Calcineurin phosphatase and phospholipase C are required for developmental and pathological functions in the citrus fungal pathogen *Alternaria alternata*

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INTRODUCTION

The fungal pathogen *Alternaria alternata* causes brown spot disease in citrus. This pathogen produces a host-selective toxin that kills host cells before invasion, and acquires nutrients primarily from dead tissues (Akimitsu et al., 2003). *Alt. alternata* colonization in leaves of the citrus causes lipid peroxidation, increased accumulation of hydrogen peroxide ($H_2O_2$) and cell death (Lin et al., 2011). Although toxin is absolutely required for fungal pathogenicity, *Alt. alternata* colonization in leaves revealed that application of CaCl$_2$ or neomycin 24 h prior to inoculation provided protection against *Alt. alternata*. These data indicate that a dynamic equilibrium of cellular Ca$^{2+}$ is critical for developmental and pathological processes of *Alt. alternata*.

Excessive Ca$^{2+}$ or compounds interfering with phosphoinositide cycling have been found to inhibit the growth of the tangerine pathotype of *Alternaria alternata*, suggesting a crucial role of Ca$^{2+}$ homeostasis in this pathotype. The roles of *PLC1*, a phospholipase C-coding gene and *CAL1*, a calcineurin phosphatase-coding gene were investigated. Targeted gene disruption showed that both *PLC1* and *CAL1* were required for vegetative growth, conidial formation and pathogenesis in citrus. Fungal strains lacking *PLC1* or *CAL1* exhibited extremely slow growth and induced small lesions on calamondin leaves. *Δplc1* mutants produced fewer conidia, which germinated at slower rates than wild-type. *Δcal1* mutants produced abnormal hyphae and failed to produce any mature conidia, but instead produced highly melanized bulbous hyphae with distinct septae. Fluorescence microscopy using Fluo-3 dye as a Ca$^{2+}$ indicator revealed that the *Δplc1* mutant hyphae emitted stronger cytosolic fluorescence, and the *Δcal1* mutant hyphae emitted less cytosolic fluorescence, than those of wild-type. Infection assessed on detached calamondin leaves revealed that application of CaCl$_2$ or neomycin 24 h prior to inoculation provided protection against *Alt. alternata*. These data indicate that a dynamic equilibrium of cellular Ca$^{2+}$ is critical for developmental and pathological processes of *Alt. alternata*.

Abbreviations: CaM, calmodulin; G-protein, GTP-binding protein; IP$_3$, inositol 1,4,5-triphosphate; MAPK, mitogen-activated protein kinase; PIP$_2$, phosphatidylinositol 4,5-bisphosphate; PKA, cAMP-dependent protein kinase A.

The GenBank/EMBL/DDBJ accession numbers for the sequence data reported in this article are KF306218 (*CAL1*) and KF306219 (*PLC1*).

One supplementary table and three supplementary figures are available with the online version of this paper.

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Cytosolic free Ca\textsuperscript{2+} plays an important role in cell signalling and controls a wide array of physiological functions and cell development in all organisms. Ca\textsuperscript{2+}/calmodulin (CaM)-mediated signalling is mediated via inositol 1,4,5-triphosphate (IP\textsubscript{3}) metabolism and has been well documented to be involved in various cellular responses to external stimuli in animal cells (Berridge, 1993; Berridge et al., 2000). Studies in fungi suggest that IP\textsubscript{3}-activated Ca\textsuperscript{2+} release may occur in fungi (Ohsumi & Anraku, 1983; Miller et al., 1990; Jackson & Heath, 1993). Ca\textsuperscript{2+} could be cytotoxic and thus, the level of cytoplasmic Ca\textsuperscript{2+} must be tightly regulated, often by the intricate interplay of multiple processes. The cytosolic Ca\textsuperscript{2+} equilibrium can be achieved by controlling Ca\textsuperscript{2+} influx channels in the cell membrane and/or the release of Ca\textsuperscript{2+} from intracellular stores (Berridge et al., 2000). Disturbing Ca\textsuperscript{2+} homeostasis often leads to reduced growth in fungi (Chung et al., 2006; da Silva Ferreira et al., 2007; Rho et al., 2009). IP\textsubscript{3}-mediated Ca\textsuperscript{2+} signalling is closely associated with G-proteins (Wendland, 2001). Upon perceiving environmental cues, a membrane-bound receptor triggers formation of GTP from GDP in the G\textsubscript{sub} subunit of a G-protein, leading to dissociation of G\textsubscript{sub}/G\textsubscript{alpha} subunits (Li et al., 2007). The released G\textsubscript{sub} subunit activates phospholipase C, which hydrolyses inositol-1,4-bisphosphate (IP\textsubscript{2}) to generate two secondary messengers, diacylglycerol (DAG) and IP\textsubscript{3} (Berridge, 1993). The role of IP\textsubscript{3} is to maintain a dynamic equilibrium of cellular Ca\textsuperscript{2+} by stimulating its release from intracellular stores in vacuoles or other organelles. Inhibition of phospholipase C activity interferes with IP\textsubscript{3} metabolism and cytosolic Ca\textsuperscript{2+} distribution. Both neomycin and lithium have multiple inhibitory effects on phosphoinositide cycling and IP\textsubscript{3}-mediated Ca\textsuperscript{2+} release in animal cells. Neomycin has been known to inhibit phospholipase C activity and phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2}) synthesis (Schacht, 1976; Gabev et al., 1989). Lithium impacts IP\textsubscript{3} dephosphorylation by inhibiting inositol-1-phosphatase activity (Inhorn & Majerus, 1988). Ca\textsuperscript{2+} signalling has been demonstrated to be involved in hyphal extension and branching, mycelial dimorphism, conidium and appressorium formation, photomorphogenesis and fungal pathogenicity in various fungi (Muthukumar & Nickerson, 1984; Robson et al., 1991; Hyde & Heath, 1997; Shaw & Hoch, 2000). The Ca\textsuperscript{2+}/CaM signalling system also mediates the biosynthesis of secondary metabolites in fungi (Kim et al., 1998; Rao & Subramanyam, 1999; Chung 2003)

