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Video article

Mitotic stopwatch for the blast fungus *Magnaporthe oryzae* during invasion of rice cells

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ABSTRACT

To study nuclear dynamics of *Magnaporthe oryzae*, we developed a novel mitotic reporter strain with GFP-NLS (localized in nuclei during interphase but in the cytoplasm during mitosis) and H1-tdTomato (localized in nuclei throughout the cell cycle). Time-lapse confocal microscopy of the reporter strain during host cell invasion provided several new insights into nuclear division and migration in *M. oryzae*: (i) mitosis lasts about 5 min; (ii) mitosis is semi-closed; (iii) septal pores are closed during mitosis; and (iv) a nucleus exhibits extreme constriction (approximately from 2 μm to 0.5 μm), elongation (over 5 μm), and long migration (over 16 μm). Our observations raise new questions about mechanisms controlling the mitotic dynamics, and the answers to these questions may result in new means to prevent fungal proliferation without negatively affecting the host cell cycle.

1. Introduction

The filamentous ascomycete *Magnaporthe oryzae* causes an economically important blast disease on rice (*Oryza sativa*). On the rice leaf surface, a three-celled conidium (one nucleus per cell) produces a germ tube that differentiates into an appressorium, which mechanically breaches an epidermal rice cell and subsequently produces invasive hyphae (IH) that secrete effectors and proliferate inside host cells (Khang et al., 2010; Khang and Valent, 2010). Cell cycle regulation has been shown to be important for the appressorium development and IH proliferation. The conidial nucleus, which gives rise to a nucleus that migrates into the appressorium, must enter S-phase for the initial differentiation of the appressorium and subsequently G2/mitosis for further appressorium maturation (Saunders et al., 2010a, 2010b; Veneault-Fourrey et al., 2006). Once inside the rice cell, IH cell cycle is under control of a transketolase-mediated metabolic checkpoint (Fernandez et al., 2014). IH colonize host tissues by filling the first-invaded cell and then moving into neighbor cells via IH pegs that likely pass through plasmodesmata (Kankanala et al., 2007).

To facilitate the study of the cell cycle, we developed a fluorescence-based mitotic reporter strain of *M. oryzae* that expresses GFP-NLS together with H1-tdTomato. Using four dimensional (4D) confocal microscopy of the reporter strain, we provide evidence that *M. oryzae* IH undergo semi-closed mitosis accompanied by septal pore closure and also that the mitotic nucleus exhibits extreme constriction and elongation during migration through the narrow IH peg.

2. Results and discussion

2.1. M. oryzae mitotic reporter strain CKF1962

During live cell imaging of IH expressing mCherry fused to a nuclear localization signal (mCherry-NLS), we serendipitously observed that the nuclear-localized fusion protein dispersed into the cytoplasm and subsequently re-localized into two newly divided nuclei (Fig. 1A). A similar pattern has been observed in *Aspergillus nidulans*, in which fluorescent protein (FP)-NLS localizes in nuclei during the interphase but disperses into the cytoplasm at mitotic entry and remains there until mitotic exit (De Souza et al., 2004; Shen et al., 2014). This led us to reason that the dynamic localizations of FP-NLS can be used as a stopwatch to study mitosis in *M. oryzae*. We thus generated an *M. oryzae* mitotic reporter strain, named CKF1962, expressing GFP-NLS together with histone H1 fused to tdTomato (H1-tdTomato) that remains associated with DNA throughout the cell cycle.

Using 4D confocal microscopy (images taken every ~13 s over 17 min; 1.5 μm z-sections over 30 μm) of CKF1962 growing inside a rice cell, we observed three nuclear divisions, two of which were

