Short Communication

EGFP-Rhm51 foci enable the visualization and enumeration of DNA double-strand breaks in *Magnaporthe oryzae*

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Abbreviations: DSBs, double-strand breaks; HR, homologous recombination; ORF, open reading frame.

Abstract

In order to detect and enumerate DNA double-strand breaks (DSBs) during the life cycle of *Magnaporthe oryzae*, an expression vector for GFP-Rhm51 fusion protein was constructed and introduced into the strain Ina86-137. Rhm51-GFP foci were detected and the number of foci in mitomycin-C-treated mycelia was higher than in untreated samples, indicating that the foci visualized DSBs occurred during the life cycle. Rhm51-GFP foci were observed in all stages of the asexual life cycle including the invasive hyphae formed in an intact rice leaf sheath, demonstrating that *M. oryzae* suffers DSBs during vegetative and infective growth.

Keywords: *Magnaporthe oryzae*, DNA double-strand breaks, infective growth, rice blast disease.

Cells proliferate mainly by meiosis and mitosis and these processes need to be tightly coordinated to preserve genome integrity and favor faithful genome propagation. Coordination of DNA replication with DNA-damage sensing, repair and cell cycle progression ensures genome integrity during cell divisions, thus preventing mutations and DNA rearrangements (Aguilera and Gomez-Gonzalez 2008). Mutations and chromosomal rearrangements in *Magnaporthe oryzae* (Couch), the causal agent of rice blast disease have been shown to drive genetic variation and evolution at the molecular level (Dean et al. 2005). For example, the appearance of new races of *M. oryzae* which can infect previously resistant varieties of rice can be attributed to recombinational events such as deletion of AVR genes (Miki et al. 2009). Changes in genetic linkage of two DNA fragments such as homologous recombination (HR)-mediated events and end-joining between non-homologous DNA fragments may result in genomic rearrangements (Aguilera and Gomez-Gonzalez 2008). The common substrate of these rearrangement events are DNA double-strand breaks (DSBs) (Agmon et al. 2009; Dudas and Chovanec 2004; Ljungman and Lane 2004; Symington 2002).

In plants, the induction of hypersensitive cell death is important in preventing pathogen development (Heath 2000). Reactive Oxygen Species (ROS) which play crucial roles in both symbiotic (Tanaka et al. 2006) and pathogenic (Egan et al. 2007) interactions is the signal molecule in higher plants that triggers a variety of defense responses, such as expression of defense-related genes and hypersensitive cell death (Bourett and Howard 1990; Koga et al. 2004; Ono et al. 2001; Tanabe et al. 2009). ROS is implicated in DNA damage including the induction of DSBs in human cells (Bennett 2001; Cadet et al. 2004). For *Magnaporthe* to cause disease, it must overcome the defense-related responses

mounted by the host. Information as to whether defense responses induce DSBs in *Magnaporthe* during infection is unknown. It was therefore important to enumerate DSBs during vegetative and infective growth to ascertain the contribution of the infection process to pathogenic variability in *M. oryzae*.

Rad51p, the recombinational repair protein that forms a nucleofilament involved in homology search and strand invasion plays a key role in DSB repair by HR (Mimitou and Symington 2008; Rossi and Mazin 2008). In the absence of DNA damage, Rad51p is not induced, but the constitutively expressed proteins are diffusely distributed within the nucleus. In response to DNA damage, Rad51p is induced and re-localizes to the sites of DNA damage to form distinct foci, the spots of GFP fluorescence which corresponds to the DSBs, which are visualized by direct or indirect fluorescence (immuno-staining). The number of Rad51 foci in a cell is an indication of the number of DSBs in that cell (Gospodinov et al. 2009). GFP-RAD51 and RAD51-GFP have been used for the visualization of foci respectively in *Ustilago maydis* (Kojic et al. 2006) and *Sordaria macrospora* (Tesse et al. 2003). In this study we aimed to establish and visualize DSBs in *M. oryzae*, with the recombinant fusion protein of *Rhm51* (the *RAD51* homolog in *M. oryzae*) and EGFP. This fusion protein was expected to form protein foci.