Calcineurin is a serine/threonine protein phosphatase highly conserved in all eukaryotes and plays a key role in Ca\textsuperscript{2+} signalling (Rusnak & Mertz, 2000; Fox & Heitman, 2002; Stie & Fox, 2008). Calcineurin is a heterodimer composed of a catalytic subunit and a regulatory subunit. The activity of calcineurin is dependent upon Ca\textsuperscript{2+}/CaM. The catalytic subunit contains a regulatory subunit-binding domain, a CaM-binding domain and an autoinhibitory domain in the C terminus. The binding of Ca\textsuperscript{2+}/CaM in the C-terminal region of the catalytic subunit leads to a conformational change, which in turn nullifies the inhibitory domain and thus activates calcineurin. Upon binding to Ca\textsuperscript{2+}/CaM, calcineurin activates the Zn-finger transcription factor Crz1p/Tcn1p that subsequently regulates the genes encoding a P-type ATPase involved in cation transport (Stathopoulos & Cyert, 1997). In the budding yeast Saccharomyces cerevisiae, calcineurin and CAM-dependent PKA antagonistically regulate Crz1p/Tcn1p, a process which is involved in the regulation of cell wall biosynthesis and ion homeostasis (Hirata et al., 1995; Mathews et al., 1997; Stathopoulos & Cyert, 1997).

We report the cloning and functional characterization of a phospholipase C-coding gene and a calcineurin-coding gene in Alt. alternata to explore their roles in the maintenance of cytosolic Ca\textsuperscript{2+} and fungal development. We demonstrate the significance of Ca\textsuperscript{2+}-mediated regulation in the context of vegetative growth, asexual development and pathogenesis in the tangerine pathotype of Alt. alternata.

**METHODS**

**Fungal strains and tests for chemical sensitivity.** The wild-type EV-MIL31 strain of Alt. alternata (Fr.) Keissler, used as both a recipient host for transformation and in the mutagenesis experiments, was single-spore isolated from diseased leaves of Minneola tangelo in Florida. Fungi were cultured on potato dextrose agar (PDA; Difco) at 28 °C. For DNA or RNA isolation, fungal strains were grown on sterile cellophane overlaid on PDA. Chemical sensitivity was assessed by transferring hyphae/conidia as a toothpick point inoculation onto a medium containing the test compound. The diameter of colonies was measured at 4–7 days. Cultured mycelia were suspended in sterile water using a disposable pestle (Fisher Scientific) and evenly spread onto agar medium. Conidia were collected by flooding with sterile water and centrifugation (5000 g) from fungal cultures grown on PDA in light for 3–4 days. Conidia were examined with a Leitz Laborlux phase-contrast microscope (Leitz Wetzlar). Conidia were germinated on glass slides incubated in a moist chamber.

**Assays for intracellular Ca\textsuperscript{2+}.** Fluo-3/AM (Biotium), a Ca\textsuperscript{2+}-binding probe, was used to measure cytosolic Ca\textsuperscript{2+}. Fungi were cultured in liquid PDB (pH 4.2) containing 150 µM Fluo-3/AM dissolved in DMSO and 4 % N,N,N-trimethylbenzylamine. The level of fluorescence was determined using the ImageJ program (http://rsb.info.nih.gov/ij/). The corrected total cell fluorescence was calculated by subtracting mean fluorescence of background readings from integrated density as described by Burgess et al. (2010).

