ± 12	35 ± 4	74 ± 5.4	18 ± 3
Δplc-1	253 ± 3 (***)	34 ± 6.1 (**)	126 ± 11(***)	55 ± 3.1 (**)	144.2 ± 20.2 (**)	49.4 ± 8 (**)
ΔsplA2	255 ± 12.2 (***)	46 ± 6 (*)	169 ± 3.1 (**)	41 ± 5 (*)	177.4 ± 14 (***)	38.1 ± 9 (*)
Δcpe-1	235 ± 6.1 (**)	50 ± 11.2 (*)	175 ± 17.2 (***)	48 ± 4 (*)	160.1 ± 10 (***)	47 ± 2 (***)

[a] Results are shown as mean ± standard deviation for three independent experiments (n=3) with *P-values* < 0.05 (*), < 0.01 (**), and < 0.001 (***) compared with the wild type strain as measured by one-way ANOVA test.

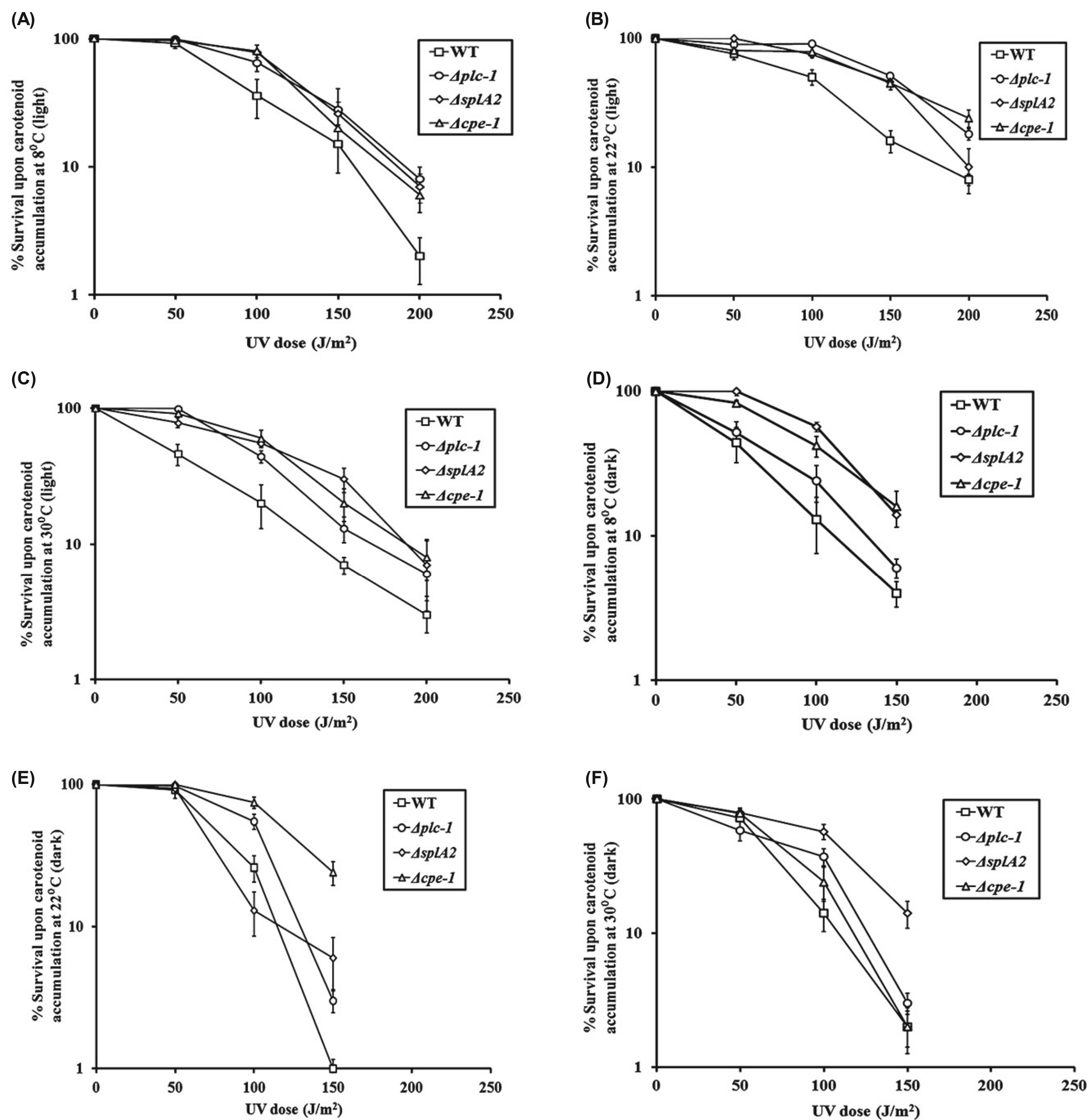


Fig. 3. **Assay for UV sensitivity relative to carotenoid accumulation.** Dose-response curves of the wild-type and mutant strains after exposure to UV radiation after carotenoid accumulation for 48 h in the dark at 8°C, 22°C or 30°C followed by 24 h either in light illumination (A), (B), and (C) or in continuous darkness (D), (E), and (F). Error bars indicate standard deviations calculated from the data for three independent experiments.

treated with 5 μM A23187, followed by DiBAC incubation showed no fluorescence, suggesting that the effect of A23187 on the germlings of *Δplc-1*, *ΔsplA2*, and *Δcpe-1* mutants was not due to dissipation of membrane potential (Supplementary data Fig. S3).

Assessment of conidiation, carotenoid accumulation, and survival after exposure to UV light in *Δplc-1*, *ΔsplA2*, and *Δcpe-1*

Conidia amounts in the *Δplc-1*, *ΔsplA2*, and *Δcpe-1* mutants were similar to the wild-type, suggesting that these mutations do not affect asexual sporulation in *N. crassa* (Supplementary data Fig. S4). We also measured carotenoid amounts in the three mutants. The characteristic orange pigmentation of *N. crassa* strains is caused by the accumulation of the xanthophyll neurosporoxanthin and variable amounts of carotenoid precursors (Zalokar, 1954; Avalos *et al.