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MoCDC14 is important for septation during conidiation and appressorium formation in *Magnaporthe oryzae*

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SUMMARY

As a typical foliar pathogen, appressorium formation and penetration are critical steps in the infection cycle of *Magnaporthe oryzae*. Because appressorium formation and penetration are closely co-regulated with the cell cycle, and Cdc14 phosphatases have an antagonistic relationship with cyclin-dependent kinases (CDKs) on proteins related to mitotic exit and cytokinesis, in this study, we functionally characterized the *MoCDC14* gene in *M. oryzae*. The *Mocdc14* deletion mutant showed significantly reduced growth rate and conidiation. It was also defective in septum formation and nuclear distribution. Septation was irregular in *Mocdc14* hyphae and hyphal compartments became multinucleate. Mutant conidia often showed incomplete septa or lacked any septum. During appressorium formation, the septum delimiting appressoria from the rest of the germ tubes was often formed far away from the neck of the appressoria or not formed at all. Unlike the wild-type, some mutant appressoria had more than one nucleus at 24 h. In addition to appressoria, melanization occurred on parts of the germ tubes and conidia, depending on the irregular position of the appressorium-delimiting septum. The *Mocdc14* mutant was also defective in glycogen degradation during appressorium formation and appressorial penetration of intact plant cells. Similar defects in septum formation, melanization and penetration were observed with appressorium-like structures formed at hyphal tips in the *Mocdc14* mutant. Often a long fragment of mutant hyphae was melanized, together with the apical appressorium-like structures. These results indicate that *MoCDC14* plays a critical role in septation, nuclear distribution and pathogenesis in *M. oryzae*, and correct septum formation during conidiogenesis and appressorium formation requires the MoCdc14 phosphatase.

Keywords: cell cycle, conidiation, cytokinesis, plant infection, *Pyricularia oryzae*, rice blast.

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INTRODUCTION

Magnaporthe oryzae (synonym: *Pyricularia oryzae*) is the causal agent of rice blast, one of the most important diseases threatening rice production worldwide, and a model system for the study of fungal–plant interactions (Dean *et al.*, 2012; Ebbole, 2007; Xu *et al.*, 2007). Although it also produces single-celled microconidia (Kato *et al.*, 1994; Zhang *et al.*, 2014), pyriform macroconidia (commonly referred to as conidia) are the primary inoculum and main source of disease dissemination (Ebbole, 2007; Wilson and Talbot, 2009). Young conidia are single-celled and contain one nucleus migrating from the conidiophore. After one round of mitosis and formation of a septum, developing conidia become two-celled. The tip compartment will then undergo one more round of mitosis and cytokinesis to form mature, three-celled conidia with one nucleus in each compartment (Howard and Valent, 1996; Liu *et al.*, 2010). Various mutants with conidiogenesis defects have been identified in *M. oryzae*, and a number of them, such as the *con1*, *con7*, *com1*, *cdc15* and *chs1* mutants, are defective in plant infection (Goh *et al.*, 2011; Kong *et al.*, 2012; Shi and Leung, 1995; Yang *et al.*, 2010).

Infection by *M. oryzae* begins with the attachment and germination of conidia on plant surfaces (Wilson and Talbot, 2009). Germ tubes emerging from conidia undergo one round of mitosis and develop dome-shaped appressoria at the tip (Osés-Ruiz *et al.*, 2016). In *M. oryzae*, cytokinesis is spatially uncoupled from mitosis during appressorium formation (Saunders *et al.*, 2010b). A special septum is formed at the neck of a developing appressorium after one daughter nucleus migrates into appressorium (Osés-Ruiz *et al.*, 2016). When appressoria mature, conidial compartments and germ tubes undergo autophagic cell death (Liu *et al.*, 2007, 2012; Veneault-Fourrey *et al.*, 2006). Carbon storage in conidia is mobilized and degraded in appressoria to generate enormous turgor pressure, which is used by *M. oryzae* to physically penetrate the cuticle and underlying plant cells (Ebbole, 2007; Saunders *et al.*, 2010a; Wilson and Talbot, 2009). Appressorium-like structures can also be formed at the hyphal tips, which also involves the formation of a special septum at the neck for delimitation (Kong *et al.*, 2013). Both appressoria formed by germ tubes and appressorium-like structures formed at hyphal tips are heavily

melanized, which is necessary for the generation of high intracellular turgor pressure and plant penetration (Howard and Valent, 1996; Martin-Urdiroz *et al.*, 2016; Wilson and Talbot, 2009). The septum that separates appressoria or appressorium-like structures from the rest of the germ tubes or hyphae must be complete and have no septal pore when intracellular turgor builds up. Indeed, melanization occurs at this special septum, together with appressoria and appressorium-like structures, but not in the rest of the germ tubes or hyphae.

In eukaryotic organisms, reversible phosphorylation of proteins by protein kinases and phosphatases is known to regulate various growth and developmental processes. As a model for the study of fungal–plant interactions, *M. oryzae* has been extensively studied with regard to the molecular mechanisms regulating plant infection processes. A number of protein kinases involved in well-conserved signal transduction pathways have been shown to regulate surface recognition, appressorium formation, penetration and invasive hyphae in this important pathogen (Li *et al.*, 2012; Zhao *et al.*, 2007). Appressorium morphogenesis is also tightly regulated by the cell cycle in *M. oryzae* (Saunders *et al.*, 2010a,b; Veneault-Fourrey *et al.*, 2006). Premitotic DNA replication and one round of mitosis are essential for appressorium formation. Hydroxyl urea treatment before germination or expression of a temperature-sensitive NimA kinase blocks appressorium development (Saunders *et al.*, 2010a). Expression of a temperature-sensitive allele of the *SEP1* kinase gene that is involved in the determination of the position and frequency of cell division results in increased septation and nuclear division in the germ tubes and in defects in appressorium formation and plant infection (Saunders *et al.*, 2010b). Protein kinases involved in cell cycle progression also probably play pivotal roles in the differentiation and growth of bulbous invasive hyphae inside plant cells in *M. oryz*ae (Goh *et al.*, 2011; Perez-Martin *et al.*, 2006; Saunders *et al.*, 2010a). Similar observations of distinct cell cycle regulation during infectious growth have been observed in *Ustilago maydis* and *Fusarium graminearum* (Liu *et al.*, 2015; Perez-Martin *et al.*, 2006; Sgarlata and Perez-Martin, 2005).

