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 Ca²⁺/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 $\Delta plc-1$, $\Delta splA2$, and $\Delta cpe-1$ 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 Ca²⁺ 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, $\Delta plc-1$, $\Delta splA2$, and $\Delta cpe-1$ 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 [Ca²⁺]_c, carotenoid accumulation, survival under stress conditions, and acquisition of thermotolerance induced by heat shock.

Keywords: calcium signaling, Ca²⁺/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 Ca²⁺ to target proteins changes their conformation, charge, and thereby, governs protein functions (Clapham, 2007). Thus, Ca²⁺ 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). Ca²⁺ signaling is typically triggered by a transient increase in cyto-

solic 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 μ M 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 Ca²⁺ in organelles (Bowman et al., 2011). In N. crassa, Ca²⁺-signaling is known to be involved in regulating a variety of processes such as Ca²⁺ 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 Ca²⁺-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 (IP₃) and diacylglycerol (DAG) (Chae et al., 2007). In animal cells, IP₃ diffuses through the cytosol and binds to the IP₃ receptors, inducing Ca²⁺ 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 IP₃ receptors has been identified in N. crassa and both of its PKC proteins lack a C2 domain with Ca²⁺ 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 stressrelated 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 Ca²⁺ 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, Ca²⁺ 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 Ca²⁺ dependent manner, thereby, liberating fatty acids and lysophospholipids (Murakami and

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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 PLA₂ 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 sPLA₂s, sPlaA and sPlaB exhibit distinct physiological properties such as pH optimum for enzyme activity, Ca² 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

Taple 1. Neurospora crassa strains used in this study								
SI. No.	FGSC no. (a/A) or strain no.	NCU no. or strain type	Type of protein	Source				
1	11248/11249	7075.2	Ca ²¹ /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 Ca²⁺-signaling proteins, Ca²⁺ exchangers that pump Ca²⁺ out of the cell and transport Ca²⁺ into the intracellular Ca²⁺ storage organelles with the simultaneous exchange of positive ions across membranes (Zelter *et al.*, 2004; Tamuli *et al.*, 2013). The *N. crassa* Ca²⁺-signaling machinery possesses six novel Ca^{2+}/H^+ exchangers and two putative Ca^{2+}/H^+ Na⁺ exchangers, and all of these exchangers contain conserved Ca²⁺ 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 Ca²⁺ in the dense vacuolar fraction, and therefore, CAX could be involved in maintaining intracellular Ca²⁺ levels in N. crassa (Bowman et al., 2011). Phylogenetic analysis has revealed that one of the N. crassa Ca²⁺/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 NCU06366encoded Ca^{2+}/H^+ exchanger has remained largely unknown. In this work, we describe the cellular roles of *plc-1*, *splA2*, 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), Ca²⁺ signaling mutants Δ NCU06245.2 a (FGSC 11411), ANCU06650.2 A (FGSC11247), ANCU-06650.2 a (FGSC11247), ΔNCU06366.2 A (FGSC 11408), Δ NCU06366.2 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 × 10⁶ 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 ($\mu g/g$) = [Total absorbance × Total carotenoid content ($\mu g/g$)] = [Total absorbance ×

Total volume of extract $(1 \text{ ml}) \times 10^4$] / [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