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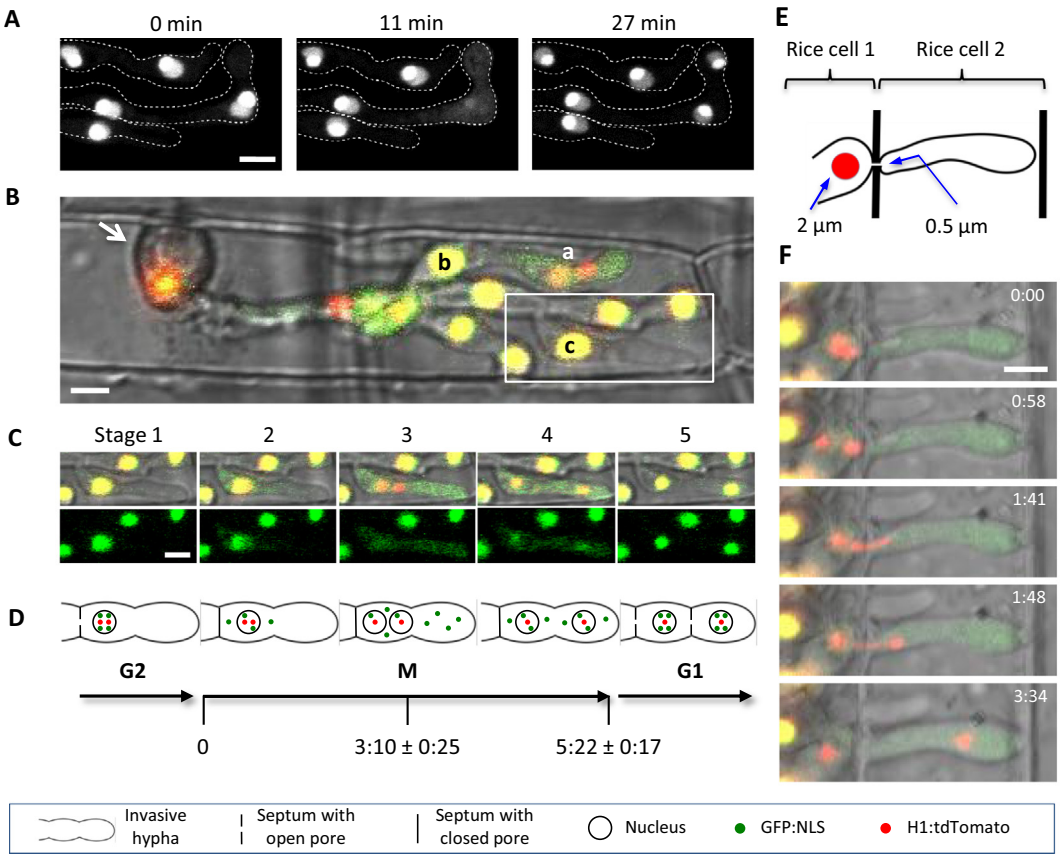


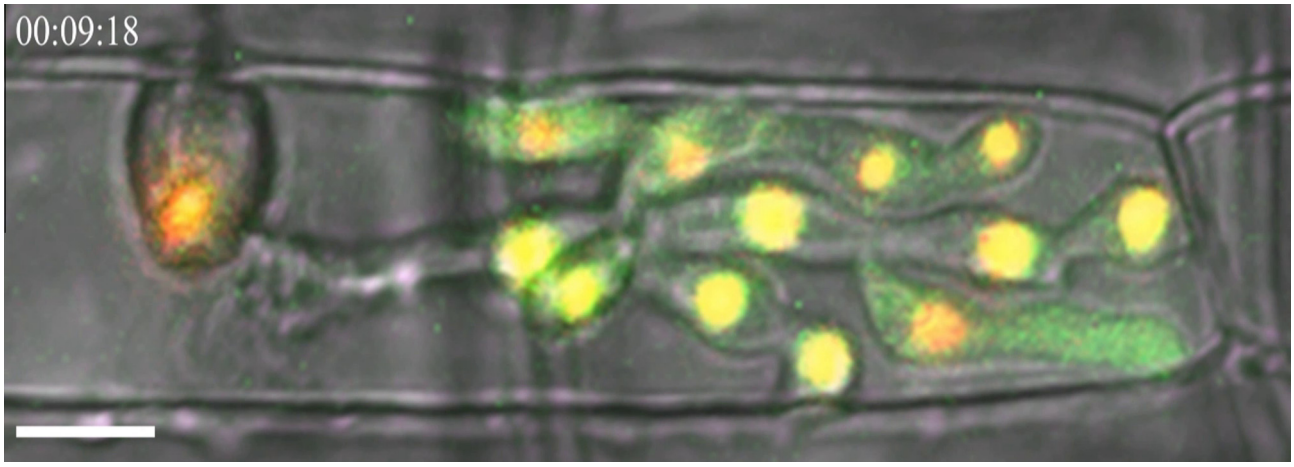
Fig. 1. Mitosis in *M. oryzae* invading rice cells. Bars = 5 µm. (A) Time-lapse confocal fluorescence images of *M. oryzae* CKF1468 invasive hyphae (IH) growing inside a rice cell at 30 h post inoculation (hpi). mCherry-NLS is pseudo-colored in white. Dotted lines outline IH. (B) Video 1 still image at $t = 0$ (31 hpi) indicating three nuclei (a–c in order of division) of *M. oryzae* transformant CKF1962 that undergo mitosis during Video 1. The arrow indicates the appressorium on the rice cell surface. The inset in (B) denotes the region corresponding to (C), which shows five sequential fluorescence pattern stages in both merged bright-field and fluorescence (top), and green fluorescence alone (bottom). (D) Schematic diagram of CKF1962 fluorescence patterns in IH undergoing mitosis with relative average times of stages 3 and 4 with time zeroed at mitotic entry (stage 2) for six rounds of mitosis from two separate infections. Times are shown in minutes:seconds ± std. (E) Diagram illustrating the question of how the IH nucleus (~2 µm diameter) migrates through the constricted IH peg (~0.5 µm diameter) in order to nucleate the IH cell that has spread from Rice cell 1 to Rice cell 2. (F) Selected time-lapse confocal images from Video 2 showing nuclear division and migration concerning the scenario in (E). Times are shown in minutes:seconds.