*, 2013). Carotenoid biosynthesis is affected by light and temperature, with neurosporoxanthin biosynthesis greatly enhanced upon illumination at low temperature whereas illumination at normal temperature results in the accumulation of carotenoid precursors (Harding *et al.*, 1969; Harding, 1974; Estrada *et al.*, 2008; Díaz-Sánchez *et al.*, 2011). Based on this, we determined carotenoid contents in light and dark at three different temperatures, 8°C, 22°C, and 30°C in the three mutants. Our results demonstrated that the three mutants had higher carotenoid content than wild type at all three temperatures, and this was statistically significant (ANOVA, $P < 0.05$) (Table 2).

It has been reported in the literature that the antioxidant activity of carotenoids against reactive oxygen species provides protection against UV damage in fungi and humans (Luque *et al.*, 2012). Therefore, we tested whether the increased carotenoid accumulation in the *Δplc-1*, *ΔsplA2*, and *Δcpe-1* mutants was linked to protection against harmful UV radiation. To test this, *N. crassa* strains were grown under conditions similar to those used for the carotenoid analysis. We found that conidia from these mutants produced at 8°C, 22°C, or 30°C in light or dark, showed increased survival relative to wild type after UV irradiation (Fig. 3). Extrapolating from our analysis of carotenoids profile, the increased UV survival of these mutants at 8°C, 22°C, and 30°C in light illumination could be due to higher carotenoid accumulation. In contrast, the UV-dose response curves of *Δplc-1*, *ΔsplA2*, and *Δcpe-1* mutants were similar to wild type under conditions that did not induce carotenoid accumulation (i.e. incubation at 30°C for 48 h followed by illumination under light at room temperature for 96 h; data not shown). These results support the notion that the increase in carotenoid content in the *N. crassa* mutants of *plc-1*, s*plA2*, and *cpe-1* is contributing to their increased UV survival.