Functions of pla	lc-1, splA2 and c	pe-1genes in N. crassa	229
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(A)	. 200 . 200 V .	220 . 240 .	340 • 390 • 400	
		SZU 340		. 104
FERGATIO 1 PLC-1 AC STAUSONET	SAVAG	YORSON VOID LEGERCLE TOWN FERS		. 177
FORO1013 1 FLC-1 TE SLOSOCOTPTOPDCATLEASAN CNTEKDORLK	TASAMPOPEDTRAYEROUSIENEN EN YE THIS SENTYL TO OT	YORSOTT WANT LOOPPOLET FURNING VIDS	SSEPSETNEEDDHKKWHKKPRFKLOKESSGT FST SST SDP	. 202
XF 001818411.2 FLC-1 AC : SIKALPGVDFDDERFTINDGSNNGVVDPLASIAALPDV	FASS	T D DASAVE VAING BRYZIEWERCERSSES	ASADDTESSSSDSISERKIKOGSKREELESNAKRESELGSMSTK	: 197
ANZ23795.1 FLC-1 NC : ANNOTOTICA POR	SAANSTAPPRSCDISVPLSEVENSENTVLTCO	SOD STYPENE VILLEGERCLEWEWERESDERSED	CARSUTESSESS-DEAYGSGEEGIASKLERKANPELPSFLGGRK	: 213
XE 003345792.1 FLC-1 SM : ANADSVTKA RCE	SAANSTAPPEVOSB	STER AD WAVLERGROTING WOWDOWD SDEESTDONDOD	CAASVTSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSS	: 220
XF 006692835.1 PLC-1 CT : TEAGSSS-A TPECUAA /PDWKTSDSEILHELADGKPKDFER	AVSSAVATA CKC OVO PUSSYEN SENTING ON	SEDERADE YEAR ALE GERCVENEWER SOTESEDDO	CAKSTTEESESESESESESESESESESESESESESESESESE	: 213
XF 003712896.1 FLC-1 MO : SETDENTGE PEDOLAT PVV GEDGAEPOS	PESSIIGEAKDCDIST PLASTERSSENTYLTCHOL	YOLSSTERVIN UPBORRCLEVEN HORDASCARV	SSVSSDDDDPDKYAKEKEEVGOIKDKIPKSL	: 188
XF 009226183.1 FLC-1 GG : ABCHKDSOT SEECVAT //RHV-GCAESDSPLAGRKDLEVSG / AF	FASSFIGSAKDCDATE PLASYFICSSENTYLTCHOL	VENSER Y WILLEGERCIEICVWDGDESDADT	SSISSASDS-DKYAKEKEEVNEVKEKLPRTL	: 194
AAV97932.1 FLC-1 CTP : CHADT-SCA ERGCAET + RCT-AGDENGSAEGLEADLATKDELDEND + RAT	SCATSAVTPL NACTOGRASSIN SSYLHESSENTYLTONOL	SETASTONY WILLEGERCIENCYNDEDESDSEA	SGYSSSDLEDEDPKKAAAA PAKEKAKVDKAKAKIPKSV	: 225
XF 007595984.1 PLC-1 CF : VEAGCODER HEE OLAS VENUSAEETPECIVANTSEPGAAASPPEELDENG INT	SASAITREVEEE	YERESTOR YON VIVE GERCIE ICVNDGDOSCNED	GTSVSSSDEEAVAAAAAAPGCAXPKKEKRSKLANLKDXHPGSL	: 240
XF 009657148.1 PLC-1 VD : STAGADG-R OCEEIAS	BASSAITAPPKDCDIG PPESYFE SENTYLTCHOL	YSESSTORY ANYLLEGERCIEICVHDGDDSDSE	SSISSDEEGSAFAASAPKKKESRLORLKDKVFNPL	: 201
KEZ44817.1 FLC-1 SA : ARSOGTOCH TAEOLSA CTEL REGGAAIPTSLLAASEEOGOGLDFEG	SATALIAFPKEEDISTPLANYFYSSSBNTYLTONOL	SEDSEAN YANVLLEGERCIEVEVHOODDSDSE	DSSSDEEKEAAEGGELKKKKKKCSTFEMLKSKLKGET	: 202
EMT64116.1 FLC-1 FO : SIADREDKT SKERLTS FROMORNSSFAVOELLARDELDLDO	SEAAATTPPKPCDISYPLASYPISSENTYLIGNOL	SEVER THE VITE LINCERCIE ICVWDGDE DPEG	TSSPSSSDEERGVKKVVKKTKKRSTFGKLK	. : 322