Cdc14 belongs to a family of highly conserved dual-specificity phosphatases that are present in fungi and animals, but absent in plants (Kerk *et al.*, 2008; Mocciaro and Schiebel, 2010). Cdc14 enzymes play a direct role in the promotion of cytokinesis by acting on components of the contractile actomyosin ring and cell separation machineries (Meitinger *et al.*, 2012). In the budding yeast *Saccharomyces cerevisiae*, *CDC14* is an essential gene that regulates mitotic exit, cytokinesis and septation (Bardin and Amon, 2001; Meitinger *et al.*, 2012). Dephosphorylation of Iqg1 by Cdc14 at its cyclin-dependent kinase (CDK) phosphorylation sites promotes the formation of the actomyosin ring that is necessary for cytokinesis (Miller *et al.*, 2015). Endoplasmic reticulum (ER) export of the Chs2 chitin synthase also requires its dephosphorylation by Cdc14 at the CDK phosphorylation sites to ensure septum formation following mitosis (Chin *et al.*, 2012). In the fission yeast *Schizosaccharomyces pombe*, the Cdc14 orthologue Clp1 functions together with the septation initiation network (SIN) to coordinate cytokinesis with nuclear division, but is not essential for mitosis and growth (Trautmann *et al.*, 2001). Clp1 reverses Cdk1-mediated phosphorylation of the mitotic inducer Cdc25 and promotes its degradation by the anaphase-promoting complex at the end of mitosis (Esteban *et al.*, 2004; Wolfe and Gould, 2004). In the filamentous fungi *Aspergillus nidulans*, *F. graminearum* and *Beauveria bassiana*, the *CDC14* orthologues are also dispensable for viability, but important for asexual development (Li *et al.*, 2015; Son and Osmani, 2009; Wang *et al.*, 2013). In the human pathogen *Candida albicans*, deletion of *CaCDC14* has no effect on growth rate, but results in defects in cell separation, mitotic exit and morphogenesis (Clemente-Blanco *et al.*, 2006).

The *M. oryzae* genome has one distinct orthologue of *CDC14*, but its functions during appressorium morphogenesis and invasive growth have not been characterized. Because of the relationship between appressorium morphogenesis and cell cycle regulation, and the function of Cdc14 phosphatases as major antagonists of CDKs (Mocciaro and Schiebel, 2010; Queralt and Uhlmann, 2008), in this study, we characterized the *MoCDC14* phosphatase gene in *M. oryzae*. The *Mocdc14* mutant showed significantly reduced growth rate and conidiation. It was also defective in septum formation and nuclear distribution during vegetative growth, conidiogenesis and appressorium formation. Mutant conidia often showed incomplete septa or lacked any septum, and produced appressoria with abnormal appressorium-delimiting septation and melanization. In addition, the *Mocdc14* mutant was defective in appressorial penetration and infection through wounds. Similar defects in septation, melanization and penetration were observed with appressorium-like structures formed by *Mocdc14* at hyphal tips. Taken together, our results show that *MoCDC14* plays a critical role in septation, nuclear distribution and pathogenesis in *M. oryzae*, and correct septum formation during conidiogenesis and appressorium formation requires the MoCdc14 phosphatase.

RESULTS

MoCDC14 is important for normal vegetative growth and nuclear distribution in hyphae

The *MoCDC14* gene (MGG_04637) is orthologous to *CDC14* of *S. cerevisiae* and *clp1* of *Sc. pombe*, and has the typical HCKAGLGR catalytic sequence. To determine its function in *M. oryzae*, we generated the *Mocdc14* deletion mutant by the split-marker approach (Catlett *et al.*, 2003). Putative *Mocdc14* deletion mutants were identified by polymerase chain reaction (PCR). Four *Mocdc14* mutants, M1, M49, M51 and M69 (Table 1), with the

same phenotypes (described below) were confirmed by Southern blot analysis (Fig. S1, see Supporting Information).

The *Mocdc14* mutant showed a reduced growth rate of approximately 75% (Table 2) and formed colonies with limited aerial hyphae on oatmeal agar (OTA) plates (Fig. 1A), indicating the importance of *MoCDC14* during vegetative growth. To determine whether deletion of *MoCDC14* affects cytokinesis, we transformed the H1-GFP fusion construct (Luo *et al.*, 2014) into Guy11 and the *Mocdc14* mutant M1. In Guy11, septation occurred regularly in hyphae and each hyphal compartment had one nucleus (Fig. 1B). In the *Mocdc14* mutant, hyphae had fewer and unevenly distributed septa, and individual hyphal compartments often contained multiple nuclei (Fig. 1B). These results suggest that the *Mocdc14* mutant is defective in mitotic exit and nuclei continue to divide in the absence of cytokinesis in vegetative hyphae.