Effects of the *Δplc-1*, *ΔsplA2*, and *Δcpe-1* mutations on survival under oxidative stress and acquisition of thermotolerance

We tested the viability of the Δ*plc-1*, Δ*splA2*, and Δ*cpe-1* mutants upon exposure to10 mM hydrogen peroxide-induced oxidative stress and found that all had reduced survival percentage compared to the wild type that was statistically significant (ANOVA, $P < 0.05$) (Fig. 4A). The resistance to hydrogen peroxide-induced oxidative stress tolerance followed the trend wild type>*Δcpe-1*>*ΔsplA2*>*Δplc-1*. These results are supported by results in *A. oryzae* for splA2 homologs demonstrating that *ΔsplaB* displays increased sensitivity to peroxide and *splaA* is strongly upregulated by oxidative stress (Nakahama *et al.*, 2010).

We also investigated roles for *plc-1*, s*plA2* and *cpe-1* in thermotolerance-to a lethal temperature. Previous work has shown that incubation of cells at a sublethal (heat shock) temperature leads to increased survival after subsequent exposure to a lethal temperature and this phenomenon has been called induced thermotolerance (Kapoor *et al.*, 1990). *N. crassa* cells when incubated at sub lethal heat shock temperatures leads to synthesis of heat shock proteins which in turn protects the cells from lethal temperature (Kapoor *et al.*, 1995; Yang and Borkovich, 1999). In contrast, uninduced thermotolerance refers to survival of cells after exposure to a lethal temperature, without preincubation at a heat shock tempera-

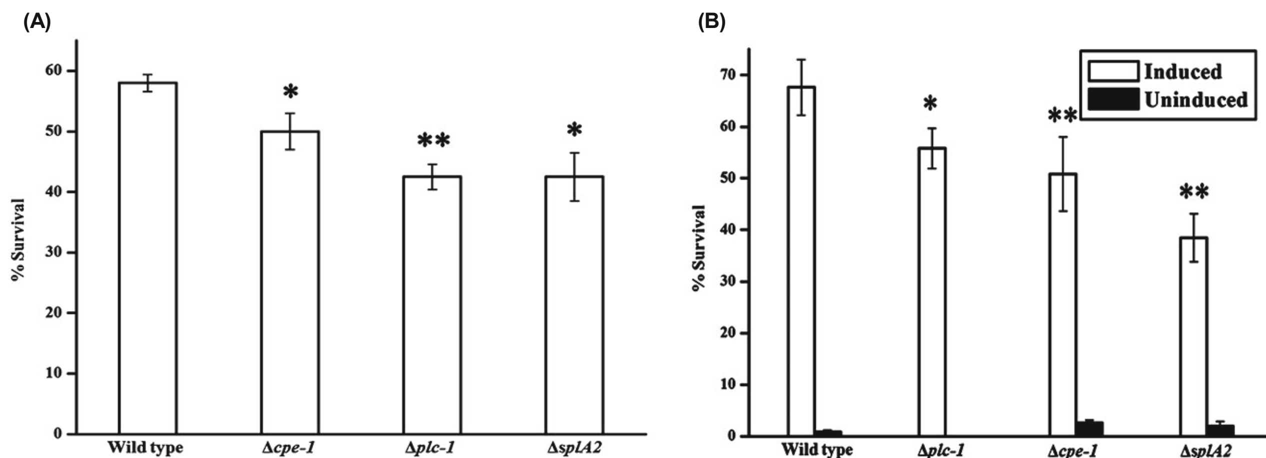


Fig. 4. Oxidative stress and thermotolerance assay. (A) Germlings (2 h old) were incubated in medium containing 10 mM H_2O_2 at 30°C and percent survival determined. (B) Viability of 2 h old germlings after exposure to 52°C lethal temperature with (induced) or without (uninduced) pre-exposure to a sublethal heat shock temperature of 44°C. Error is calculated as the standard deviations, using data from three independent experiments (n=3). Statistically significant values are indicated by asterisks, $^{*}P < 0.05$; $^{**}P < 0.01$.