KFE48906.1 FLC-1 ArC : SEAG-ADGR GNDOIKA : RDISREEISGTAAAAAATAAAASEDIVTLEELDFAR	SESAITNAAPPTDIS PRSSIFESSENTYLSG OL	SEDSWEETYONVLISGERCVEVEVWDGDESDYSS	ESSTDDEAPEK-SKKEKKPSRIQSITGKLCRSLTSRF	: 205
XF 007823279.1 FLC-1 KA : SHAD-ADGE TLGDIAR OCH KNSDKTHAAKLLOSTEIDLSA OF	SEDSNITERNNOSDISTPLSSYFIESSENTYLSGNOL	YEDSTICK YONVLINGERCVEICVHDGDESDLSA	SSTESSADERADDEAVPTKSKREGTFDRLEQRIPDSL	: 192
KDB16403.1 PLC-1 VV : SSAG-COGR SAECARR BOG BSEEGTFAAANLIGGADVELCA CT	SEDSDITLECKDADIST PLSSYYISSSENTYLSGECL	YGDS TORYANULINGERCVEICVWDGDESDLSA	SSASSSCAEEPPAEEPLT KPKBNSTFDPLPCKMPGSL	: 195
CCE31671.1 PLC-1 CVP : THAD-ODGR SAGOIRR CEW SNDCATFAAAKLYGEDDGEGRIEVND AN	SEDSAITLENKTCDIST PLPSYFISSSENTYLSGNOL	TOSTICA YENVLLIGERCVEICVNOGDESGLSS	ESSSDDEAPEASDESEKPKKPEAFSKLKEIIPGSL	: 202
XF 008595238.1 PLC-1 BB : ABAG-SDKY TASOTSO PLYKEDQIFDDEADMFSLDLAT	ASSEAAAILPRDDEDIST PUSATFEESSENTYLTENOL	TODSTTER VANULABOGREVEIEVNDOTOSCAES	SE-SDSDDEDTVKADKKDSESTLDRFAKLKSPVPESV	1 : 196
XF 003715760.1 PLC-1 NO : KYAN-ANEM SRDOLGI EHT HEDENGISSHITDKAGHSLREL DA	ASPSGNALEKAAPCDIGLEPISDYFISSSENTYLTCNOL	SEDSEVENTIL FORRCIE ICVHDGEERFLAG	FESDCAENERYFAAREAGEEETKPGPTYFVTFKDKM	1 : 197
XF 001938227.1 FLC-1 PT : DISKHYDLTTKEGOLPHLTEEG CSGKDAELIADGSFSE ANAF	SDSASVMKPPLPL	SELASVERY AND MEGGREVE IE VADGEVLSDSS	SDEEAAMGNPGADSLRKEKKKSKSGLSIRKRL	: 200
9 15	3 11 P6 Y5IssSENTYL3GngI	S S Y VL GCRC6E6DVWDG		
		**		
* 420 * 440 * 460	480 + V500 +	520 * 540 *	560 * 580 * 600	
EEE21554.1 PLC-1 PB : LDOFSKGSATKPCTASTETAAGTTATNAVSGAVCAGV	AAAI AVEPRIJAS MILES AND BUILT IN THE	DY TILT IS SAME HIMAN ASL SOLVE THE T AT AG	SEVPEDIEAKLENGEINAN S GEN NAMMANN AU CLEADCANT	: 341
EEE04719.1 FLC-1 AC : LDELSEGSTACPO		TEDBARYTHOLPITUSLEVEACLEOOKUMUDITTO ANEON	HELFADIEAKLERGEIEHA S GDA REILERVE AV ETASCA	: 329
EGE01013.1 FLC-1 TE : FDELGTENTED	WROPP WROPP WEEP WAR AV FROWOYT	TRANARYTSOLFVINSLEVEASEECOEMUDINTS WEG	WELTPENCACIANGOLSHISN GON NETHERVE VS DK	: 330
XF 001818411.2 FLC-1 AO : LGGLIGRKSSPED		IPDSA SVTSDITVIVSLEVEASLOOCANUDIND ANKON	VEVTPEKEATOP A EDA REALISVE VA TS	: 326
AA223795.1 PLC-1 NC : KSKDKKVPEPTTTTPPPPPFAAATTTEAAFVGLSAET-NPLER	KSGGSASLFOTLSRKSTREF WIEGTLET DU FRIVOZ	INDYCENWIDLPLIVSLEVECSNEOOT/MUDINROPHED!	HEELAND T ED GANVANA A NN	: 369
XF 003345792.1 FLC-1 SM : KSKDKKIRGFNAATTTTTTTTTTTTTTTTTTTTTAAEVPAASVAATFATATTNEAAAFAGSLSADTINFLEK	SKTGGSASLEPTLSRKSTREP WLEGYTET DI FREVOZA	INDYGEAT IDI PLIVSLEVECSA SOCERMULIMRO THODI	TPEPEECAKOL T EEV GAILVAVA AT ED	: 401
XF 006692835.1 FLC-1 CT : ERKEREKREKKEKKEKEKKQKKSRFASLTSRFEKTSLTDTPSSEACA	TENASTOCEAPPERKSTRREP WEGETLT AVEFRIVOEN	INDYGRVTTO FLIVSLEVECSPECCETATE MEDINKOR	VND	: 375