The *Mocdc14* mutant is defective in conidiogenesis

The *Mocdc14* mutant also showed significantly reduced conidiation. The average number of conidia produced by the mutant on each OTA plate was $(2.2 \pm 1.1) \times 10^3$, which was reduced by more than 100-fold in comparison with the wild-type (Table 2). Microscopic examination showed that most of the mutant conidiophores were aberrant, bearing no conidia (Fig. 2A). Conidia formed by the *Mocdc14* mutant were abnormal in morphology. Instead of forming typical three-celled conidia, over 99% of mutant conidia were single-celled (Fig. 2B). No septum or only incomplete septation was observed in almost all the conidia formed by the *Mocdc14* mutant (Fig. 2C), further indicating the importance of *MoCDC14* in septation in *M. oryzae*. Whereas wild-type conidia had three nuclei (one nucleus in each compartment), mutant conidia had only one or two nuclei (Fig. 2C). No three-celled conidia or conidia with three nuclei were produced by the *Mocdc14* mutant, indicating that *MoCDC14* plays a critical role in mitosis and cytokinesis in developing conidia.

Table 1 Wild-type and mutant strains of *Magnaporthe oryzae* used in this study.

Strain	Brief description	Reference
Guy11	Wild-type	Chao and Ellingboe (1991)
M1	*Mocdc14* deletion mutant of Guy11	This study
M49	*Mocdc14* deletion mutant of Guy11	This study
M51	*Mocdc14* deletion mutant of Guy11	This study
M69	*Mocdc14* deletion mutant of Guy11	This study
C1	*Mocdc14*/*MoCDC14*-GFP transformant of M1*	This study
C2	*Mocdc14*/*MoCDC14*-GFP transformant of M1	This study
HG1	Transformant of Guy11 expressing H1-GFP	This study
HG2	Transformant of Guy11 expressing H1-GFP	This study
HM1	Transformant of M1 expressing H1-GFP	This study
HM2	Transformant of M1 expressing H1-GFP	This study

*All of the green fluorescent protein (GFP) fusion constructs were integrated ectopically in the *M. oryzae* genome.

Deletion of *MoCDC14* affects septation to delimit appressoria on germ tubes

When assayed for appressorium formation on hydrophobic surfaces, although *Mocdc14* showed defects in conidium morphology, mutant conidia were normal in germination and still produced melanized appressoria at the tips of germ tubes (Fig. 3A). However, appressorium formation was slightly reduced in the mutant. Whereas over 95% of wild-type germ tubes formed appressoria after incubation for 24 h on hydrophobic surfaces, appressorium formation was observed in only 79% of the germ tubes in the *Mocdc14* mutant under the same conditions (Table 2). Interestingly, parts of the germ tubes and conidia were often melanized in the *Mocdc14* mutant (Fig. 3A). When stained with calcofluor white (CFW), the wild-type formed a septum at the neck of appressoria. In the mutant, the septum that delimited the appressorium from the rest of the germ tubes was not formed, or formed on germ tubes far away from the appressoria (Fig. 3A). The part of the germ tubes delimited together with appressoria was also heavily melanized (Fig. 3A). If there was no septum formation or septation was incomplete in germ tubes and conidia, the entire germ tube together with conidial compartments became melanized (Fig. 3A). These results suggest that *MoCDC14* is important for the formation of the septum delimiting the appressorium from the rest of the germ tubes.

To determine the effects of *MoCDC14* deletion on nuclear behaviour during appressorium formation, transformants expressing the H1-GFP construct were stained with CFW. In the wild-type, one nucleus moved back into the conidia after a single round

Table 2 Defects of the *Mocdc14* mutant in growth, conidiation and appressorium formation

Strain	Growth rate (mm/day)	Conidiation ($\times 10^3$ spores/plate)	Germination (%)*	Appressorium formation (%)†
Guy11	3.4 ± 0.1^A	2475.0 ± 353.2^A	98.4 ± 0.5^A	95.8 ± 0.9^A
M1	0.9 ± 0.1^B	2.2 ± 1.1^B	91.3 ± 2.0^A	79.0 ± 4.5^B
C1	3.3 ± 0.1^A	2183.3 ± 222.9^A	99.1 ± 0.1^A	96.7 ± 1.0^A

*Percentage of conidia germinating by 24 h.

†Percentage of germ tubes forming appressoria by 24 h.

Means and standard deviations were calculated from three independent measurements. Data were analysed with the protected Fisher's least significant difference (LSD) test. Different letters indicate significant difference ($P = 0.05$).