ture. The *Δplc-1, ΔsplA2*, and *Δcpe-1* mutants had decreased survival relative to wild type under induced thermotolerance. In contrast survival was slightly greater than wild type for the *ΔsplA2* and *Δcpe-1* mutants under uninduced thermotolerance conditions (Fig. 4B). In addition, statistical significance was achieved for the thermotolerance assay, according to variance analysis (ANOVA, $P < 0.05$). Interestingly, no viable colony was observed in the *Δplc-1* mutant under uninduced thermotolerance conditions. These results suggested that *plc-1*, s*plA2*, and *cpe-1* may play a role in survival at lethal temperatures mediated by prior expression of heat shock proteins. Of interest, *splaB* in *A. oryzae* is weakly upregulated by heat shock (Nakahama *et al.*, 2010).

Conclusion

The *N. crassa* strains lacking *plc-1*, s*plA2*, and *cpe-1* displayed growth defects in response to increases in $[Ca^{2+}]_c$ induced by the Ca^{2+}ionophore A23187. Moreover, the carotenoid profile in the *plc-1*, s*plA2*, and *cpe-1* mutants was altered and the increased carotenoid amount was linked to UV-survival of the strains. Furthermore, *plc-1*, s*plA2*, and *cpe-1* mutants had decreased survival after exposure to hydrogen peroxide-induced oxidative stress and in induced thermotolerance experiments. These phenotypes may result from a disruption in calcium homeostasis in the mutants, suggesting that calcium signaling regulates numerous cellular pathways in *N. crassa*.

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References

Alcántara-Sánchez, F., Reynaga-Peña, C.G., Salcedo-Hernández, R., and Ruiz-Herrera, J. 2004. Possible role of ionic gradients in the apical growth of *Neurospora crassa*. *Antonie van Leeuwenhoek* **86**, 301–311.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410.

Avalos, J., Luis, M., and Corrochano, L.M. 2013. Carotenoid Biosynthesis in *Neurospora*, pp. 227–241. *In* Kasbekar, D.P. McCluskey, K. (eds.), Neurospora: genomics and molecular biology. Caister Academic Press, Norfolk, UK.

Balestrieri, B., Maekawa, A., Xing, W., Gelb, M.H., Katz, H.R., and Arm, J.P. 2009. Group V secretory phospholipase A2 modulates phagosome maturation and regulates the innate immune response against *Candida albicans*. *J. Immunol.* **182**, 4891–4898.

Berridge, M.J., Bootman, M.D., and Lipp, P. 1998. Calcium–a life and death signal. *Nature* **395**, 645–648.

Boilard, E., Lai, Y., Larabee, K., Balestrieri, B., Ghomashchi, F., Fujioka, D., Gobezie, R., Coblyn, J.S., Weinblatt, M.E., Massarotti, E.M., *et al.* 2010. A novel anti-inflammatory role for secretory phospholipase A_2 in immune complex-mediated arthritis. *EMBO Mol. Med.* **2**, 172–187.

Bootman, M.D., Collins, T.J., Peppiatt, C.M., Prothero, L.S., MacKenzie, L., De Smet, P., Travers, M., Tovey, S.C., Seo, J.T., Berridge, M.J., *et al.* 2001. Calcium signalling–an overview. *Semin. Cell Dev. Biol.* **12**, 3–10.

Borkovich, K.A., Alex, L.A., Yarden, O., Freitag, M., Turner, G.E., Read, N.D., Seiler, S., Bell-Pedersen, D., Paietta, J., Plesofsky, N., *et al.* 2004. Lessons from the genome sequence of *Neurospora crassa*: tracing the path from genomic blueprint to multicellular organism. *Microbiol. Mol. Biol. Rev.* **68**, 1–108.

Bowman, B.J., Abreu, S., Margolles-Clark, E., Draskovic, M., and Bowman, E.J. 2011. Role of four calcium transport proteins, encoded by *nca-1*, *nca-2*, *nca-3*, and *cax*, in maintaining intracellular calcium levels in *Neurospora crassa*. *Eukaryot. Cell* **10**, 654–661.