**Cloning and sequencing.** All oligonucleotide primers used in this study are listed in Table S1 (available in the online Supplementary Material). The Alt. alternata phospholipase C gene (PLC1) fragment was amplified by PCR with two degenerate primers PLC-FA and PLC-R that are complementary to PLC gene homologues of fungi, from genomic DNA of the tangerine pathotype of Alt. alternata. Similarly, a calcineurin phosphatase gene 1 (CAL1) fragment was amplified with the primers Calcin-F and Calcin-R (Table S1). Fungal DNA was isolated using a DNeasy Plant kit (Qiagen). The 5' and 3' untranslated regions of the gene were amplified by PCR from a chromosome library of Alt. alternata that was constructed from genomic DNA cleaved with four different endonucleases (DraI, EcoRI, PvuI and Stul), using a Universal GenomeWalker kit (BD Biosciences). PCR fragments were purified using a DNA cleanup kit (GenScript) and sequenced at Eton.
BioScience (Research Triangle, NC, USA). ORF and exon/intron positions were predicted using Softberry gene-finding software (http://www.softberry.com). Fungal RNA was purified with Trizol reagent (Molecular Research Center), denatured, blotted onto a nylon membrane and hybridized to a digoxigenin (DIG)-11-dUTP (Roche Applied Science)-labelled DNA probe. The probe was labelled by PCR with gene-specific primers (Figs S1 and S2). The probe was detected by immunological assay using CSPD (disodium 3-[4-methoxyxpirol]-1,2-dioxetane-3,2-(5’-chloro)tricyclo (3.3.1.1)decan-4-yl)phenyl phospho) as a chemiluminescent substrate (Roche Applied Science) for alkaline phosphatase.

Genetic modification of fungi. The *Alt. alternata* PLC1 gene was inactivated by inserting a bacterial phosphotransferase B gene (HYG) cassette under control of the *Aspergillus nidulans* trpC gene promoter and terminator conferring resistance to hygromycin. As illustrated in Fig. S1, a 5’PLC::HYG (1.1–1.2 kb) fragment was amplified by two-round PCR with the primers PLC-pro2F, PLC::M13R, M13R and hyg3 as described previously (Catlett et al., 2003; Yang et al., 2009; You et al., 2009). A 9CAL::3’PLC (1.8 + 1.0 kb) fragment was amplified with the primers: hyg4, M13F, M13F::PLC and PLC-TAG.

Similar approaches were carried out to produce 5’CAL::HYG (0.8 + 1.2 kb) and hYG::3’CAL (1.8 + 0.9 kb) fragments for disruption of the *Alt. alternata* CAL1 gene with the primers: Calcin-pro2F, Calc::M13R, M13R, hyg3, hyg4, M13F, M13F::Calc and Calc-TAG (Fig. S2). PCR fragments (10 μl each) were mixed and transformed into protoplasts prepared from the EV-MIL31 strain, using CaCl2 and polyethylene glycol (Chung et al., 2002). Fungal transformants were recovered from medium containing 250 μg hygromycin ml−1 (Calbiochem). Putative mutants specifically disrupted at the PLC1 locus were examined by PCR with the primer PLC-pro1F, whose sequence is not present in the split marker fragments, paired with the primer 1800R or the primer hyg3. Similarly, putative mutants specifically disrupted at the CAL1 locus were examined by PCR with the primers Calcin-ATG and Calcin-TAG, and verified further with the primers hyg3 and Calcin-pro1F, whose sequence is not present within the split marker fragments.

Phenotypes observed for PLC1 or CAL1 mutation were complemented by co-transforming a functional PLC1 or CAL1 gene under control of its own promoter with the pCB1532 plasmid carrying a dominant Sur gene cassette conferring resistance to sulfonylurea as described previously (Lin et al., 2009). Transformants were recovered from medium amended with 5 μg sulfonylurea ml−1 (Chem Service).

**Virulence tests.** Fungal virulence was assayed on detached calamon leaves by inoculating with a conidial suspension (104 conidia ml−1) onto detached leaves. Fungal mycelial mass (3.2 mm) was incubated in a moist chamber for 3–5 days for lesion development. *CaCl2* (0.6 M) or neomycin (1 mM) was sprayed onto calamon leaves using a mini-sprayer 24 h prior to the inoculation of the wild-type strain of *Alt. alternata*. The size or area of necrotic lesions was determined using the ImageJ program. The significance of treatments was determined by analysis of variance and treatment means separated by Student’s t-test (P<0.05).

**RESULTS**

**Imbalance of Ca2+ suppresses growth of *Alt. alternata***

Ca2+ or compounds interfering with Ca2+ balance were added to medium to determine the effect of external Ca2+ on fungal growth. On PDA, the addition of CaCl2 or calcium nitrate at concentrations lower than 100 mM promoted fungal growth slightly (Fig. 1a, b), suggesting that *Alt. alternata* is capable of regulating Ca2+ uptake. When applied at high concentrations (>150 mM), two salts of Ca2+ inhibited fungal growth. Both lithium chloride and neomycin have multiple inhibitory activities on the phosphoinositide signalling system. The addition of either compound greatly reduced fungal growth (Fig. 1c, d).