XF 003712896.1 FLC-1 NO : TSRFRESSLAKRLGQFVDSKTEPKQPPISTSNSQGSGGPSPSALTRGADED	STNDCNLSALCKTOTEEAFVISET WHEG TLT EVERSIVOYA	IRDDAFTASDIFLIVSLEVECGPLOOLONUDINE ABOGY	VFKFAECAAVE S EAS NEEDENVE A / VD	: 355
XF 009226183.1 PLC-1 GG : TTRFKDTSLGRRLGEFVESKTEPKOOFSSSLSFASTESNSASGGGGTEA	CEPKAVLCKSPTFVG-ANEF WYHGYTLINDVEREVOYA	VEENAFTASDEPLIVSLEVHCREGOCCOMUDINCOASKGY	POPEECATAR ALEAS NELLENVE A GL	: 356
AAV97932.1 PLC-1 CrP : LOKLEOTSLGKKLEKYVEKKTEPKAFASPSTSSAAAAAAAFAFAFAAAKDDSKVKVASG	SSSFAPPIVERLEALAAAVISE WEGTLITEVSFELVOLA	INEYABAWIDI PLIVSLEVBAGP COEDMUNIAN THAGE	DDP	: 399
XF 007595984.1 FLC-1 CF : ASBLEKTSLGKENDERIEKSTSPPTETATTREAVIAAAPTFAIATTIASVAESTIATTIPKET	APRTEOPPLOKTPSPFMFPIEROVFHOVTLT EVERENOET	IF DYGERSAU PLIVSLEVECSA LOOPIN, NIMEDINAGI	APT PCVCAPEL R ADEMONITYAWA A PG	: 418
XF 009657148.1 FLC-1 VD : ARKLKELSLTGSEAAAKPTSSETKAFAASS	EPSALKKTPSPOLAIVEPFWLBGTTLTF EVERINGVT	IRDYARKTEDTPL DSLEVECSAROCSEMUNINK VHARE	SPI PDTEPTON O GON NEISVEWS AP GS	: 342
KEZ44817.1 PLC-1 SA : QKADEKITQPREFVAATQEGSKKVKND	GIFALKPCAVEF WIRGYTLT, DITFRIVEVA	IPDSA BRUSDIEVIUSLEVECTP CODENCEINERABRON	VPIPEGEPTEL A COM NELEVEVE APP PG	: 334
ENT64116.1 PLC-1 FO :EKLAKTSLRDKDESSDFASGQQFAPCAD	PESTEPEAAIVEP WISGTLIT EISTEAVOEA	IFZ9 HVVSDEFVIVSLEVHCKQDQQLANS FINK VHGDE	APE	: 456
KFE48906.1_FLC-1_ATC : ERTSRGKELDETAAKAGDPTSTAGDDGKQEDAEH		TAKSA HAVID PLIVSLEVHCSFAQQATMUNIMETHADY	PEPOREPRAN S ARM NEMANENTS AT OS	: 345
XF_007823279.1_FLC-1_KA : TSKLEKTSLAKTRELKETGKENTDTANGALPDGD	SKIVPEVALVEPEWEBGTTLTKEVSFREVON	IFDSAFTVSDIFLISLEVECGAEQQVMANINXIVESGM	WTEDEIKPOLE S EDA RELLINVE AT DT	: 331
KDB16403.1 FLC-1 VV : SABLQBASLGTSPEGKETGSHDFERCAGAVGDDDHDDH	DDHDDHDNEVFAVAVVEFEVFBGTLTREVEFEVONA	IFES AVA TO PLIVSLEVECTERONMY TIMO PAKON	TE CANTEGAL S ADA PETAHAVA A ADA	: 344
CCE31671.1 FLC-1 CVP : SARLEPTSLGKERKPRSTSKGELEAEFVASLEDG	AFNIVSEVAAFEFFWLEGTTLTREVSFFEVORA	TEDS HIVSDIPLIISLEVECGADOOINMININXIAHKDA	RE	: 342
XF_008595238.1_FLC-1_BB : VSRLSRTGALRRKLERTTSERAADTCNESPTRLE	SSSQSSLANEF WIEGITLTREVEFRIVOAT	IF KEAFEASDIPLIESLEVECNE COAVANDIME ANGOV	AVVAFFEDCVFAILIS CAL GRELVEVEYAFEDK	: 336
XF 003715760.1 PLC-1 MO : IIKAABKANNKFDFVDPEGRTIDDR		VEYABAVSDIPLIVSLEVECSPICONACCIME AWEEP	PPTPEEDPTSI S ADI PKILIFVE VAENK	: 324
XE 001938227.1 PLC-1 PT : AAKFSKKEEPEEKSEEASPPPQDGMQKIEP	WESASFTOPALER VERGETLET DITERAVOAT	IBDYAFTVSNIPLIVSLEVHTCPEQCETMUEINNEYHKGM	PEEPLEPAQDLDSVHATIKDPERKIIVAVARGETTEVAPTSQP	: 347
	EP V BG TLT FR VC	6 AF 11P6I SLEVE QQ MV I6 5	6 Ip p 6 4666KVK pp	

