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

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Excessive Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ 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 CaCl₂ or neomycin 24 h prior to inoculation provided protection against *Alt. alternata*. These data indicate that a dynamic equilibrium of cellular Ca²⁺ is critical for developmental and pathological processes of *Alt. alternata*.

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 (H₂O₂) and cell death (Lin et al., 2011). Although toxin is absolutely required for fungal pathogenicity, *Alt. alternata* also relies on effective detoxification of H₂O₂ and other reactive oxygen species (ROS) to ensure survival and colonization in the host plant (Chung, 2012).

*Alt. alternata* lacks a sexual phase and propagates mainly through the production of conidia. Conidia are disseminated by rain splashes and are required for initiating *Alternaria* brown spot disease in citrus. Our previous studies have demonstrated that the cytoplasmic cAMP level, controlled by GTP-binding proteins (G-proteins), plays an important role in conidial formation by *Alt. alternata* (Wang et al., 2010). Conidial formation by the wild-type strain of *Alt. alternata* was suppressed by cAMP or its inhibitors (atropine, theophylline or 3-isobutyl-1-methylxanthine). An *Alt. alternata* strain impaired for the G₁₅ subunit failed to produce significantly fewer conidia than wild-type; however, application of cAMP or its inhibitors partially restored conidial formation by the G₁₅ mutant. In contrast, the *Alt. alternata* strain impaired for the G₁₅-dependent protein kinase A (PKA) catalytic subunit produced twofold more conidia than wild-type, and a strain lacking the PKA regulatory subunit failed to produce any conidia, suggesting a negative function of PKA for conidiation (Tsai et al., 2013). Studies with *Alt. alternata* revealed that conidial formation was also regulated by the FUS₃ and SLT₂ mitogen-activated protein kinase (MAPK)-mediated signalling pathways as well as NADPH oxidase (NOX), because fungal strains impaired for FUS₃, SLT₂ or NOX produced no conidia or significantly fewer than wild-type (Lin et al., 2010; Yago et al., 2011; Yang & Chung 2012, 2013).

Abbreviations: CaM, calmodulin; G-protein, GTP-binding protein; IP₃, inositol 1,4,5-trisphosphate; MAPK, mitogen-activated protein kinase; PIP₂, phosphatidylinositol 4,5-bisphosphate; PKA, cAMP-dependent protein kinase A.

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One supplementary table and three supplementary figures are available with the online version of this paper.
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 Gx subunit of a G-protein, leading to dissociation of Gx from the Gβγ subunits (Li et al., 2007). The released Gx subunit activates phospholipase C, which hydrolyses inositol-1,4,5-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 cAMP-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; Matheos 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 sterilized 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 by 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 % Triton X-100, with gentle shaking for 12–24 h. Images of Ca\textsuperscript{2+} green fluorescence were examined with the aid of a Nikon microscope equipped with a 450–490 nm excitation filter and a 520 nm emission filter as previously described (Chung, 2003). 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 1 (PLC1) fragment was amplified by PCR with two degenerate primers PLC-F 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 (EcoRI, PvuII, PvuI and SstI), 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-methoxyspiro[1,2-dioxetane-3,2’-(5’-chloro)tricyclo (3.3.1)decan-4-yl]phenyl phosphonate} 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, hyg3, 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, Calcin::M13R, M13R, hyg3, hyg4, M13F, M13F::Calcin and Calcin-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 sulfonyleurea as described previously (Lin et al., 2009). Transformants were recovered from medium amended with 5 µg sulfonyleurea ml⁻¹ (Chem Service).