**Targeted disruption of the calcineurin- and phospholipase C-coding genes in *Alt. alternata***

Growth reduction of *Alt. alternata* on medium containing high concentrations of Ca2+ could also be due to osmotic stress. Apart from the impacts on phosphoinositide cycling, neomycin and lithium chloride could affect other biochemical and physiological processes in cells. To determine the roles of Ca2+, the gene sequences encoding a calcineurin (CAL1) and a phospholipase C (PLC1) gene were independently obtained by PCR amplification from genomic DNA of *Alt. alternata*. Conceptual translation revealed that the *Alt. alternata* PLC1 gene has a 3315 bp intronless ORF and encodes a 1104-amino acid polypeptide that shows a strong structural similarity to fungal 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterases. PLC1 contains a pleckstrin homology (PH) domain, an EF-hand Ca2+-binding domain, a catalytic X domain, a catalytic Y domain and a C2 domain. The PLC1 gene was functionally disrupted by transforming two DNA fragments (5’PLC::HYG and hYG::3’PLC), overlapping within the HYG cassette, into protoplasts of the EV-MIL31 strain of *Alt. alternata* (Fig. S1). Because the HYG gene was split into separate fragments, the gene was not functional until homologous recombination had occurred between 5’PLC::HYG and hYG::3’PLC.

Of 36 transformants recovered, 18 showed severe growth reduction. Transformants were screened by PCR with the primers PLC-2F and PLC-TAG, revealing that a 3.9 kb fragment was amplified from genomic DNA of *Alt. alternata* but no product was obtained from wild-type. Northern blot hybridization of wild-type RNA to a PLC1-specific probe identified a 3.5 kb transcript that was absent in both D25
and D35 transformants (Fig. S1), indicating successful disruption of *PLC1* in the genome of *Alt. alternata*.

Conceptual translation revealed that the *Alt. alternata* *CAL1* gene has a 1899 bp ORF interrupted with four introns (101, 47, 4 and 49 bp) and encodes a 551 aa polypeptide that shows a strong similarity to the serine/threonine-protein phosphatase 2B catalytic subunit A of fungi. The amino terminal domain of *CAL1* has a phosphatase active site. The carboxyl terminus encompasses a regulatory subunit-binding domain, a calmodulin-binding domain and an autoinhibitory region. The *CAL1* gene was functionally disrupted by transforming two DNA fragments (5′*CAL*::HYg and hYG::3′*CAL*) into protoplasts of the wild-type strain (EV-MIL31) of *Alt. alternata* (Fig. S2). Of over 20 transformants recovered, 12 displayed a marked growth reduction on PDA. Transformants were first screened by PCR with the primers calcin-ATG and hyg3, revealing that a 2 kb fragment was amplified from genomic DNA of all slow-growing strains, but not from that of wild-type and fast-growing strains (data not shown). PCR examination of two randomly selected transformants (*D*cal*D62* and *D*cal*D69*) using two different primer sets confirmed that the integration of the selective HYG marker gene cassette occurred within *CAL1*.

The primers calcin-ATG and calcin-TAG amplified a 2 kb fragment from the genomic DNA of the wild-type and a 4 kb fragment from that of D62 and D69. The primer calcin-1F, which is not present in the split marker fragments, was paired with the primer hyg3 in PCR to validate *CAL1*-specific integration. The two primers amplified a 2.7 kb fragment from genomic DNA of D62 and D69, but failed to amplify any products from wild-type (Fig. S2).

**Both calcineurin and phospholipase C are required for normal morphogenesis, vegetative growth and conidial formation**

On PDA, growth of the *Δplc1* mutants was reduced by an average of 65% compared with wild-type at day 7 (Fig. 2a). Reintroduction of a *PLC1* fragment with its own promoter into protoplasts prepared from the D25 strain resulted in a strain that exhibited radial growth comparable to that of wild-type, confirming the role of *PLC1* in fungal growth. Microscopic examination revealed that the *Δplc1* mutant produced hyphae resembling those produced by wild-type on PDA (Fig. 2b). However, when cultured in liquid medium, the hyphae of the *Δplc1* mutant, but not of the wild-type, became swollen, showing distinct bulbous

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**Fig. 1.** Maintaining Ca$^{2+}$ homeostasis is required for vegetative growth. The wild-type EV-MIL31 strain of *Alt. alternata* was grown on PDA supplemented with (a) CaCl$_2$, (b) calcium nitrate, (c) lithium chloride or (d) neomycin at various concentrations. The radius of colonies was measured at 3 days. Each point represents the mean ± SD of the colony radius from two independent experiments, with at least three replicates.

![Fig. 1. Maintaining Ca$^{2+}$ homeostasis is required for vegetative growth.](image-url)
segments (data not shown). To evaluate conidial formation, cultured mycelia were ground in sterile water, spread on PDA and incubated in light for 5–7 days. Δplc1 mutants reduced conidial formation by ∼77% of that of wild-type (Fig. 2c). Exogenous application of Ca2+ failed to restore the wild-type levels of both radial growth and conidial