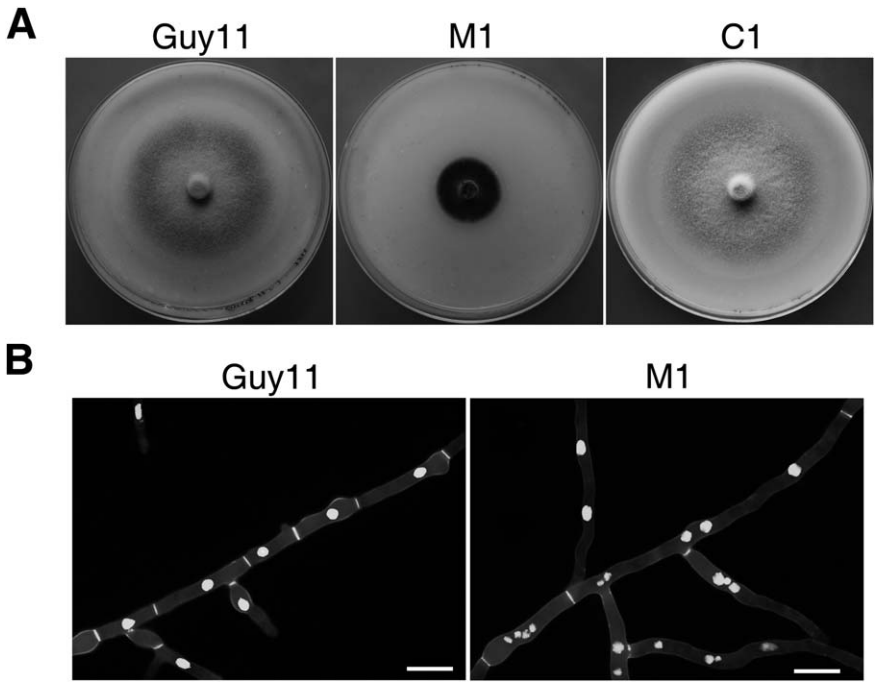

Fig. 1 Growth and nuclear distribution defects of the *Mocdc14* mutant. (A) Ten-day-old oatmeal cultures of the wild-type (Guy11), *Mocdc14* mutant (M1) and *Mocdc14*/*MoCDC14* complemented transformant (C1). (B) Hyphae of transformants of Guy11 and *Mocdc14* mutant expressing the H1-GFP construct were stained with calcofluor white (CFW) and examined by epifluorescence microscopy. Bar, 10 μm.

of mitosis had occurred in the germ tube, and the other moved into developing appressoria (Saunders *et al.*, 2010b). Each wild-type appressorium contained a single nucleus (Fig. 3B). However, approximately 10.4 ± 3.0% of the appressoria formed by the *Mocdc14* mutant had two or more nuclei (Fig. 3B). Therefore, deletion of *MoCDC14* affects mitotic division and cytokinesis (septation) during appressorium formation in *M. oryzae*.

MoCDC14 is also important for the delimitation of appressoria at hyphal tips

In addition to the formation of appressoria at the tips of germ tubes, *M. oryzae* also forms appressorium-like structures at hyphal tips (Kong *et al.*, 2013). On artificial hydrophobic surfaces, hyphal tips of the *Mocdc14* mutant still developed appressorium-like structures. However, fragments of hyphae were often delimited and melanized together with appressorium-like structures in the *Mocdc14* mutant (Fig. 4A). Unlike the wild-type, septation in the mutant occurred far away from the neck of appressorium-like structures along the hyphae. Therefore, similar to its septation defects during appressorium formation on germ tubes, the *Mocdc14* mutant was defective in the formation of the septum to delimit appressorium-like structures at the hyphal tips (Fig. 4B). *MoCDC14* must also be important for the formation of the septum delimiting the appressorium-like structures on hyphae.

We also examined the number of nuclei in appressorium-like structures formed at the hyphal tips of transformants expressing the H1-GFP construct. Like the wild-type, the *Mocdc14* mutant had a single nucleus in appressorium-like structures (Fig. 4C).

MoCDC14 is important for plant infection

To determine the effect of *MoCDC14* deletion on virulence, 2-week-old seedlings of rice cultivar CO-39 were used for spray infection assays. At 7 days post-inoculation (dpi), numerous typical blast lesions were observed on leaves inoculated with Guy11 (Fig. 5A). Under the same conditions, 63.0 ± 6.4% of the leaves sprayed with the *Mocdc14* mutant had no typical blast lesions. Although some leaves had rare small black spots, blast lesions with extensive necrotic zones were never observed on leaves inoculated with the *Mocdc14* mutant (Fig. 5A), indicating that *MoCDC14* plays a critical role in plant infection and lesion development.

MoCDC14 is important for appressorium penetration and invasive growth

In penetration assays with onion epidermal cells, whereas 67.3 ± 4.2% of Guy11 appressoria penetrated and formed invasive hyphae by 72 hpi, only 5.2 ± 3.6% of the appressoria formed by the *Mocdc14* mutant penetrated onion epidermal cells (Fig. 5B). The majority of *Mocdc14* appressoria appeared to be melanized, together with parts of or entire germ tubes on onion epidermis, and failed to penetrate underlying plant cells. Furthermore, in comparison with the wild-type, invasive hyphae formed by the *Mocdc14* mutant in rare onion epidermal cells penetrated by mutant appressoria were narrower and less bulbous (Fig. 5B).

Similar results were obtained in penetration assays with barley epidermal cells. Whereas 74.6 ± 5.1% of Guy11 appressoria penetrated and formed invasive hyphae by 48 hpi, only 9.9 ± 3.2% of the appressoria formed by the *Mocdc14* mutant