complete rounds as one had already begun to divide at the start of imaging (Video 1 and Fig. 1B). Based on the fluorescence patterns, we defined the following sequential stages (Fig. 1C): (1) co-localization of H1-tdTomato and GFP-NLS in pre-mitotic nuclei, (2) initial dispersion of GFP-NLS into the cytoplasm, (3) separation of the tdTomato-tagged chromosomes, (4) initial re-localization of GFP-NLS into mitotic nuclei, and (5) complete co-localization of H1-tdTomato and GFP-NLS in the two divided nuclei. Given that beginning of dispersion or re-localization of FP-NLS marks mitotic entry or exit, respectively, the first and last stages (1 and 5) represent late G2 and early G1, respectively, and the three middle stages (2–4) represent mitosis (Fig. 1C and D). Based on the three nuclear divisions (Video 1; Fig. 1B and C), we estimated the duration of mitosis to be approximately 5 min, which was consistent with another observation of three nuclear divisions at a different infection site (Fig. 1D) and is comparable to that in *A. nidulans* (Bergen and Morris, 1983).

2.2. Proposed semi-closed mitosis and mitotic closure of septal pores in *M. oryzae*

Increasing evidence suggests that various forms of open mitosis occur in many fungi that were previously considered to undergo completely closed mitosis (De Souza and Osmani, 2007). For example, in the basidiomycete *Ustilago maydis*, as the mitotic spindle forms in the daughter bud, the nuclear envelope (NE) breaks down locally with most of the NE remaining in the mother cell (Straube et al., 2005). During mitosis, *A. nidulans* undergoes partial disassembly of nuclear pore complexes (NPCs) (De Souza et al., 2004). Nuclear-localized FP-NLS disperses into the cytoplasm during mitosis when NE permeability increases due to partial NPC disassembly (De Souza et al., 2004). Similarly to *A. nidulans*, our FP-NLS data (Fig. 1A–C) suggests that *M. oryzae* maintains a partially disassembled NPC during mitosis, resulting in the dispersal of fluorescence into the cytoplasm. Consistent with this, cytoplasmically-expressed FP was shown to equilibrate between the cytoplasm and nucleoplasm during mitosis (Bourett et al., 2002). Furthermore, $DiOC_6$ staining revealed that the mitotic NE remains intact (Saunders et al., 2010b). Taken together, we hypothesize that *M. oryzae* undergoes a form of semi-closed mitosis where the NE remains intact, but NPCs partially dissociate during mitosis.

Interestingly, we noticed that when FP-NLS dispersed throughout apical cells during mitosis, it did not diffuse into adjacent cells (Fig. 1A–C), suggesting the mitotic closure of septal pores. We also noticed non-synchronized mitosis between the apical cell and its adjacent cell (the first two nuclear divisions in Fig. 1B and Video 1). Shen et al. (2014) observed a similar phenomenon in *A. nidulans* and suggested that opening and closing of septal and nuclear pores are under cell cycle regulation to be out of phase with each other during cell cycle progression. This provides an explanation of how the mitotic state of apical cells does not affect the cell cycle in adjacent cells in *A. nidulans* (Shen et al., 2014). Our results



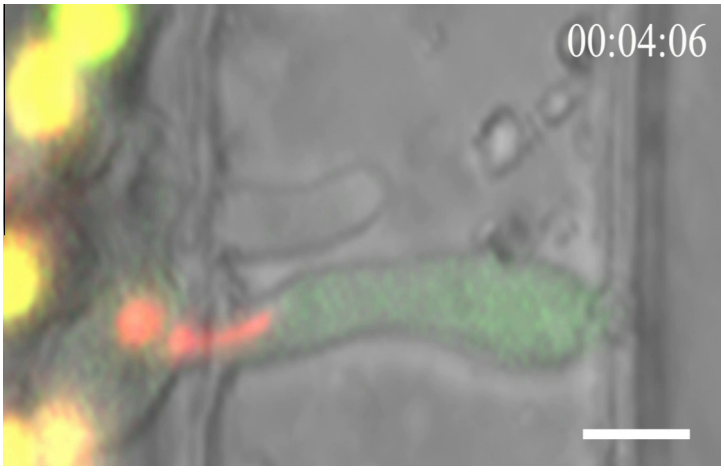
Video 1. Time-lapse movie of CKF1962 IH growing within the first invaded rice cell at 31 hpi. CKF1962 expresses GFP-NLS (green) and H1-tdTomato fusion proteins (red). Movie consists of 79 images taken every ~13 s over a duration of 17 min 38 s. Original images contained 20 z-sections (covering 30 μm).

suggest that *M. oryzae* (one nucleus per cell) shares similar mitotic dynamics, namely, semi-closed mitosis and mitotic closure of septal pores, with *A. nidulans* (multiple nuclei per cell). It will be interesting to continue to investigate how such dynamics are regulated in these and other fungi.