**Fig. 2.** Phospholipase C plays an important role in vegetative growth and formation and germination of conidia in *Alt. alternata*. (a) Time-course changes in radius increase of wild-type (WT), two independent Δplc1 mutants (D25 and D35) lacking phospholipase C and the genetically complemented strain (Δplc/PLC) grown on PDA. An example of images showing fungal growth on the test medium for 5 days is also shown. (b) Hyphae of the wild-type and the Δplc1 mutant. (c) Quantitative determination of conidial formation. Conidiation was evaluated by growing fungal strains on PDA in light for 3 days. (d) Germination of conidia producing single or multiple germ tubes on glass slides. The percentage of germination is indicated above the columns. Each point or column represents the mean ± SD of the colony radius from two independent experiments, with at least three replicates.
formation in Δplc1 mutants (data not shown). The strain re-expressing a functional PLC1 produced conidia at quantities comparable to those of wild-type. Assays on a glass slide indicated that conidia collected from both D25 and D35 germinated at rates slower than wild-type and the complementation strain (Fig. 2d). Conidia collected from the wild-type strains often produced multiple germ tubes. Less than 30% of conidia examined produced a single germ tube. Mutational inactivation of PLC1 had impacts primarily on the production of multiple germ tubes.

On PDA or minimum medium (Chung, 2003), Δcall mutants exhibited severe growth reduction by over 95% of that of wild-type (Fig. 3a). Δcall mutants produced no appreciable aerial hyphae and formed compact colonies with a dark, rugged appearance and depressed margins, and were discernible from wild-type (Fig. S3). A dense, cone-shaped, mycelial mass appeared in the centre of the colony after prolonged incubation (~20 days). Growth and colony morphology were fully restored to wild-type levels by transforming the D62 protoplasts with a functional CAL1. Unlike wild-type, Δcall mutants produced short-branched, dark-pigmented hyphae (Figs 3b and S3). Disruption of the CAL1 gene not only resulted in severe growth retardation, but also changed hyphal morphology and completely abolished conidial formation. Δcall mutants produced aberrant, enlarged hyphae that formed distinct cell clusters but never developed into mature conidia (Fig. 3c). The genetically complemented strains produced ovoid, multiple-cell conidia that were morphologically similar to those produced by wild-type (Fig. 3c), confirming the essential role of calcineurin phosphatase for conidial formation.

**Cytosolic Ca²⁺ is affected by calcineurin and phospholipase C**

The level of intracellular Ca²⁺ was assessed using Fluo-3 fluorescent dye. After Ca²⁺ binding, Fluo-3 emitted green fluorescence. Microscopic examination revealed that the wild-type hyphae emitted weak fluorescence (Fig. 4). Bright green fluorescence was observed in the hyphal cytoplasm of D25 and D35 lacking PLC1. When hyphae were immersed in the Fluo-3 solution, the hyphae of the Δplc1 mutant, but not the wild-type, became swollen, showing distinct bulbous segments and bright green fluorescence. The fluorescence coalesced as distinct patches distributed along the fungal hyphae, suggesting that there was accumulation of Ca²⁺ within cytoplasmic compartments. The Cpl strain, regaining a functional copy of PLC1, displayed very weak emission signals similar to those seen in wild-type.

In contrast, hyphae of the Δcal-D62 and Δcal-D69 strains lacking calcineurin emitted barely detectable green fluorescence, showing intensities apparently weaker than those seen in the hyphae of wild-type (Fig. 5). The complementation strain, re-expressing a wild-type copy of CAL, emitted Fluo-3/Ca²⁺ green fluorescence at intensities similar to those of wild-type. Quantitative analysis of the level of fluorescence also confirmed that the Δplc1 mutant hyphae emitted stronger cytosolic fluorescence and the Δcall mutant hyphae emitted less cytosolic fluorescence than those of wild-type (Fig. 6).

**Phospholipase C and calcineurin are required for fungal virulence**

Because both Δplc1 and Δcall mutants produced very few or no conidia, fungal pathogenicity was evaluated by placing fungal mycelium on calamondin leaves (Fig. 7a). Inoculation of citrus leaves with wild-type resulted in visible dark-pigmented lesions at 3 days post-inoculation (days p.i.). In contrast, similar inoculation of the Δplc1 mutants resulted in small or no lesions at 3 days p.i. (Fig. 7b). The genetically reverted Cp1 strain induced necrotic lesions at rates and magnitudes similar to wild-type on wounded or unwounded leaves of citrus. Similarly, infection assessed on detached calamondin leaves indicated that Δcall mutants exhibited weak virulence on both wounded and unwounded calamondin leaves at 3 days p.i. (Fig. 7c). Complementation of the Δcall mutant with the full-length CAL1 cassette restored full virulence to levels equivalent to those of wild-type.

**Application of Ca²⁺ or neomycin prior to inoculation decreases lesion formation on citrus**

Infection assessed on detached calamondin leaves revealed that application of CaCl₂ at 0.6 M or neomycin at 1 mM 24 h prior to inoculation provided protection against _Alt. alternata_, showing a drastic reduction of necrotic lesions compared with the leaves pre-treated with water (Fig. 8). Neomycin provided greater protection against _Alt. alternata_ than did CaCl₂.