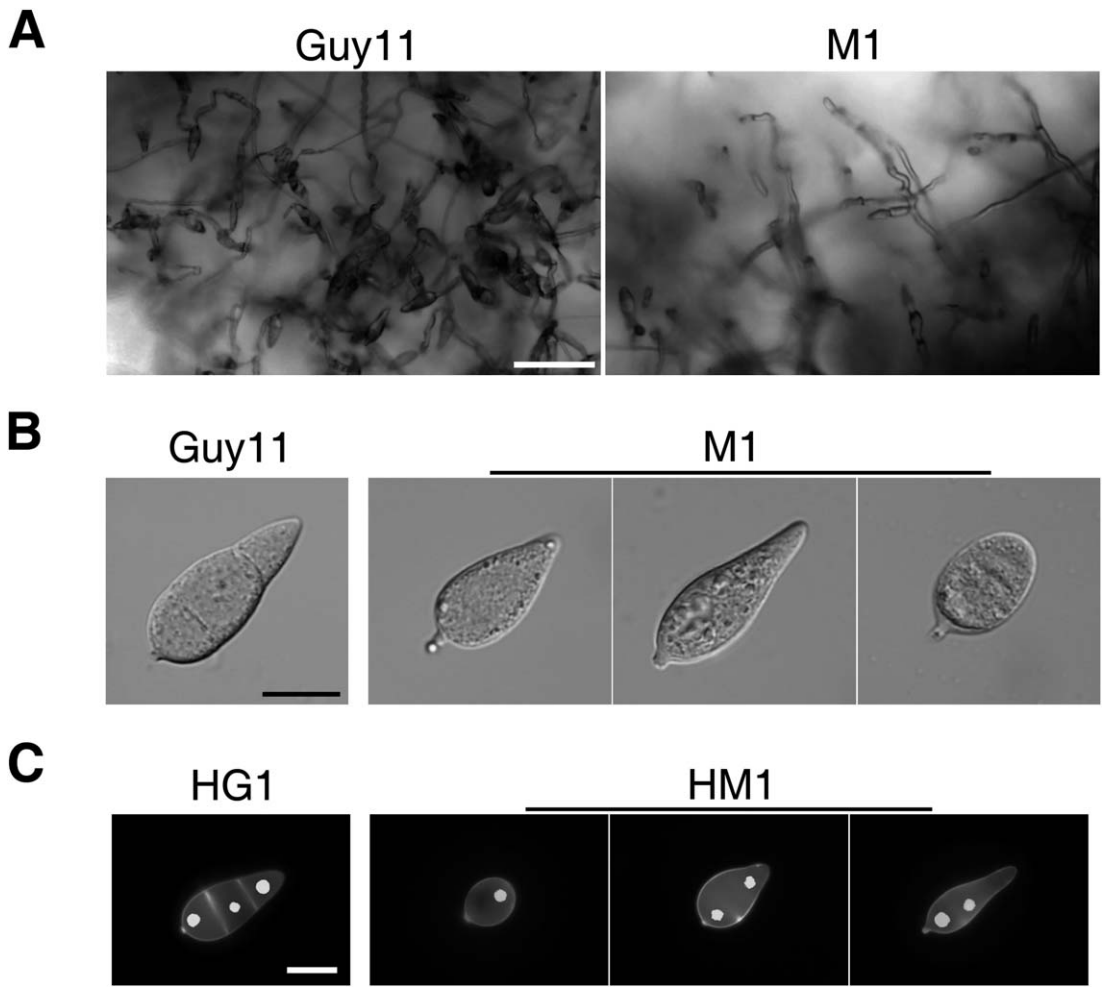

Fig. 2 Defects of the *Mocdc14* mutant in conidiogenesis. (A) Ten-day-old oatmeal cultures of the wild-type (Guy11) and *Mocdc14* mutant (M1) were examined for conidia and conidiophores under a dissection microscope. Bar, 50 μm. (B) Conidia of Guy11 and *Mocdc14* mutant were examined by differential interference contrast (DIC) microscopy. Bar, 10 μm. (C) Conidia of transformants of Guy11 (HG1) and *Mocdc14* mutant (HM1) expressing the H1-GFP construct were stained with calcofluor white (CFW) and examined by epifluorescence microscopy. Bar, 10 μm.

penetrated barley epidermal cells (Fig. 5C). The *Mocdc14* mutant also showed delayed spread to neighbouring cells. By 48 hpi, wild-type invasive hyphae had spread from the penetrated cells to neighbouring barley epidermal cells. Under the same conditions, invasive hyphae of the mutant were limited to the penetrated cell (Fig. 5C). These results indicate that the *Mocdc14* mutant is defective in penetration and infectious growth after penetration.

MoCDC14 is required for appressorium-like structure-mediated penetration and infection

We also conducted infection assays with culture blocks (Liu *et al.*, 2010) to determine the effect of *MoCDC14* deletion on penetration by appressorium-like structures formed at hyphal tips. On intact barley leaves inoculated with the *Mocdc14* mutant, only limited necrosis was observed directly beneath culture blocks (Fig. 6A). On wounded leaves, the *Mocdc14* mutant also caused only limited blast lesions outside the wounding sites (Fig. 6A). Under the same conditions, Guy11 caused extensive necrosis on both intact and wounded leaves surrounding the inoculation sites (Fig. 6A).

In barley epidermal cell penetration assays with culture blocks, whereas the wild-type penetrated into host cells through appressorium-like structures and developed invasive hyphae by 48 hpi (Fig. 6B), the majority of the appressorium-like structures formed by *Mocdc14* failed to penetrate under the same conditions. Even at 72 hpi, only less than 2% of the appressorium-like structures formed by the *Mocdc14* mutant were able to penetrate and showed limited invasive growth in the penetrated cells. Under the same conditions, Guy11 showed extensive invasive growth in the initial penetrated and neighbouring cells (Fig. 6B). These results indicate that the *Mocdc14* mutant is defective in