Cavazzini, D., Meschi, F., Corsini, R., Bolchi, A., Rossi, G.L., Einsle, O., and Ottonello, S. 2013. Autoproteolytic activation of a symbiosis-regulated truffle phospholipase A_2. *J. Biol. Chem.* **288**, 1533–1547.

Chae, S.W., Kim, J.M., Yun, Y.P., Lee, W.K., Kim, J.S., Kim, Y.H., Lee, K.S., Ko, Y.J., Lee, K.H., and **Rha, H.K.** 2007. Identification and analysis of the promoter region of the human PLC-δ4 gene. *Mol. Biol. Rep.* **34**, 69–77.

Chin, D. and Means, A R. 2000. Calmodulin: a prototypical calcium sensor. *Trends Cell Biol.* **10**, 322–328.

Choi, J., Kim, K.S., Rho, H.S., and Lee, Y.H. 2011.Differential roles of the phospholipase C genes in fungal development and pathogenicity of *Magnaporthe oryzae*. *Fungal Genet. Biol.* **48**, 445–455.

Chung, H.J., Kim, M.J., Lim, J.Y., Park, S.M., Cha, B.J., Kim, Y.H., Yang, M.S., and **Kim, D.H**. 2006. A gene encoding phosphatidyl inositol-specific phospholipase C from *Cryphonectria parasitica* modulates the *lac*1 expression. *Fungal Genet. Biol.* **43**, 326–336.

Clapham, D.E. 2007. Calcium signaling. *Cell* **131**, 1047–1058.

Cornelius, G. and Nakashima, H. 1987. Vacuoles play a decisive role in calcium homeostasis in *Neurospora crassa*. *J. Gen. Microbiol.* **133**, 2341–2347.

Davis, R.H. and De Serres, F.J. 1970. Genetic and microbial research techniques for *Neurospora crassa*. *Methods Enzymol.* **17**, 79–143.

Deka, R., Kumar, R., and Tamuli, R. 2011. *Neurospora crassa* homologue of Neuronal Calcium Sensor-1 has a role in growth, calcium stress tolerance, and ultraviolet survival. *Genetica* **139**, 885–894.

Deka. R. and Tamuli, R. 2013. *Neurospora crassa ncs-1*, *mid-1* and *nca-2* double-mutant phenotypes suggest diverse interaction among three Ca^{2+}-regulating gene products. *J. Genet.* **92**, 559–563.

Díaz-Sánchez, V., Estrada, A. F., Trautmann, D., Limón, M. C., Al-Babili, S., and Avalos, J. 2011. Analysis of *al-2* mutations in *Neurospora*. *PLoS ONE* **6**, e21948.

Estrada, A.F., Youssar, L., Scherzinger, D., Al-Babili, S., and Avalos, J. 2008. The *ylo-1* gene encodes an aldehyde dehydrogenase responsible for the last reaction in the *Neurospora* carotenoid pathway. *Mol. Microbiol.* **69**, 1207–1220.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.

Flick, J.S. and Thorner, J. 1993. Genetic and biochemical characterization of a phosphatidyl-inositol specific phospholipase C in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* **13**, 5861–5876.

Galagan, J.E., Calvo, S.E., Borkovich, K.A., Selker, E.U., Read, N.D.,

Jaffe, D., FitzHugh, W., Ma, L.J., Smirnov, S., Purcell, S., *et al*. 2003. The genome sequence of the filamentous fungus *Neurospora crassa*. *Nature* **422**, 859–868.

Gavric, O., Becker dos Santos, D., and Griffiths, A. 2007. Mutation and divergence of the phospholipase C gene in *Neurospora crassa*. *Fungal Genet. Biol.* **44**, 242–249.

Guttery, D.S., Pittman, J.K., Freñal, K., Poulin, B., McFarlane, L.R., Slavic, K., Wheatley, S.P., Soldati-Favre, D., Krishna, S., Tewari, R., *et al.* 2013. The *Plasmodium berghei* Ca^{2+}/H^{+} exchanger, PbCAX, is essential for tolerance to environmental Ca^{2+} during sexual development. *PLoS Pathog.* **9**, e1003191.