**DISCUSSION**

Cytosolic Ca²⁺ has been proposed to regulate and coordinate many developmental processes in fungi. In this work, we have demonstrated that the maintenance of intracellular Ca²⁺ associated with the calcineurin- and phospholipase C-mediated pathways is critical for hyphal elongation, conidial formation and pathogenesis of _Alt. alternata_, consistent with the notion that Ca²⁺ controls a multitude of cellular processes. Cytoplasmic free Ca²⁺ concentrations are generally maintained around 100–350 nM within fungal cells (Halachmi & Eilam, 1989; Miller et al., 1990). Because Ca²⁺ is cytotoxic at high concentrations, _Alt. alternata_ must have developed mechanisms to maintain low intracellular Ca²⁺. This assumption is supported further by the observation that Ca²⁺ at concentrations less than 100 mM has no adverse effects on fungal growth. Ca²⁺ ions can enter the cytoplasm through membrane-associated channels (Miller et al., 1990). The appropriate concentration of intracellular Ca²⁺ could be maintained by pumping Ca²⁺ out of the cell through the
plasma membrane or by storing it in vacuoles or other cytosolic organelles. In addition, Ca\(^{2+}\) could be incorporated into the plasma membrane or bound to Ca\(^{2+}\)-binding proteins (e.g. CaM) present in the cytoplasm. Although Ca\(^{2+}\) uptake via membrane-bound transporter systems, such as a Ca\(^{2+}\)-ATPase and an

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**Fig. 3.** Calcineurin phosphatase is required for growth, hyphal elongation and conidial formation. (a) Time-course changes in radius increase of wild-type (WT), two independent Δcal1 mutants (D62 and D69) lacking calcineurin and the genetically complemented strain (Δcal/CAL) grown on PDA. Each point represents the mean ± SD of the colony radius from two independent experiments, with at least three replicates. An example of images showing fungal growth on the test medium for 5 days is also shown. (b) The CAL1-deficient mutants (D62 and D69) produced short-branched, dark-pigmented hyphae. (c) Wild-type strain produces elliptical conidia, whereas Δcal1 mutants do not produce any conidia. Fungal hyphae obtained from Δcal1 mutants, showing deformed, highly melanized hyphae with distinct septae in chains. Bars, 20 μm.
ATPase-driven H\(^+\)/Ca\(^{2+}\) antiport, has been demonstrated in animal cells, it is not yet experimentally proven that fungi utilize similar mechanisms to maintain cytoplasmic Ca\(^{2+}\) homeostasis (Jackson & Heath, 1993). The mechanism underlying Ca\(^{2+}\) export in fungi also remains largely unknown, even though fungi have multiple Ca\(^{2+}\) exchangers (Zelter et al., 2004).

Maintaining Ca\(^{2+}\) homeostasis is crucial for fungal growth. The IP\(_3\)-mediated signalling system is one of the key pathways in regulating cytosolic Ca\(^{2+}\) homeostasis. Ca\(^{2+}\) stored in vacuoles could be released back into the cytoplasm, in response to the activation of IP\(_3\) in animal cells (Berridge, 1993). As with animal cells, the vacuole of fungal cells is an important organelle for Ca\(^{2+}\) storage (Cornelius et al., 1989; Halachmi & Eilam, 1989). IP\(_3\) is generated from a membrane-bound lipid, PIP\(_2\) via the activity of phospholipase C. Lithium disrupts the phosphoinositide cycling and blocks IP\(_3\)-mediated activation of Ca\(^{2+}\) release (Inhorn & Majerus, 1988). Likewise, neomycin interferes with internal Ca\(^{2+}\) release through inhibiting phospholipase C activity, binding to phosphoinositides and inhibiting PIP\(_2\) synthesis (Schacht, 1976; Gabev et al., 1989). In addition, neomycin affects inositol phospholipid metabolism by suppressing phosphoinositide kinase and inhibits the activity of phosphatidylinositol-phospholipase D (Liscovitch et al., 1991; Wang et al., 1996).

In the present study, we showed that exogenous application of excessive Ca\(^{2+}\) in the form of CaCl\(_2\) or calcium nitrate, lithium or neomycin suppresses vegetative growth of *Alt. alternata*. As with many pharmacological inhibitors for Ca\(^{2+}\), neomycin and lithium chloride may lack target specificity. To more precisely determine the roles of Ca\(^{2+}\) in fungal development, we identified a phospholipase C-coding gene (*PLC1*) of *Alt. alternata* and characterized its product acting as a regulator of maintaining Ca\(^{2+}\) homeostasis. PLC1-mediated Ca\(^{2+}\) homeostasis plays a critical role in the hyphal elongation, conidial formation and germination pattern of conidia in *Alt. alternata*. Phospholipase C has also been shown to be required for conidium and appressorium formation and pathogenicity in *Botrytis cinerea* (Schumacher et al., 2008) and in the rice blast pathogen *Magnaporthe oryzae* (Rho et al., 2009; Choi...
Ca$^{2+}$ signalling in *Alternaria alternata*

**Fig. 6.** Quantitative measurement of Ca$^{2+}$-Fluo-3 fluorescence within fungal hyphae of the wild-type and the Δplc1 (a) and Δcal (b) mutants of *Alt. alternata* shown in Figs 4 and 5. The intensity of fluorescence was determined using the ImageJ program. Each column represents the mean ± SD intensity of fluorescence measured from at least 19 spots. Means (separation by Student’s t-test, P≤0.05) marked by the same letter are not significantly different.