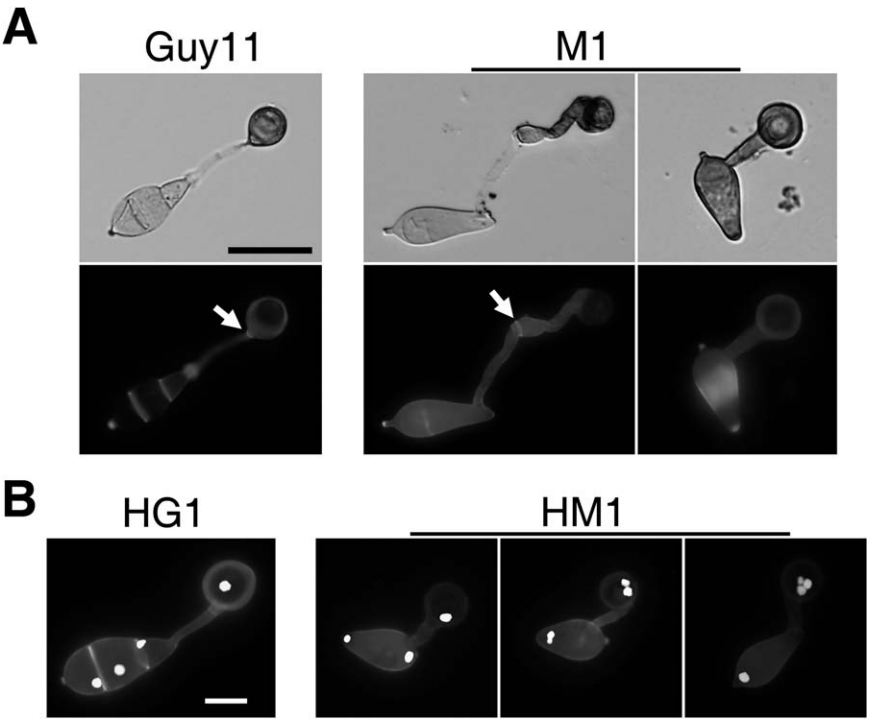

Fig. 3 Appressorium formation assays with conidia of the *Mocdc14* mutant. (A) Conidia of the wild-type (Guy11) and *Mocdc14* mutant (M1) were incubated on hydrophobic surfaces for 24 h and examined for appressorium formation after staining with calcofluor white (CFW). Septa in the germ tubes are marked with arrows. Bar, 20 μm. (B) Appressoria formed by the H1-GFP transformants of Guy11 (HG1) and *Mocdc14* (HM1) at 12 h were stained with CFW and examined by epifluorescence microscopy. Bar, 10 μm.

penetration by appressorium-like structures and *MoCDC14* is also important for invasive growth after penetration.

Complementation and localization of *MoCdc14*

For complementation assays, the *MoCDC14*-GFP fusion construct was generated by the yeast gap repair approach (Zhou *et al.*, 2011a) and transformed into the *Mocdc14* deletion mutant. The resulting *Mocdc14*/*MoCDC14*-GFP transformant C1 (Table 1) showed normal growth (Fig. 1A), conidiation (Table 2) and plant infection (Figs 5A and 6A). When examined by epifluorescence microscopy, green fluorescent protein (GFP) signals were found mainly in the nucleus in conidia and hyphae of transformant C1 (Fig. 7). Each nucleus had one bright spot probably caused by the localization of MoCdc14-GFP proteins to the spindle pole body (SPB). These results indicate that the expression of *MoCDC14*-GFP complements the *Mocdc14* mutant and MoCdc14 is localized to the nucleus.

Mocdc14 mutants show altered mobilization of glycogens

Defects in conidium morphology and appressorium melanization may affect appressorium turgor generation. To test this hypothesis, we assayed the mobilization and degradation of glycogen stored in conidia. Like the wild-type, the *Mocdc14* mutant had abundant glycogen in conidia (Fig. 8A). However, the mobilization of glycogen to developing appressoria was notably delayed in the *Mocdc14* mutant after incubation on hydrophobic surfaces for 8 h (Fig. 8A). At 24 h, a significantly higher percentage of mutant conidia still contained glycogen (Fig. 8B). At 48 h, many appressoria formed by the *Mocdc14* mutant still contained glycogen (Fig. 8C). These data indicate that the mobilization and degradation of glycogen are affected by the deletion of *MoCDC14*.

Deletion of *MoCDC14* reduces the expression levels of *CON1* and *CON7*

Because the *Mocdc14* mutant produced morphologically abnormal conidia and showed significantly reduced conidiation, we assayed the expression of several genes that are known to be important for conidiogenesis in *M. oryzae*, including *CON1*, *CON2*, *CON7*, *COM1* and *HTF1* (Liu *et al.*, 2010; Shi and Leung, 1995; Shi *et al.*, 1998; Yang *et al.*, 2010), using RNA isolated from 7-day-old OTA cultures incubated at 25°C under fluorescent light. Whereas the expression of *CON1*, *CON2*, *CON7* and *COM1* was down-regulated, the expression of *HTF1* was up-regulated in the *Mocdc14* mutant (Fig. S2, see Supporting Information). Nevertheless, in comparison with Guy11, only the expression levels of *CON1* and *CON7* were reduced over two-fold in the *Mocdc14* mutant (Fig. S2). Reduced expression of *CON1* and *CON7* may contribute to the conidiation defects of the mutant, because these genes are important for the development of conidiophores and normal conidium morphology (Odenbach *et al.*, 2007; Shi and Leung, 1995; Zhou *et al.*, 2009).

