Fungal Genetics and Biology 91 (2016) 55–65

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

Cytosolic free calcium dynamics as related to hyphal and colony growth in the filamentous fungal pathogen *Colletotrichum graminicola*

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ARTICLE INFO

Article history:
Received 15 September 2015
Revised 2 April 2016
Accepted 4 April 2016
Available online 5 April 2016

Keywords:
Aequorin luminometry
Colletotrichum graminicola
Calcium signalling
Hyphal tip growth
Yellow cameleon imaging

ABSTRACT

Tip growth of pollen tubes and root hairs of plants is oscillatory and orchestrated by tip-focussed variations of cytosolic free calcium ($[Ca^{2+}]_{cyt}$). Hyphae of filamentous fungi are also tubular tip-growing cells, and components of the Ca^{2+} signalling machinery, such as Ca^{2+} channels and Ca^{2+} sensors, are known to be important for fungal growth. In this study, we addressed the questions if tip-focussed $[Ca^{2+}]_{cyt}$ transients govern hyphal and whole-colony growth in the maize pathogen *Colletotrichum graminicola*, and whether colony-wide $[Ca^{2+}]_{cyt}$ dynamics rely on external Ca^{2+} or internal Ca^{2+} stores. Ratiometric fluorescence microscopy of individual hyphae expressing the Ca^{2+} reporter Yellow Cameleon 3.6 revealed that Ca^{2+} spikes in hyphal tips precede the re-initiation of growth after wounding. Tip-focussed $[Ca^{2+}]_{cyt}$ spikes were also observed in undisturbed growing hyphae. They occurred not regularly and at a higher rate in hyphae growing at a medium-glass interface than in those growing on an agar surface. Hyphal tip growth was non-pulsatile, and growth speed was not correlated with the rate of spike occurrence. A possible relationship of $[Ca^{2+}]_{cyt}$ spike generation and growth of whole colonies was assessed by using a codon-optimized version of the luminescent Ca^{2+} reporter Aequorin. Depletion of extracellular free Ca^{2+} abolished $[Ca^{2+}]_{cyt}$ spikes nearly completely, but had only a modest effect on colony growth. In a pharmacological survey, some inhibitors targeting Ca^{2+} influx or release from internal stores repressed growth strongly. However, although some of those inhibitors also affected $[Ca^{2+}]_{cyt}$ spike generation, the effects on both parameters were not correlated. Collectively, the results indicate that tip growth of *C. graminicola* is non-pulsatile and not mechanistically linked to tip-focused or global $[Ca^{2+}]_{cyt}$ spikes, which are likely a response to micro-environmental parameters, such as the physical properties of the growth surface.

1. Introduction

Tip-growing cells are found in animals and plants, as well as in fungi and oomycetes. In pollen tubes and root hairs of plants, apical growth rates and cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) in the apex display oscillatory variations which are tightly linked by a feedback loop (Monshausen et al., 2008; Steinhorst and Kudla, 2013). In those systems, variations in apical $[Ca^{2+}]_{cyt}$ regulate actin (de-) polymerisation and cell wall flexibility and hence orchestrate the elongation process (Hepler and Winship, 2010; Wang et al., 2008; Feijó et al., 2001; Cárdenas et al., 2008). A multitude of studies have demonstrated that Ca^{2+}-related processes are also involved in the regulation of filamentous fungal growth. For instance, the Ca^{2+} sensor protein calmodulin (CaM) and the CaM-dependent kinases 1 and 2 are crucial for hyphal extension (Nguyen et al., 2008; Kumar and Tamuli, 2014). The Ca^{2+}- and Ca^{2+}/CaM-regulated phosphatase calcineurin and its target Crz1 have also been shown to be important for hyphal growth in a number of fungi (Prokisch et al., 1997; da Silva Ferreira et al., 2007; Juvvadi et al., 2011; Zhang et al., 2012; Tsai and Chung, 2014; Schumacher et al., 2008). As in plants, Ca^{2+} thus appears to regulate growth of fungi. This provokes the question whether there are similarities with respect to spatial and temporal patterns of $[Ca^{2+}]_{cyt}$ dynamics in relation to the kinetics of tip growth.