The plasmid (pBARST-PPR-GFP-Rhm51A, Fig. 1) had an *Rhm51* promoter (PPR, 1121 bp) linked directly to EGFP gene followed by *Rhm51* open reading frame (ORF, Accession No. AB562330). This plasmid was used for the transformation of strain Ina86-137 and its *rhm51* deletion mutant Ina86-137Δ*rhm51* (Ndindeng et al. unpublished). After the protoplast-PEG transformation, the transformants with pBARST-PPR-GFP-Rhm51A were selected based on the resistance to bialaphos (4 μg/ml). The functionality

of GFP-Rhm51 fusion as Rhm51was assessed by the complementation of the growth defects (reduction in mycelial growth, conidiation, appressoria formation) of Ina86-137Δ*rhm51* in the pBARST-PPR-GFP-Rhm51A transformants of the mutant (data not shown). Although the functionality of the transgenic fusion protein was checked, the expression level was not determined and this may pose some risk when using such genes for imaging experiments. However, the phenotypes of Ina86-137-GFP-Rhm51A were similar to those of wild type Ina86-137 except for the constitutive expression of GFP (Fluorescent signal) during normal growth in Ina86-137-GFP-Rhm51A. One transformant of the wild type Ina86-137 named Ina86-137-GFP-Rhm51A was used for further analysis.

Conidia of the transformant, Ina86-137-GFP-Rhm51A were used for the inoculation of liquid medium (2YEG, 10 g/l glucose and 2 g/l yeast extract) and incubated at 27^{o}C for 3 days. Mycelia were collected by filtration and stained with DAPI (4', 6-diamidino-2-phenylindole, 2 μg/ml) for 1 h followed by fluorescence microscopy (Fig. 2). GFP signal co-localized with DAPI signal without formation of foci in hyphae showing distinct and compact nuclei. On the other hand, GFP foci were detected in the hyphae, without the distinct and compact DAPI-stained nuclei (nuclei appeared fragmented). The distinct nuclei have been revealed as mitotic nuclei, and the absence of the distinct nuclei indicated the G_2-arrest in *Aspergillus nidulans* (Westfall and Momany 2002). Based on the fact that DSBs are revealed as the cause of G_2-arrest in other organisms, the GFP foci detected in the vegetative hyphae of the transformant were expected as caused by DSBs, which occurred in *M. oryzae* vegetative hyphae.

DNA DSBs can be induced by addition of DNA-damaging chemicals; therefore the number of foci is expected to be increased by addition of such reagents. The number of foci was then detected in the germinating conidia, germ tubes and growing hyphae, with or without treatment by 0.1 μM mitomycin C (Wako, Osaka, Japan), a DNA-DSB inducing agent. The liquid cultured hyphae were not used because the tightly entangled hyphae prevented the accurate enumeration of foci. A conidial suspension (containing 0.1 μM mitomycin C when applicable) was spotted on a hydrophilic, frosted glass slide to allow a maximum number of conidia to grow and form branching hyphae, then incubated at 27^{o}C in the dark for 5 or 10 h. As expected, foci were observed in conidia, germ tube and branching hyphae and the number of foci were higher in treated samples ($P > 0.05$) although foci were detected both in Mitomycin-C -treated and untreated samples (Fig. 3 and 4). These results indicated that the detected GFP foci correspond to DSBs which occurred in the life cycle of *M. oryzae*.

Then conidia suspension of the transformant Ina86-137-GFP-Rhm51A was spotted on the hydrophobic surface of Gelbond film (Lonza, Rockland, ME, USA) and incubated at 27^{o}C, in order to observe the Rhm51 foci during the appressoria formation (Fig. 5). Foci were observed in the appressoria suggesting that DSBs also occur during appressorium formation.