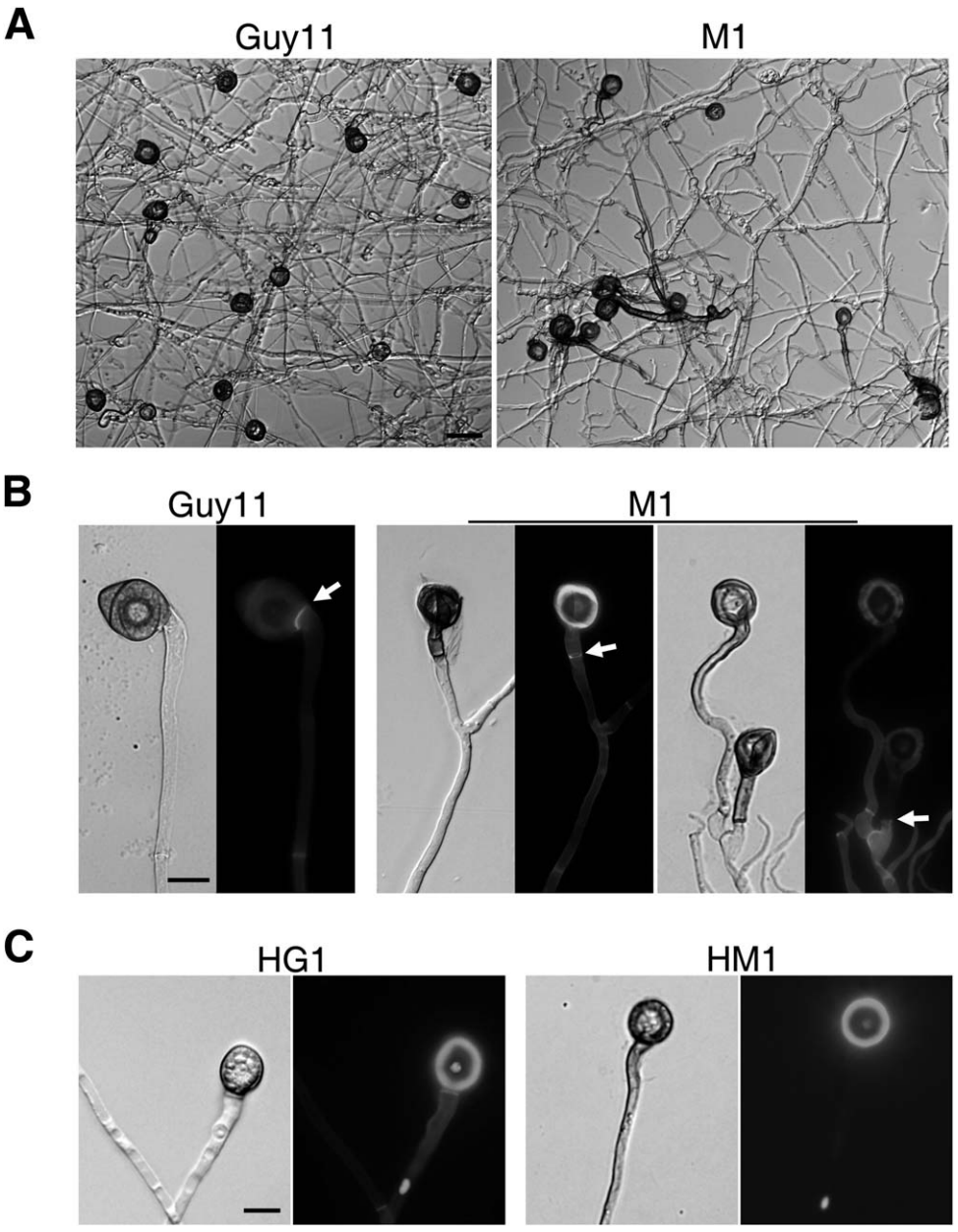

Fig. 4 Formation of appressorium-like structures at hyphal tips. (A) Appressorium-like structures formed on glass cover slips by hyphal tips of Guy11 and *Mocdc14* mutant M1. Bar, 20 μm. (B) Appressorium-like structures formed at hyphal tips were stained with calcofluor white (CFW) and examined by differential interference contrast (DIC) (left) and epifluorescence (right) microscopy. Septa are marked with arrows. Bar, 10 μm. (C) Hyphae and appressorium-like structures formed by the H1-GFP transformants of Guy11 (HG1) and *Mocdc14* (HM1) were examined by DIC and epifluorescence microscopy. Bar, 10 μm.

DISCUSSION

The Cdc14 protein phosphatases are well conserved in fungi for the promotion of mitotic exit and cytokinesis by dephosphorylation of their substrates at sites phosphorylated by CDKs (Bloom *et al.*, 2011; Chen *et al.*, 2008). However, whether or not the single-copy *CDC14* gene is essential for growth varies among different fungi. In *S. cerevisiae*, *CDC14* is an essential gene, but its orthologue in *Sc. pombe* is not. In *M. oryzae*, *MoCDC14* encodes a typical Cdc14 phosphatase protein, with the HCX5R catalytic site sequence (Mocciaro and Schiebel, 2010). The *Mocdc14* mutant is viable, but shows a significantly reduced growth rate, which is similar to the *cdc14* mutants in *F. graminearum* and *B. bassiana* (Li *et al.*, 2015; Wang *et al.*, 2013). However, deletion of the *CDC14* orthologue produces no obvious defects in growth rate in *A. nidulans* (Son and Osmani, 2009) or *C. albicans* (Clemente-Blanco *et al.*, 2006). In *M. oryzae*, in addition to a reduced growth rate, the *Mocdc14* mutant produces colonies with enhanced pigmentation and reduced aerial hyphae. Vegetative hyphae show fewer and unevenly distributed septa and individual hyphal compartments often contain multiple nuclei. Therefore, it is likely that deletion of *MoCDC14* affects cytokinesis and nuclear distribution in vegetative hyphae, which is similar to the defects of the