Harding, R.W. 1974. The effect of temperature on photo-induced carotenoid biosynthesis in *Neurospora crassa*. *Plant Physiol.* **54**, 142–147.

Harding, R.W., Huang, P.C., and Mitchell, H.K. 1969. Photochemical studies of the carotenoid biosynthetic pathway in *Neurospora crassa*. *Arch. Biochem. Biophys.* **129**, 696–707.

Kapoor, M., Sreenivasan, G.M., Goel, N., and Lewis, J. 1990. Development of thermotolerance in *Neurospora crassa* by heat shock and other stresses eliciting peroxidase induction. *J. Bacteriol.* **172**, 2798–2801.

Kapoor, M., Curle, C.A., and Runham, C. 1995. The *hsp70* gene family of *Neurospora crassa*: cloning, sequence analysis, expression, and genetic mapping of the major stress-inducible member. *J. Bacteriol.* **177**, 212–221.

Kazmierczak, J., Kempe, S., and Kremer, B. 2013. Calcium in the early evolution of living systems: a biohistorical approach. *Curr. Org. Chem.***17**, 1738–1750.

Köhler, G.A., Brenot, A., Haas-Stapleton, E., Agabian, N., Deva, R., and Nigam, S. 2006. Phospholipase A2 and phospholipase B activities in fungi. *Biochim. Biophys. Acta.***1761**, 1391–1399.

Kumar, R. and Tamuli, R. 2014.Calcium/calmodulin-dependent kinases are involved in growth, thermotolerance, oxidative stress survival, and fertility in *Neurospora crassa*. *Arch. Microbiol.* **196**, 295–305.

Lev, S., Desmarini, D., Li, C., Chayakulkeeree, M.,Traven, A., Sorrell, T.C., and Djordjevic, J.T. 2013. Phospholiase C of *Crytococcus neoformans* regulates homeostasis and virulence by providing inositol trisphosphate as a substrate for Arg1 Kinase. *Infect. Immun.* **81**, 1245–1255.

Lew, R.R., Abbas, Z., Anderca, M.I., and Free, S.J. 2008. Phenotype of a mechanosensitive channel mutant, *mid-1*, in a filamentous fungus, *Neurospora crassa*. *Eukaryot. Cell* **7**, 647–655.

Luque, E.M., Gutierrez, G., Navarro-Sampedro, L., Olmedo, M., Rodríguez-Romero, J., Ruger-Herreros, C., Tagua, V.G., and Corrochano, L.M. 2012. A relationship between carotenoid accumulation and the distribution of species of the fungus *Neurospora* in Spain. *PLoS ONE* **7**, e33658.

McCluskey, K., Wiest, A., and Plamann, M. 2010. The fungal genetics stock center: a repository for 50 years of fungal genetics research. *J. Biosci.* **35**, 119–126.

Murakami, M. and Kudo, I. 2002. Phospholipase A2. *J. Biochem.* **131**, 285–292.

Nakahama, T., Nakanishi, Y., Viscomi, A.R., Takaya, K., Kitamoto, K., Ottonello, S., and Arioka, M. 2010. Distinct enzymatic and cellular characteristics of two secretory phospholipases A_2 in the filamentous fungus *Aspergillus oryzae*. *Fungal Genet. Biol.* **47**, 318–331.

Nakashima, S., Wakatsuki, S., Yokoyama, T., Arioka, M., and Kitamoto, K. 2003. Identification and characterization of Scp15, a novel protein from *Streptomyces coelicolor* A3(2) with neurite-inducing activity in PC12 cells. *Biosci. Biotechnol. Biochem.* **67**, 77–82

Nelson, G., Kozlova-Zwinderman, O., Collis, A.J., Knight, M.R., Fincham, J.R., Stanger, C.P., Renwick, A., Hessing, J.G., Punt, P.J., Van den Hondel, C.A., *et al.* 2004. Calcium measurement in living filamentous fungi expressing codon-optimized aequorin. *Mol. Microbiol.* **52**, 1437–1450.