The intact leaf sheath assay (Koga et al. 2004) was used to observe Rhm51 foci during fungal invasion of compatible rice (cultivar: Shin2) by Ina86-137-GFP-Rhm51A strain. Conidia (1.5×10^5 /ml) of Ina86-137-GFP-Rhm51A suspended in distilled water were used to inoculate intact leaf sheaths. Foci were observed and enumerated after 9, 18, 24 and 48 h post inoculation under fluorescent bright light at 25^{o}C. Foci were observed

during conidia germination, germ tube elongation and appressoria formation and the amount was comparable to those observed when the strain was grown on frosted glass slides without mitomycin-C treatment. Foci were also observed in the appressoria during penetration (data not shown) and in infective hyphae (Fig. 5) suggesting that *Rhm51* was induced during infection. These results demonstrated that *M. oryzae* suffers DSBs during infective growth although effective enumeration of foci in the infective hyphae was hindered by their embedded nature inside the plant cell.

This is the first report of the enumeration of DSBs in *M. oryzae*. The functionality of EGFP-Rhm51 fusion protein enables us to detect DSBs *in vivo* without further staining or other treatment, and is applicable for further studies on DSB repair and its importance for the pathogen's growth and pathogenicity. *M. oryzae* is revealed to suffer DSBs during multiple stages in its life cycle, and recombinational repair of DSBs may contribute to pathogen's genome variability.

Acknowledgements

The authors are grateful to the Islamic Development Bank for supporting Ndindeng's study at Hokkaido University through the Merit Scholarship Programme for High Technology (MSP).

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Figure legends

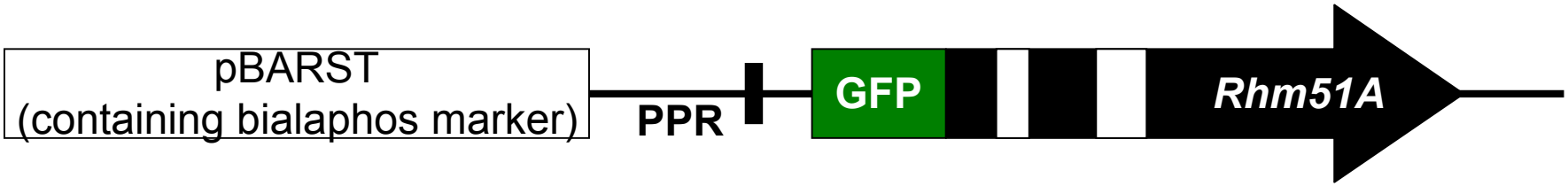
Fig. 1. Construction of pBARST-PPR-GFP-Rhm51A vector for the detection of Rhm51 foci in *Magnaporthe oryzae*. The vector was constructed by Gateway system (Invitrogen, Carlsbad, CA). PPR (*Rhm51* Putative Promoter, 1121 bp), EGFP (Green Fluorescent Protein), *Rhm51*ORF were ligated into pENTR-D-TOPO (Invitrogen, Carlsbad, CA, USA), and the reporter structure insert was subcloned into bialaphos resistant destination vector pBARST (Abe et al. 2006) using LR clonase (Invitrogen, Carlsbad, CA, USA). White boxes in *Rhm51* ORF indicates the introns. The pBARST-PPR-GFP-Rhm51A was digested with *Xba* I to be linearized prior to the transformation of fungal cells.

Fig. 2. Detection of Rhm51-foci in *Magnaporthe oryzae* during vegetative growth in liquid media. Conidia were prepared from Ina86-137 wild-type strain carrying pBARST-PPR-GFP-Rhm51A. Conidia were inoculated in 2YEG and incubated at 27^{o}C for 3 days and then treated with DAPI (4', 6-diamidino-2-phenylindole). The results presented are based on analysis from three independent samples and 150 different images. Arrows show points of foci. Scale bars = 10 μm.