In a series of imaging experiments, López-Franco et al. (1994) provided experimental evidence for an oscillatory rhythm of hyphal elongation in a taxonomically diverse range of fungi. Oscillation frequencies differed between species, and amplitudes were generally in the nanometer range. However, such pulsatile growth kinetics of fungal hyphae have been put into question in subsequent theoretical considerations (Jackson, 2001) and experimental analyses (Sampson et al., 2003). Similar to the kinetics of hyphal growth, information on spatial and temporal $[Ca^{2+}]_{cyt}$ dynamics

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http://dx.doi.org/10.1016/j.fgb.2016.04.001
1087-1845/

in the hyphal apex is limited. In early studies, evidence for a tip-focussed $[Ca^{2+}]_{cyt}$ gradient in *Neurospora crassa*, supposedly generated by Ca^{2+} release from internal stores, was obtained by Fluo-3 dual dye ratio imaging and electrophysiological analyses (Levina et al., 1995; Silverman-Gavrila and Lew, 2003, 2002; Lew, 1999). However, temporal dynamics of the tip-high $[Ca^{2+}]_{cyt}$ gradient were not analysed at that time. More recently, a genetically encoded Ca^{2+} sensor, Yellow Cameleon 3.6 (YC3.6), was employed to image $[Ca^{2+}]_{cyt}$ in several fungi with high spatial and temporal resolution (Kim et al., 2012). In that work, tip-high $[Ca^{2+}]_{cyt}$ pulses were revealed in hyphae of *Fusarium oxysporum*, *Fusarium graminearum*, and *Magnaporthe oryzae*. However, those $[Ca^{2+}]_{cyt}$ gradients were not permanent, as would have been expected from the earlier studies, but of a transient nature. In a follow-on study, it was shown for *F. oxysporum* that those $[Ca^{2+}]_{cyt}$ transients varied with growth conditions (Kim et al., 2015). Strikingly, the authors observed that there were periods in which the hyphae grew normally in the absence of a $[Ca^{2+}]_{cyt}$ gradient.

The recent imaging experiments of Kim et al. (2012, 2015) seriously questioned the dogma of a key regulatory role for $[Ca^{2+}]_{cyt}$ in undisturbed tip growth of fungi. Except for those analyses, temporally and spatially resolved $[Ca^{2+}]_{cyt}$ dynamics have not been determined in relation to hyphal tip growth. In the present study, we have analysed the relationship of $[Ca^{2+}]_{cyt}$ dynamics and hyphal growth in *Colletotrichum graminicola*, a hemi-biotrophic pathogen of maize (*Zea mays*) that infects the plant from seedling stage to post-harvest residues (Münch et al., 2008). *C. graminicola* has provoked dramatic yield losses in the past (Bergstrom and Nicholson, 1999), and the identification of processes that may serve as fungicide targets is highly desirable. By employing the YC3.6 reporter, we have initially addressed the question whether growing hyphae of this fungus generate a tip-high $[Ca^{2+}]_{cyt}$. To macroscopically analyse the correlation of $[Ca^{2+}]_{cyt}$ elevations and colony growth, we established a whole-colony protocol based on the luminescent Ca^{2+} reporter aequorin. A pharmacological survey revealed that an alteration of colony-wide $[Ca^{2+}]_{cyt}$ spike occurrence by a range of agents was not reflected in analogous growth differences. Our results indicate that, unlike in tip-growing plant cells, growth of *C. graminicola* is not dependent on rhythmic tip-focussed $[Ca^{2+}]_{cyt}$ elevations and not correlated with global $[Ca^{2+}]_{cyt}$ spikes.

2. Materials and methods

2.1. Fungal strain and general growth conditions

The filamentous fungus *C. graminicola* M2 (M1.001) and transformants derived from this strain were grown on modified Leach's complete medium (mLCM) agar at 23 °C and 65% relative humidity (rh) (Lange et al., 2014a). To induce the production of falcate (sickle-shaped) conidia the fungus was grown on oat meal agar (OMA; Koneman et al., 1997). OMA was composed of 50 g ground oat flakes (Baukhof, Uelzen, Germany), 12 g agar and 1 L deionised water. The mixture was heated in the microwave, homogenized with a hand-held blender, and autoclaved for 45 min at 121 °C. Inoculated OMA plates were sealed with micropore tape (3M, Saint Paul, MN, USA) and incubated under diurnal conditions in a growth cabinet (Adaptis A-1000, Conviron, Winnipeg, Canada) equipped with fluorescent light tubes (HE21W/840/T5, Osram, Munich, Germany) and set to 16 h light (120–150 μmol m^{-2} s^{-1}, 22 °C, 65% rh) and 8 h darkness (18 °C, 65% rh).