Nicholas, K.B. and Nicholas, H.B. 1997. GeneDoc: a tool for editing and annotating multiple sequence alignments. Distributed by the author. http://www.psc.edu/biomed/genedoc.

Rho, H.S., Jeon, J., and Lee, Y.H. 2009. Phospholipase C-mediated calcium signalling is required for fungal development and pathogenicity in *Magnaporthe oryzae*. *Mol. Plant Pathol.***10**, 337–346.

Rodriguez-Amaya, D.B. and Kimura, M. 2004. Harvest plus handbook for carotenoid analysis. *Harvest Plus Technical Monograph 2*. International Food Policy Research Institute (IFPRI) and International Center for Tropical Agriculture (CIAT), Washington, DC, USA.

Rzhetsky, A. and Nei, M. 1992. Statistical properties of the ordinary least-squares, generalized least-squares, and minimum-evolution methods of phylogenetic inference. *J. Mol. Evol.* **35**, 367–375.

Schumacher, J., Viaud, M., Simon, A., and Tudzynski, B. 2008. The G alpha subunit BCG1, the phospholipase C (BcPLC1) and the calcineurin phosphatase co-ordinately regulate gene expression in the grey mould fungus *Botrytis cinerea*. *Mol. Microbiol.* **67**, 1027–1050.

Soragni, E., Bolchi, A., Balestrini, R., Gambaretto, C., Percudani, R., Bonfante, P., and Ottonello, S. 2001. A nutrient-regulated, dual localization phospholipase A(2) in the symbiotic fungus *Tuber borchii*. *EMBO J.* **20**, 5079–5090.

Tamuli, R., Kumar, R., and Deka, R. 2011. Cellular roles of neuronal calcium sensor-1 and calcium/calmodulin-dependent kinases in fungi. *J. Basic Microbiol.* **51**, 120–128.

Tamuli, R., Kumar, R., Srivastava, D.A., and Deka, R. 2013. Calcium signaling, pp. 35–57. *In* Kasbekar, D.P. and McCluskey, K. (eds.), Neurospora: genomics and molecular biology. Caister Academic Press, Norfolk, UK.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28**, 2731–2739.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higgins, D.G. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25**, 4876–4882.

Tsai, H.C. and Chung, K.R. 2014. Calcineurin phosphatase and phospholipase C are required for developmental and pathological functions in the citrus fungal pathogen *Alternaria alternata*. *Microbiology* **160**, 1453–1465.

VogeL, H.J. 1964. Distribution of lysine pathways among fungi: evolutionary implications. *Am. Naturalist* **98**, 435–446.

Wakatsuki, S., Arioka, M., Dohmae, N., Takio, K., Yamasaki, M., and Kitamoto, K. 1999. Characterization of a novel fungal protein, p15, which induces neuronal differentiation of PC12 cells. *J Biochem.* **126**, 1151–1160. Erratum in: *J Biochem.* **127**, 939.

Wakatsuki, S., Yokoyama, T., Nakashima, S., Nishimura, A., Arioka, M., and Kitamoto, K. 2001. Molecular cloning, functional expression and characterization of p15, a novel fungal protein with potent neurite-inducing activity in PC12 cells. *Biochim. Biophys. Acta* **1522**, 74–81.

Yang, Q. and Borkovich, K.A. 1999. Mutational activation of a Gαi causes uncontrolled proliferation of aerial hyphae and increased sensitivity to heat and oxidative stress in *Neurospora crassa*. *Genetics* **151**, 107–117.

Zalokar, M. 1954. Studies on biosynthesis of carotenoids in *Neurospora crassa*. *Arch. Biochem. Biophys.* **50**, 71–80.

Zelter, A., Bencina, M., Bowman, B.J., Yarden, O., and Read, N.D. 2004. A comparative genomic analysis of the calcium signaling machinery in *Neurospora crassa*, *Magnaporthe grisea*, and *Saccharomyces cerevisiae*. *Fungal Genet. Biol.* **41**, 827–841.