Fig. 3. Detection of Rhm51 foci in *Magnaporthe oryzae* during vegetative growth. Conidia were spotted onto frosted glass slides and incubated at 27^{o}C for 5-10 h. Foci were detected in the germinating conidia, germ tubes and growing hyphae, with or without treatment by 0.1 μM mitomycin C. Arrows show points of foci. Scale bars = 10 μm.

Fig. 4. Enumeration of Rhm51 foci. The results are means of three independent experiments. At least 50 images were analyzed at each stage of growth. Bar with different letter signify statistical difference at the 0.05 level of significance using the Student's t-test.

Fig. 5. Detection of Rhm51-foci in *Magnaporthe oryzae* during appressoria formation and plant infection. Conidia from Ina86-137-GFP-Rhm51A (1 x 10^4 /ml) were spotted on hydrophobic surface of a Gelbond film (Lonza, Rockland, ME, USA) and incubated at 27^{o}C for 24 h and foci were observed. Conidia (1.5 x 10^5 /ml) were inoculated on intact leaf sheath of compatible rice (cultivar Shin2) and incubated at 25^{o}C and 60 % relative humidity in a cultivation chamber for 48 h. GFP-Rhm51 foci were detected during plant colonization. APP = Appressorium, HI = Infective Hyphae. Arrows show points of foci. Scale bars = 10 μm



pBARST-PPR-GFP-Rhm51A digested with *Xba* I

Fig. 1

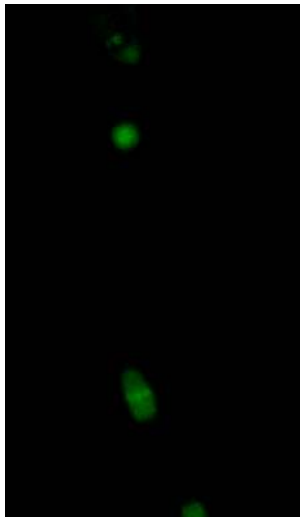
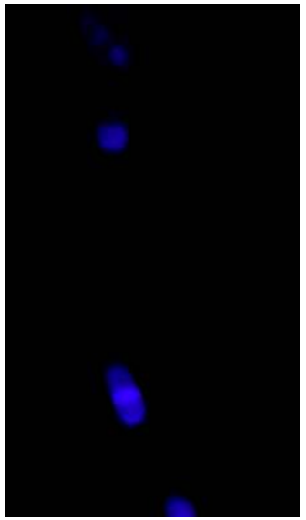
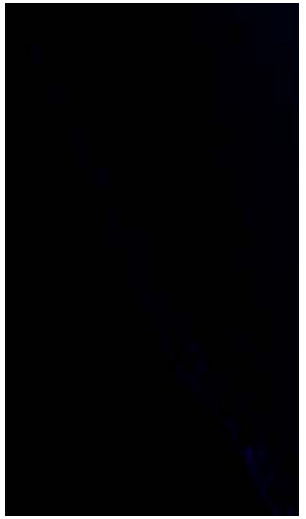
Rhm51 foci	Nuclei	GFP-Rhm51	DAPI	BF/DIC
Absent	Distinct, Compact			
Present	Not distinct			