(B)																	
			20	•	40		60		80		100		120		140		
XF_960883.1_SPIA2_NC	:							MEFFSALALS	SL PIANW	WTGSESDSTG-				ADSLFRBAET	IQQ	:	42
CCC12480.1_SPLA2_SM	:							MEFFSTLALS	SLISIAN	WAGSESDKTG-				AGSLFRRAET	100	:	42
XF_006968333.1_SPLA2_TR	:							MEFTAL	FATAFAVL	LSS				PLANBOGS	17A	:	31
XF 003006973.1 SPIA2 VA	:							METNAL	LA VFAAL	L TTDEAOT				-PKIAARQ-S	ITA	:	36
XF 001911620.1 SPLA2 FA	:							APAFT	LA AGSAL	LASTG				VLEORQ-S.	AVQ	:	31
XF 003042542.1 SPLA2 NH	:							METSLO	VSILEVAR	LAGTEDE					ANT	:	34
XF 006691563.1 SPLA2 CT	: MPESSTD	WRKFDNDDD	LEDARAYIA	EVIPEVLDCI	LSGSVSLLTG	SOEPLAGOSI	VFDLIALIN	STS-LIAN	LAVVEANE	DATLETA				-AATLKRL-S	LTE	:	102
XF 003666499.1 SPIA2 MT								APEL-STA	LOASSVI	LISTGRAD				-AATVPRO-S	ATO		35
XE 007820830 1 SPT 12 MA								MENNAT	TAUATTAL	A ASTUTT				PKRO-D	INT		33
YE 006670930 1 -PT 12 CH								WT	A-TEAUT	OT TO				DEPT N	00		26
VE CODECOGES 1 OPTAS PP									COLT DT AT	AL & CUILD				DEPEN	*¥		20
AP UU0399052.1 SPLAZ DB									SSTLELIN	A.MavvD				PROPA			23
ADBIS048.1 SPLAZ VV								ASP-NA	IRAAVAAV	STLAALQ				PETIA	LEA		32
CCX11717.1_SPLA2_PO	:							METT-TILV	S-TTIA9	L'VADIAIP	RGNVPDIVE	LGVDEFNN	WGIDH	RGKYPDVINL	ATDIVPESAVA	KDNII :	74
AAF80454.1_SPLA2_TB	:						N	VAIA-AIIL	MGULANAA	D:VSEFAALNK	RGNAEVIAE	TGDVPDFNT	QITEPTGEGD	RGDVADETNL	STDIVPETEAA:	SFA :	83
EAB70714.1_SPLA2_BN1	:							MASE-TEVVI	A-ULPESS	15			FGLFE	RGGIASPATI	EET	:	37
XF_001818634.2 SPLA2 AO	:							MENI-FVAT	GLFAAVS	SALPYT			TEVN	ENPISALÇAR.	ATTC	:	40
EAS33618.2 SPIA2 CI	:						MR	INTR-FVIL	GLAPLAPT	NEVEFR			ADAN	AGAETSLTSR	ADPC	:	44
EGC46915.1 SPLA2 AC	:							MASA-LILS	cs.c	-ALTLS			APTA	AGDLSTLTIS	AET	:	35
								6k		P							