Virulence tests. Fungal virulence was assayed on detached calamondin leaves by inoculating with a conidial suspension (1 × 10⁴ conidia ml⁻¹) or fungal mycelium grown on PDA. Conidial suspension was applied (5 µl) onto detached leaves. Fungal mycelia mass (≤1 mm) was placed onto citrus leaves with sterile toothpicks and the inoculated leaves were incubated in a moist chamber for 3-5 days for lesion development. CaCl2 (0.6 M) or neomycin (1 mM) was sprayed onto calamondin 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 EH-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 wild-type and all fast-growing transformants, but no product was identified from 18 slow-growing transformants (data not shown). A 3.2 kb fragment was amplified from wild-type and all fast-growing transformants, but no product was identified from 18 slow-growing transformants (data not shown). A 3.2 kb fragment was amplified from wild-type and all fast-growing transformants. Two slow-growing strains (*plc* D25 and *Δplc* D35) were randomly selected as putative disruptants for further characterization by PCR with a different primer set. When the primer PLC-1F, whose sequence is not present in the split marker fragments, was paired with the primer 1800R, a 1.8 kb fragment was amplified from genomic DNA of the wild-type but no product was obtained from that of two transformants (Fig. S1). When the primer PLC-1F was paired with the hyg3 primer, whose sequence is present in the hygromycin phosphotransferase gene (HYG) cassette, an expected 2.7 kb fragment was amplified from genomic DNA of transformants *Δplc* D25 and *Δplc* D35, but not 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 ($5^{\prime}$CAL::HYg and hYG::3$^{\prime}$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 ($D_{calc}D_{62}$ and $D_{calc}D_{69}$) 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 $\Delta plc1$ 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 $\Delta plc1$ mutant produced hyphae resembling those produced by wild-type on PDA (Fig. 2b). However, when cultured in liquid medium, the hyphae of the $\Delta plc1$ 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.
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 Δ*plc1* mutants (D25 and D35) lacking phospholipase C and the genetically complemented strain (Δ*plc/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 Δ*plc1* 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), Δcal1 mutants exhibited severe growth reduction by over 95% of that of wild-type (Fig. 3a). Δcal1 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 Δ62 protoplasts with a functional CAL1. Unlike wild-type, Δcal1 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. Δcal1 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 Ca^{2+} is affected by calcineurin and phospholipase C**

The level of intracellular Ca^{2+} was assessed using Fluo-3 fluorescent dye. After Ca^{2+} 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 cytoplast 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 Ca^{2+} 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 CAL1, emitted Fluo-3/Ca^{2+} 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 Δcal1 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 Δcal1 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 Δcal1 mutants exhibited weak virulence on both wounded and unwounded calamondin leaves at 3 days p.i. (Fig. 7c). Complementation of the Δcal1 mutant with the full-length CAL1 cassette restored full virulence to levels equivalent to those of wild-type.

**Application of Ca^{2+} or neomycin prior to inoculation decreases lesion formation on citrus**

Infection assessed on detached calamondin leaves revealed that application of CaCl_2 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 CaCl_2.

**DISCUSSION**

Cytosolic Ca^{2+} has been proposed to regulate and coordinate many developmental processes in fungi. In this work, we have demonstrated that the maintenance of intracellular Ca^{2+} 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 Ca^{2+} controls a multitude of cellular processes. Cytoplasmic free Ca^{2+} concentrations are generally maintained around 100–350 nM within fungal cells (Halachmi & Eilam, 1989; Miller et al., 1990). Because Ca^{2+} is cytotoxic at high concentrations, Alt. alternata must have developed mechanisms to maintain low intracellular Ca^{2+}. This assumption is supported further by the observation that Ca^{2+} at concentrations less than 100 mM has no adverse effects on fungal growth. Ca^{2+} ions can enter the cytoplasm through membrane-associated channels (Miller et al., 1990). The appropriate concentration of intracellular Ca^{2+} could be maintained by pumping Ca^{2+} 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<sup>+</sup>/Ca<sup>2+</sup> antiport, has been demonstrated in animal cells, it is not yet experimentally proven that fungi utilize similar mechanisms to maintain cytoplasmic Ca<sup>2+</sup> homeostasis (Jackson & Heath, 1993). The mechanism underlying Ca<sup>2+</sup> export in fungi also remains largely unknown, even though fungi have multiple Ca<sup>2+</sup> exchangers (Zelter et al., 2004). Maintaining Ca<sup>2+</sup> homeostasis is crucial for fungal growth. The IP<sub>3</sub>-mediated signalling system is one of the key pathways in regulating cytosolic Ca<sup>2+</sup> homeostasis. Ca<sup>2+</sup> stored in vacuoles could be released back into the cytoplasm, in response to the activation of IP<sub>3</sub> in animal cells (Berridge, 1993). As with animal cells, the vacuole of fungal cells is an important organelle for Ca<sup>2+</sup> storage (Cornelius et al., 1989; Halachmi & Eilam, 1989). IP<sub>3</sub> is generated from a membrane-bound lipid, PIP<sub>2</sub> via the activity of phospholipase C. Lithium disrupts the phosphoinositide cycling and blocks IP<sub>3</sub>-mediated activation of Ca<sup>2+</sup> release (Inhorn & Majerus, 1988). Likewise, neomycin interferes with internal Ca<sup>2+</sup> release through inhibiting phospholipase C activity, binding to phosphoinositides and inhibiting PIP<sub>2</sub> synthesis (Schacht, 1976; Gabev et al., 1989). In addition, neomycin affects inositol phospholipid metabolism by suppressing phosphoinositide kinase and inhibits the activity of phosphatidylocholine-phospholipase D (Liscovitch et al., 1991; Wang et al., 1996).