Fig. 2

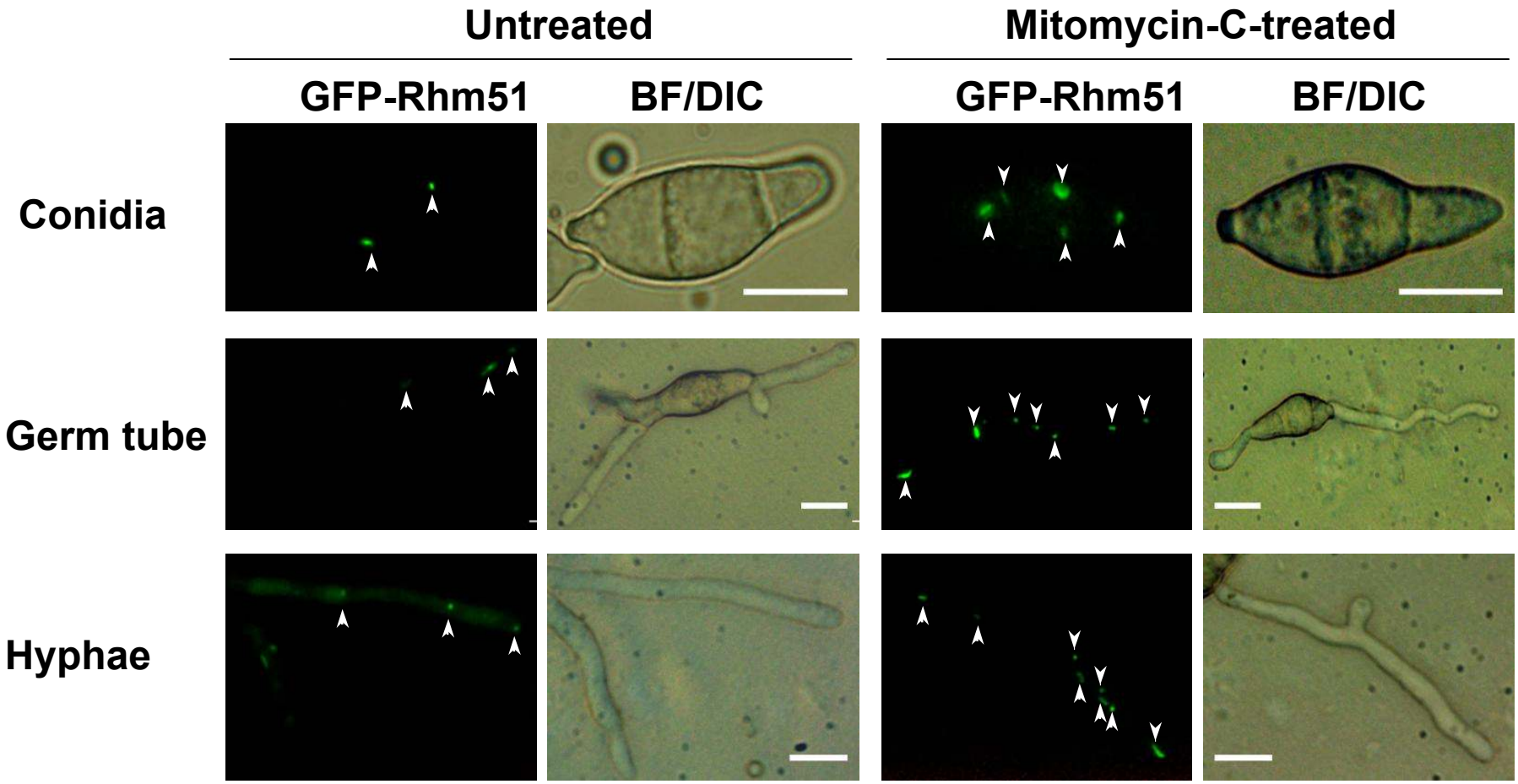

Untreated
Mitomycin-C-treated
GFP-Rhm51
BF/DIC
GFP-Rhm51
BF/DIC
Conidia
Germ tube
Hyphae