160 * 180 * 200 * 220 * 240 * 260 * 280	* 300
XF 960883.1 \$PLA2 NC :	: 155
CCC12480.1 SPLA2 SM :TOPRIMEST UPGEBVERN SEADADSSDGGYSED BUGEED BUGE	: 155
XF 006968333.1 SPLA2 TB :TELOL/BDTSLPDETTERN KNESN-LWSSDGCDOSED ELGHEVE ACVERDESCON KTONEE-ESGUNEN FF7DD/VTC-OSSSVOSVCDA ADVYNA WYA (EGGDASPCKT	EES : 152
XF 003006973.1 SPLAZ VA :	DE- : 156
XF 001911620.1 SPLAZ FA :	ET- : 151
XE 003042542.1 SPLA2 NE :	S- : 153
XF 006691563.1 SPLAZ CT :	RED : 221
XF 003666499.1 SPLA2 MT :	RDD : 144
YE 007820830 1 SP12 VA	SD- : 153
YE ODESTORED 1 SPEAD CH - TODAY SPEAD DESTORED AND	ADAASAU-POPD : 155
	AUUAGATOPEPS - 150
	PDCK : 140
	FEGA . 149
	. 202
AREVOSS. 1 SELAZ TE : ASSYSTALSEVSU	: 211
EAB/U/14.1 SPLAZ BNI :	: 156
XF 001818634.2 SPLA2 AO :SAKAFINIDASKAKAGKAKAKAKSKAKSGINGSKOLORADOWND SCOREDEGGALTOKONED KARECHIDISKOSKIKGVEGBR ADVYHDIV: B GKDDZALZTD	PEVEFEKRDEVA : 178
EAS33618.2 SPIA2 CI :	: 161
zgc46915.1_spla2_ac :	: 152
TD 5 6 F 4 P W sDgC3 spl P g5 F p C REDFGY N Q R5t 4 6D nF D c C A YY aV fG	