In the present study, we showed that exogenous application of excessive Ca<sup>2+</sup> in the form of CaCl<sub>2</sub> or calcium nitrate, lithium or neomycin suppresses vegetative growth of Alt. alternata. As with many pharmacological inhibitors for Ca<sup>2+</sup>, neomycin and lithium chloride may lack target specificity. To more precisely determine the roles of Ca<sup>2+</sup> 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<sup>2+</sup> homeostasis. PLC1-mediated Ca<sup>2+</sup> 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 et al., 2010).

**Fig. 4.** Mutational disruption of the phospholipase C-coding gene (PLC1) in Alt. alternata resulted in the elevation of cytosolic free Ca<sup>2+</sup>. Fungal hyphae, prepared from wild-type (WT), two independent Δplc1 mutants (D25 and D35) lacking phospholipase C and the genetically complemented strain (Δplc/PLC), were stained with the Ca<sup>2+</sup>-binding dye Fluo-3 and examined microscopically. The intensities of green fluorescence represent the relative amounts of free Ca<sup>2+</sup> in the cytoplasm.

**Fig. 5.** Mutational disruption of the calcineurin-coding gene (CAL1) in Alt. alternata decreases cytosolic free Ca<sup>2+</sup>. Fungal hyphae, prepared from wild-type (WT), two independent Δcal mutants (D62 and D69) lacking calcineurin and the genetically complemented strain (Δcal/CAL), were stained with the Ca<sup>2+</sup>-binding dye Fluo-3 solution and examined microscopically.
Cytoplasmic free Ca\textsuperscript{2+} can be determined by using Ca\textsuperscript{2+} fluorescent dyes, such as Fluo-3, Indo-1 and Fura-2 (Hyde, et al., 1998). Fluo-3 is a single-wavelength dye that changes fluorescent dyes, such as Fluo-3, Indo-1 and Fura-2 (Hyde, et al., 1998).

Ca\textsuperscript{2+} signalling in *Alternaria alternata*

*Fig. 6.* Quantitative measurement of Ca\textsuperscript{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, \(P \leq 0.05\)) marked by the same letter are not significantly different.

Cytoplasmic free Ca\textsuperscript{2+} can be determined by using Ca\textsuperscript{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\textsuperscript{2+} (Taylor & Wang, 1980), providing an excellent tool to measure the free Ca\textsuperscript{2+} concentration in fungi. The level of intracellular Ca\textsuperscript{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\textsuperscript{2+} appears to be accumulated primarily in cytoplasmic compartments, as the Fluo-3/Ca\textsuperscript{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\textsuperscript{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\textsuperscript{2+} gradients (Jackson & Heath, 1993). Thus, growth reduction observed in Δplc1 mutants could be attributable to the disturbance of the intracellular Ca\textsuperscript{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\textsuperscript{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\textsuperscript{2+}.

The Ca\textsuperscript{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\textsuperscript{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\textsuperscript{2+}-binding dye Fluo-3 reveals that, in contrast to Δplc1, Δcal1 mutants accumulate lower intracellular Ca\textsuperscript{2+} than wild-type. Because the Δcal1 mutants produce heavily melanized hyphae, the reduced Ca\textsuperscript{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
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). 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 assayed 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 *Candidas 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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