Fig. 3

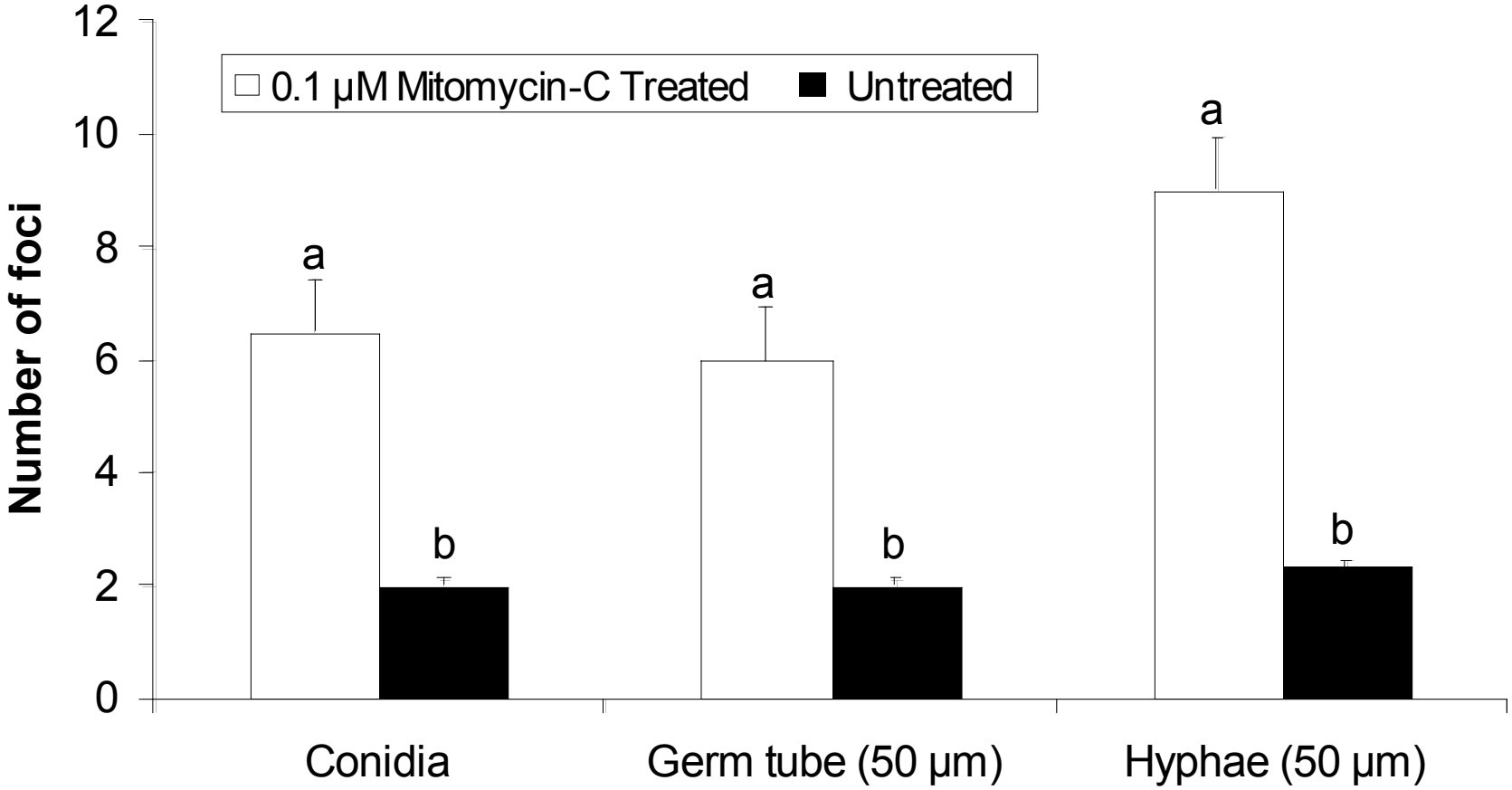

12
10
8
6
4
2
0
Number of foci
0.1 µM Mitomycin-C Treated
Untreated
a
b
a
b
a
b
Conidia
Germ tube (50 µm)
Hyphae (50 µm)