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 Ca²⁺ 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, *Ophiotar picceae*; PA, *Podospora anserine*, PB, *Paracoccidioides brasiliensis*; PO, *Pyronema omphalodes*; PT, *Pyrenophoratritici-repentis*; SA, *Scedosporium apicceae*; PA, *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 alignment

~1×10⁶ 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 H₂O₂ (test) by the number of colonies from plates not exposed to H₂O₂ (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

N. crassa NCU06245, NCU06650, and NCU06366 encode three distinct types of Ca²⁺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 Ca²⁺ 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, $\Delta splA2$ and Δcpe -1 mutants show growth defects in response to increased intracellular free Ca²⁺

We found that growth rates of the $\Delta plc-1$, $\Delta splA2$, and $\Delta cpe-1$ mutants on VGM were similar to the wild-type (Supplementary data Table S1). However, the $\Delta plc-1$, $\Delta splA2$, and $\Delta cpe-1$ 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 $\Delta splA2$ mutant. In case of the $\Delta cpe-1$ 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 Ca²⁺ 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, $\Delta splA2$, 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, $\Delta splA2$, and Δcpe -1mutants



Fig. 2. Calcium ionophore assay. *N. crassa* wild type and the three mutant strains were grown in petri dishes containing the ion-ophore 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.

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

			1							
	Carotenoids (µg/g dry wt.)ª									
Strains	8°C	2	22°C		30°	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				
$\Delta plc-1$	253 ± 3 (***)	34 ± 6.1 (**)	$126 \pm 11(***)$	55 ± 3.1 (**)	144.2 ± 20.2 (**)	49.4 ± 8 (**)				
$\Delta splA2$	255 ± 12.2 (***)	46 ± 6 (*)	169 ± 3.1 (**)	41 ± 5 (*)	177.4 ± 14 (***)	38.1 ± 9 (*)				
∆cpe-1	235 ± 6.1 (**)	50 ± 11.2 (*)	175 ± 17.2 (***)	48 ± 4 (*)	160.1 ± 10 (***)	47 ± 2 (***)				
A										

^a Results are shown as mean \pm 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.

Assessment of conidiation, carotenoid accumulation, and survival after exposure to UV light in Δplc -1, $\Delta splA2$, and Δcpe -1

Conidia amounts in the $\Delta plc-1$, $\Delta splA2$, and $\Delta cpe-1$ 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 $\Delta plc-1$, $\Delta splA2$, and $\Delta cpe-1$ 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). Extrapola-

ting 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 $\Delta plc-1$, $\Delta splA2$, and $\Delta cpe-1$ 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*, *splA2*, and *cpe-1* is contributing to their increased UV survival.

Effects of the $\Delta plc-1$, $\Delta splA2$, and $\Delta cpe-1$ mutations on survival under oxidative stress and acquisition of thermotolerance

We tested the viability of the $\Delta plc-1$, $\Delta splA2$, and $\Delta cpe-1$ 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> $\Delta cpe-1>\Delta splA2>\Delta plc-1$. These results are supported by results in *A. oryzae* for splA2 homologs demonstrating that $\Delta splaB$ displays increased sensitivity to peroxide and *splaA* is strongly upregulated by oxidative stress (Nakahama *et al.*, 2010).

We also investigated roles for *plc-1*, *splA2* 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 H₂O₂ 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.

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ture. The $\Delta plc-1$, $\Delta splA2$, and $\Delta cpe-1$ mutants had decreased survival relative to wild type under induced thermotolerance. In contrast survival was slightly greater than wild type for the $\Delta splA2$ and $\Delta cpe-1$ 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 $\Delta plc-1$ mutant under uninduced thermotolerance conditions. These results suggested that *plc-1*, *splA2*, 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*, *splA2*, and *cpe-1* displayed growth defects in response to increases in $[Ca^{2+}]_c$ induced by the Ca²⁺ionophore A23187. Moreover, the carotenoid profile in the *plc-1*, *splA2*, and *cpe-1* mutants was altered and the increased carotenoid amount was linked to UV-survival of the strains. Furthermore, *plc-1*, *splA2*, 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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