Functional inactivation of a phospholipase C gene in the chestnut blight pathogen *Cryphonectria parasitica* resulted in a strain that exhibits reduced growth and downregulation of a laccase-coding gene (Chung *et al.*, 2006). Our results further underscore the important role of Ca$^{2+}$ in fungi.

Cytoplasmic free Ca$^{2+}$ can be determined by using Ca$^{2+}$ fluorescent dyes, such as Fluo-3, Indo-1 and Fura-2 (Hyde, 1998). Fluo-3 is a single-wavelength dye that changes fluorescent intensity when bound to Ca$^{2+}$ (Taylor & Wang, 1980), providing an excellent tool to measure the free Ca$^{2+}$ concentration in fungi. The level of intracellular Ca$^{2+}$, as assessed based on the intensity of the Fluo-3 fluorescent dye, is apparently much higher within Δplc1 mutants than that seen in wild-type. Ca$^{2+}$ appears to be accumulated primarily in cytoplasmic compartments, as the Fluo-3/Ca$^{2+}$-derived green fluorescence coalesces as dense spots within fungal hyphae. Expressing a functional copy of PLC1 in a Δplc1 null mutant restores wild-type accumulation of Ca$^{2+}$, confirming that PLC1 contributes to the impaired phenotypes. Alternatively, impairment of PLC could affect cell wall composition and thus facilitate Fluo-3 uptake. A Δplc1 null mutant exhibits extremely slow growth. Fungal hyphal extension is closely related to intracellular Ca$^{2+}$ gradients (Jackson & Heath, 1993). Thus, growth reduction observed in Δplc1 mutants could be attributable to the disturbance of the intracellular Ca$^{2+}$ equilibrium, which could cause aberrant mitotic chromosome segregation as observed in the Sac. cerevisiae mutant defective for PLC (Payne & Fitzgerald-Hayes, 1993). In addition, PLC1-mediated Ca$^{2+}$ homeostasis could have a role in membrane permeability in *Alt. alternata*, because the hyphae of the Δplc1 mutant, but not the wild-type, became swollen when incubated in liquid medium or solution. Swollen hyphae apparently coincide with a loss of PLC1 and increased intracellular Ca$^{2+}$.

The Ca$^{2+}$/CaM-binding protein calcineurin plays an important role in the regulation of fungal morphogenesis, cell wall biosynthesis, cell cycles, circadian rhythms and pathogenesis (Rusnak & Mertz, 2000). Calcineurin has also been implicated in the regulation of Ca$^{2+}$ influx in budding yeasts (Muller *et al.*, 2001). Mutational inactivation of a calcineurin catalytic subunit-coding gene (*CAL1*) in *Alt. alternata* resulted in strains that exhibit severe growth retardation, produce abnormal colonies showing a dark, rugged appearance and depressed margins, and fail to produce any conidia. The observed phenotypes strongly resemble those observed in other fungal species lacking calcineurin (Prokisch *et al.*, 1997; Harel *et al.*, 2006; da Silva Ferreira *et al.*, 2007). Microscopic examination of fungal hyphae after staining with the Ca$^{2+}$-binding dye Fluo-3 reveals that, in contrast to Δplc1, Δcal1 mutants accumulate lower intracellular Ca$^{2+}$ than wild-type. Because the Δcal1 mutants produce heavily melanized hyphae, the reduced Ca$^{2+}$-Fluo-3 fluorescence seen in these isolates could also, in part, be due to the interference of melanin. The abnormalities seen with Δcal1 mutants could be complemented by re-expressing a wild-type copy of *CAL1*.

Calcineurin and cAMP-dependent PKA have opposite effects in regulating the transcription factor Crz1p/Tcn1p (Hirata *et al.*, 1995; Matheos *et al.*, 1997; Stathopoulos & Cyert, 1997). Calcineurin dephosphorylates Crz1p/Tcn1p, facilitating nuclear localization. In contrast, PKA phosphorylates Crz1p/Tcn1p, resulting in cytoplasmic localization. Antagonistic regulation between PKA and calcineurin in the context of conidiation could also occur in *Alt. alternata*. Calcineurin is absolutely required for conidial formation. In contrast cAMP-dependent PKA negatively regulates conidial formation in *Alt. alternata*, as deletion of a PKA catalytic subunit-coding gene resulted in hyper-conidiation strains (Tsai *et al.*, 2013). Moreover, calcineurin signalling pathways may selectively interact with other signalling networks to regulate conidial formation. We have observed that deletion of the FUS3 or SLT2 MAP kinase-coding genes reduces conidiation by...
**Fig. 7.** Virulence assays. (a) Fungal pathogenicity assayed on detached calamondin leaves inoculated with mycelial mass obtained from wild-type (WT), PLC deletion strains (Δplc D25 and D35) and genetically reverted strains (Δplc/PLC) of *Alt. alternata*. Inoculation of the Δplc1 mutants occasionally produced pinpoint lesions (indicated by arrows). (b) Quantitative determination of necrotic lesions on intact or pre-wounded leaves using the ImageJ program (http://rsb.info.nih.gov/ij/). Each column represents the mean ± SD of necrotic lesions measured from at least 10 spots. (c) Fungal pathogenicity assayed on detached calamondin leaves inoculated with mycelial mass obtained from wild-type, CAL deletion strains (Δcal D62 and D69) and a genetically reverted strain (Δcal/CAL). Inoculation of the Δcal1 mutants occasionally produced pinpoint lesions (indicated by arrows). The inoculated leaves were incubated in a moist chamber for lesion development. Photos were taken 4 days post-inoculation.