2.3. Nuclear dynamics during IH movement from infected cells to adjacent cells

M. oryzae IH undergo an extreme constriction when spreading into adjacent host cells (Kankanala et al., 2007). This raises an intriguing question of how the nucleus (~2 μm diameter in our measurement) migrates through the constricted IH peg (~0.5 μm diameter reported in Kankanala et al., 2007) (Fig. 1E). To answer this question, we documented the nuclear dynamics during IH cell-to-cell movement by utilizing our mitotic stopwatch strain CKF1962. This strain allows us to predict the timing of the nuclear division and subsequent migration. That is, the separation of the tdTomato-tagged chromosomes occurs within approximately 3 min from the initial dispersal of nuclear-localized GFP-NLS into the cytoplasm (Fig. 1B–D). Thus, we first identified an IH cell that had grown into the adjacent host cell and exhibited dispersed GFP-NLS without a tdTomato-tagged nucleus, and then we initiated high temporal and spatial resolution 4D confocal imaging (images taken every ~20 s; 1.5 μm z-sections over 13.5 μm; Fig. 1F and Video 2). Consistent with the prediction, within 3 min we observed the separation of the tdTomato-tagged chromosomes in the IH cell residing in the initially invaded cell. One nucleus then squeezed through the constricted region of the IH peg and stretched to over 5 μm in length. Subsequently, the elongated nucleus began to round up at the leading edge, becoming spherical as it moved to its final location 16.9 μm from the site of chromosome separation. To our knowledge, this is the first report of extreme stretch and long migration of a fungal nucleus during mitosis. It is an exciting possibility that nuclear dynamics studies with additional FP reporters, such as for cytoskeleton/motor proteins and NE/NPCs, will provide further insight into mechanisms of nuclear migration and plasticity as well as infection-related development in *M. oryzae*.

In summary, we provide the first detailed analysis of nuclear division and migration in *M. oryzae* during host invasion and also describe a FP-based reporter system that can be used to study mitosis and nuclear dynamics in other fungi. Our studies raise new mechanistic questions concerning the mitotic dynamics, and the answers to these questions may provide new targets to prevent fungal proliferation without negatively affecting the host cell cycle.



Video 2. Time-lapse movie of CKF1962 IH growing in a neighboring rice cell at 39 hpi and undergoing mitosis. Movie consists of 17 images taken every ~20 s over a duration of 5 min 32 s. Original images contained 9 z-sections (covering 13.5 μm).

3. Methods

M. oryzae wild-type strain O-137 was transformed with binary vector pBV1078 to generate transformant CKF1468 or sequentially with two binary vectors pCK1287 and pCK1288 to generate transformant CKF1962 using Agrobacterium-mediated transformation (Khang et al., 2005). pBV1078 was produced by cloning of NLS (three tandem repeats of the nuclear localization signal from simian virus large T-antigen) at the C terminus of mCherry under the BAS4 promoter in binary vector pBHt2 (hygromycin selection marker; Mullins et al., 2001). pCK1287 was produced by cloning of hH1 (histone H1 from *Neurospora crassa*) at the N terminus of tdTomato under control of the constitutive promoter from the *M. oryzae* ribosomal protein 27 gene (RP27) in binary vector pBHt2. pCK1288 was produced by cloning of NLS at the C terminus of 3XEGFP under the RP27 promoter in binary vector pBGt (G418 selection marker; Kim et al., 2011). Rice strain YT16 was grown and inoculated as previously described (Jones et al., 2016).

Confocal microscopy was performed on a Zeiss Axio Imager M1 microscope equipped with a Zeiss LSM 510 META system using Plan-Apochromat 20×/0.8 NA and Plan-Neofluor 40×/1.3 NA (oil) objectives. Excitation/emission wavelengths were 488 nm/505–530 nm (GFP), and 543 nm/560–615 nm (tdTomato). Images were acquired using LSM 510 software (Version 3.2). Images were analyzed and processed using a combination of the Zen software, Adobe Photoshop, and ImageJ (http://imagej.nih.gov/ij/).

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