Fig. 4

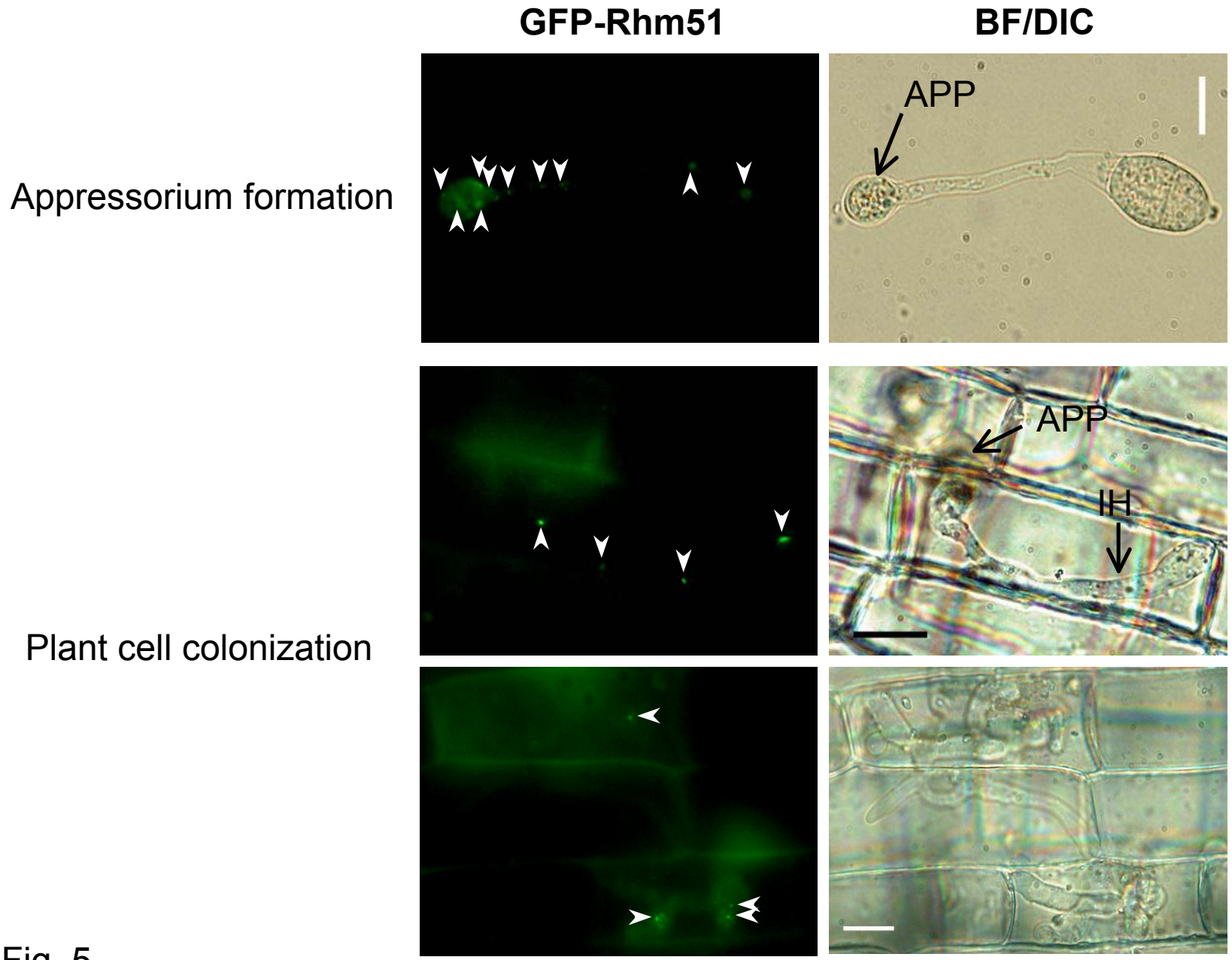

GFP-Rhm51
BF/DIC
Appressorium formation
Plant cell colonization
APP
APP
IH

Fig. 5