*Alt. alternata* (Lin et al., 2010; Yago et al., 2011; Chung, 2013). FUS3 and PKA appear to be independent pathways in the positive and negative modulation of conidial formation, respectively, because the *Alt. alternata* strain lacking FUS3 exhibits wild-type PKA activity and expression of the PKA gene (Tsai et al., 2013). In *Sac. cerevisiae*, calcineurin activity has been shown to be indirectly modulated by the p42/p44 MAPK through phosphorylation of the calcineurin regulator Rcn1 (Hilioti et al., 2004). Whether or not calcineurin interacts with FUS3, SLT2 or PKA for conidial production awaits further analysis. Another interesting aspect of calcineurin is that it is involved in regulating stress responses in some fungi (Kraus & Heitman, 2003). The activation of calcineurin has been implicated in cellular redox status. Recent studies also demonstrated a direct interaction between calcineurin and heat-shock proteins in fungi (Imai & Yahara, 2000). Because the Δcal1 mutants exhibit severe growth reduction,
it is difficult to assess the role of calcineurin in stress responses in *Alt. alternata*. Our previous studies have shown that *Alt. alternata* apparently utilizes specialized or synergistically regulated signalling pathways, including the redox-responsive activator YAP1, the HOG1 MAPK, the SKN7 regulator and the NADPH oxidase to cope with oxidative stress (Lin *et al.*, 2009; Yang *et al.*, 2009; Lin & Chung, 2010, Chen *et al.*, 2012; Yang & Chung, 2012, 2013). It will be of considerable interest to determine whether calcineurin interacts with YAP1, SKN7, HOG1 and NOX at transcriptional and/or post-translational levels in the future.

Fungal pathogenicity assay on detached citrus leaves revealed that strains impaired for PLC1 or CAL1 produce small or no necrotic lesions. Because fungal strains lacking PLC1 or CAL1 are severely defective in conidiation and radial growth, it is not surprising that these mutants exhibit considerably reduced virulence to citrus. Spray applications of CaCl2 at 0.6 M or neomycin at 1 mM onto citrus leaves 24 h prior to inoculation of the wild-type strain of *Alt. alternata* resulted in lower disease incidence compared with those pre-sprayed with water. The results further support the disruption of Ca2+ signalling in *Alt. alternata* having a profound impact on pathogenesis. Calcineurin has also been demonstrated to be required for virulence in the plant pathogens *Sclerotinia sclerotiorum* and *Ustilago maydis* (Harel *et al.*, 2006; Egan *et al.*, 2009), as well as in the human pathogens *Cryptococcus neoformans* and *Candida albicans* (Fox *et al.*, 2001; Blankenship *et al.*, 2003).

In conclusion, we have demonstrated here that maintaining Ca2+ homeostasis via phospholipase C-associated regulation and the calcineurin-mediated signalling pathway is required for vegetative growth, hyphal morphology and conidiation in the tangerine pathotype of *Alt. alternata*. Our results also highlight the important role of Ca2+ homeostasis in fungal virulence in *Alt. alternata* and further underscore an important regulatory role of Ca2+ signalling pathways in fungi.

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**Fig. 8.** Reduction of *Alternaria* Brown spot. (a) Reduction of *Alternaria* brown spot severity caused by *Alt. alternata* on detached calamondin leaves by application of Ca2+ or neomycin. Mock control was treated with water only. Application of sodium chloride or magnesium chloride (at 0.6 M) onto citrus leaves did not impact lesion formation caused by *Alt. alternata*. (b) Quantitative determination of necrotic lesions using the ImageJ program. Calamondin leaves were sprayed with Ca2+, neomycin or water and incubated in a moist chamber for 24 h. Conidial suspension (10⁶ conidia mL⁻¹) prepared from wild-type was uniformly sprayed onto the leaves. The inoculated leaves were incubated in a moist chamber for an additional 2–4 days for lesion formation. Each column represents the mean area of necrotic lesions and the standard error per leaf (n=20). Means (separation by Student’s t test, P<0.05) marked by the same letter are not significantly different.


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