2.2. Growth assays on EGTA-containing medium

To assess the effect of EGTA on hyphal growth of *C. graminicola*, a modified PAAP culture protocol was applied (Lange et al., 2014a). EGTA was solved at 500 mM in bi-distilled water, adjusted to pH 8.00 with KOH, filter-sterilized, and added at 10 mM final concentration to mLCM agar temperated at 65 °C. 55-mm Petri dishes were filled with 7.5 mL untreated or EGTA-containing mLCM agar. For PAAP cultures, 3 × 3 cm pieces of nitrocellulose membrane (Protran BA84, pore diameter 0.2 μm, Whatmann, Dassel, Germany) were sterilized in bleach solution (Rissel et al., 2014) for at least 5 min followed by 4 washes with sterile bi-distilled water and 2 washes with sterile liquid mLCM and placed onto untreated mLCM agar immediately after solidification. Plates were dried for 30 min in a laminar flow hood, and 300 falcate conidia were inoculated onto each membrane. Mycelium was grown for 48 h at 23 °C, and the colony size on four membranes was marked with a needle and then measured. Next, the remaining membranes (N = 3 per condition) were transferred with a sterile forceps to fresh mLCM plates that were either supplemented with 10 mM EGTA or un-supplemented and incubated further for 29 h. The gained colony diameter was measured as above.

To determine the total Ca^{2+} concentration of the mLCM agar, 100 μL medium were dissolved in 10 mL digestion solution (32.5% HNO_3, 6% H_2O_2) and heated for 10 min to 180 °C in a MARS 5 Xpress high pressure microwave oven (CEM, Kamp-Lintfort, Germany). Samples were analysed by ICP-OES (ULTIMA 2, HORIBA, Kyoto, Japan). Medium pH was measured by covering a pH test strip (pH-Fix 6.0–7.7, Macherey-Nagel, Düren, Germany) with a thin layer of mLCM agar. Concentrations of free Ca^{2+} were calculated with webmaxc (http://www.stanford.edu/~cpatton/webmaxcS.htm).

2.3. Growth assays on calcium-depleted medium

Synthetic Complete (SC) medium plates for the Ca^{2+} depletion experiment were made up of 20 g L^{-1} glucose, 76 mM NH_4Cl, 7.9 g L^{-1} Complete Supplement Mixture (CSM) (Formedium), and 1.5% EDTA-washed agar. Ca^{2+}-depleted SC medium contained additionally 1.8 g L^{-1} yeast nitrogen base without $(NH_4)_2SO_4$, amino acids, and $CaCl_2$ (Formedium). For Ca^{2+}-containing SC medium, 1.9 g L^{-1} yeast nitrogen base with $CaCl_2$, without $(NH_4)_2SO_4$ and without amino acids (Formedium) was used instead. EDTA-washed agar was purified from agar-agar (Kobe I, Carl Roth, Karlsruhe, Germany) as follows. 2 g agar were suspended in 50 mL Tris-EDTA solution (50 mM EDTA, 100 mM Tris base pH 8.3) and agitated in a 50-mL tube for one day at room temperature. After centrifugation for 1 min at 4000*g*, the supernatant was discarded, and the agar resuspended in Tris–EDTA as above. In total, seven washes with Tris–EDTA were done, followed by 20 washes with bidistilled water. The agar suspension was incubated shaking for 1 h between each washing step. The purified agar was lyophilized. The remaining Ca^{2+} content was determined in triplicate by ICP-OES as described in Section 2.2 using 100 mg agar. The purified agar (1.5%) contained 0.85 ± 0.14 μM Ca^{2+} (mean ± SE, N = 3).

2.4. Growth assays on inhibitor-containing media

2-APB (Roth), capsazepine (Sigma, Deisenhofen, Germany), nifedipine (Sigma), and W-7 (Sigma) were dissolved at 100 mM in DMSO (Duchefa, Haarlem, The Netherlands). U73122 (Sigma) was dissolved at 4 mM in DMSO. $GdCl_3$ (Sigma), $La(NO_3)_3$ (Roth) and TMB8·HCl (Sigma) were dissolved at 100 mM in bi-distilled water and filter-sterilized (0.2 μm, Bio One, Greiner, Frickenhausen, Germany). (±)-Verapamil·HCl (Sigma) was dissolved at 50 mM in bi-distilled water and filter-sterilized. Caffeine and ruthenium red were dissolved directly in the medium prior to autoclaving. To prepare inhibitor-containing media, mLCM agar was autoclaved. After cooling to 65 °C, 1 μL of the inhibitor stock solution or solvent control was added per 1 mL medium. In case