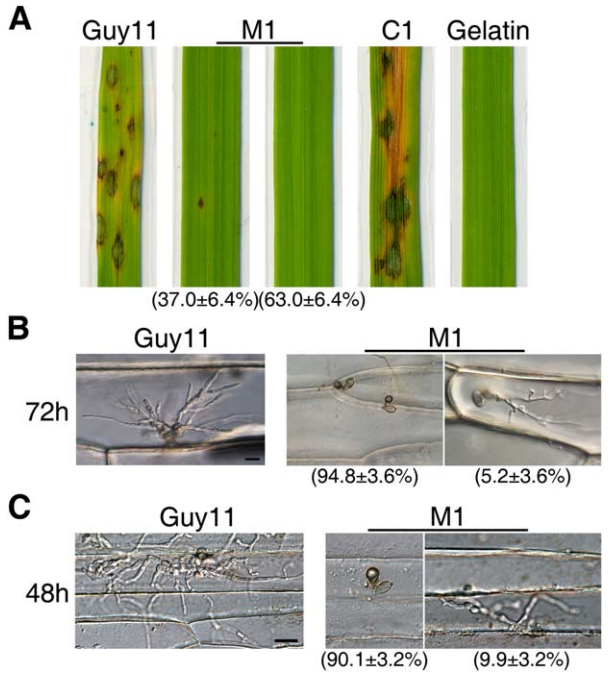

Fig. 5 Infection and penetration assays with conidia. (A) Leaves of 2-week-old rice seedlings were sprayed with conidia of Guy11, the *Mocdc14* deletion mutant M1 and the complemented transformant C1. Inoculation with gelatin solution was used as the negative control. (B) Onion epidermal cells inoculated with conidia of Guy11 and mutant M1 were examined at 72 h post-inoculation (hpi). Only 5.2 ± 3.6% of the appressoria formed by the *Mocdc14* mutant penetrated onion cells. (C) Barley epidermal cells inoculated with conidia from Guy11 and the mutant M1 were examined at 48 hpi. Only 9.9 ± 3.2% of the appressoria formed by the *Mocdc14* mutant penetrated barley cells. Bars, 20 μm.

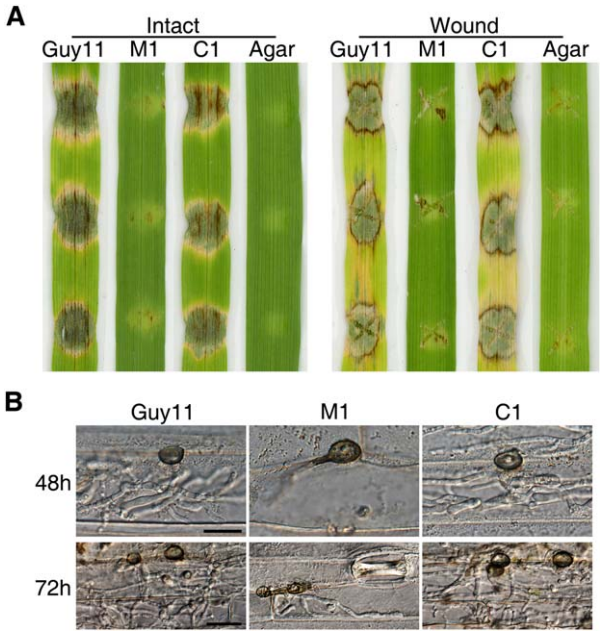

Fig. 6 Infection and penetration assays with culture blocks. (A) Intact and wounded barley leaves were inoculated with culture blocks of the wild-type Guy11, *Mocdc14* deletion mutant M1 and *Mocdc14/MoCDC14* complemented strain C1. Inoculation with oatmeal agar blocks (Agar) was used as the negative control. Typical leaves were photographed at 5 days post-inoculation (dpi). (B) Barley leaves inoculated with hyphal blocks of Guy11, M1 and C1 were examined for invasive hyphae at 48 and 72 h post-inoculation (hpi). Bars, 20 μm.

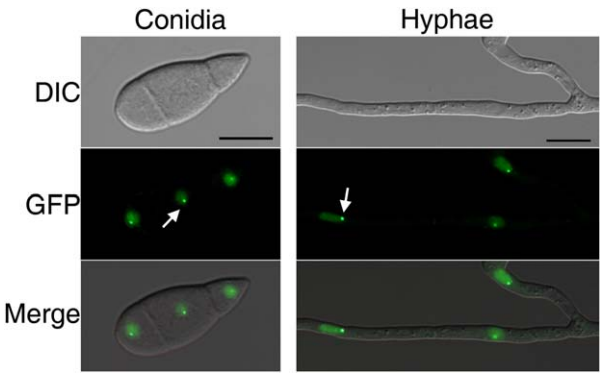

Fig. 7 Subcellular localization of the MoCdc14-GFP fusion proteins. Conidia and vegetative hyphae of the *Mocdc14/MoCDC14*-GFP transformant C1 were examined by differential interference contrast (DIC) and epifluorescence microscopy. Arrows point to the putative spindle pole body (SPB) as spots with stronger green fluorescent protein (GFP) signals. Bars, 20 μm.

Fgcdc14 mutant in *F. graminearum* (Li *et al.*, 2015). MoCdc14 may also be important for cell division and septum formation by counteracting CDK phosphorylation on its substrate in *M. oryzae* during vegetative growth.

The *Mocdc14* mutant shows reduced conidiation and the majority of mutant conidia contain only one or two nuclei without a septum or with only one incomplete septum. In the entomopathogenic fungus *B. bassiana*, the Cdc14 orthologue also acts as a positive regulator of asexual development (Wang *et al.*, 2013, 2016). In addition to reduced conidiation, the *Mocdc14* mutant is also defective in conidium morphology. In *M. oryzae*, young conidia formed at the tip of conidiophores are single-celled and contain a single nucleus. Mature three-celled conidia with uni-nucleate compartments are formed by two asymmetric mitotic divisions and septation (Liu *et al.*, 2010). The *Mocdc14* mutant may be defective in septation after the first asymmetrical division, which results in blocking of the second asymmetrical nuclear division in developing conidia. In *F. graminearum*, conidia normally have more than five septa and uni-nucleate compartments. The conidia of the *Fgcdc14* mutant show reduced septum formation and an increased number of nuclei per conidial compartment (Li *et al.*, 2015). Nevertheless, the majority of *Fgcdc14* mutant conidia still contain three or more septa. It seems that *CDC14* plays a more critical role in nuclear division and septum formation in *M. oryzae* than in *F. graminearum* during conidiogenesis.

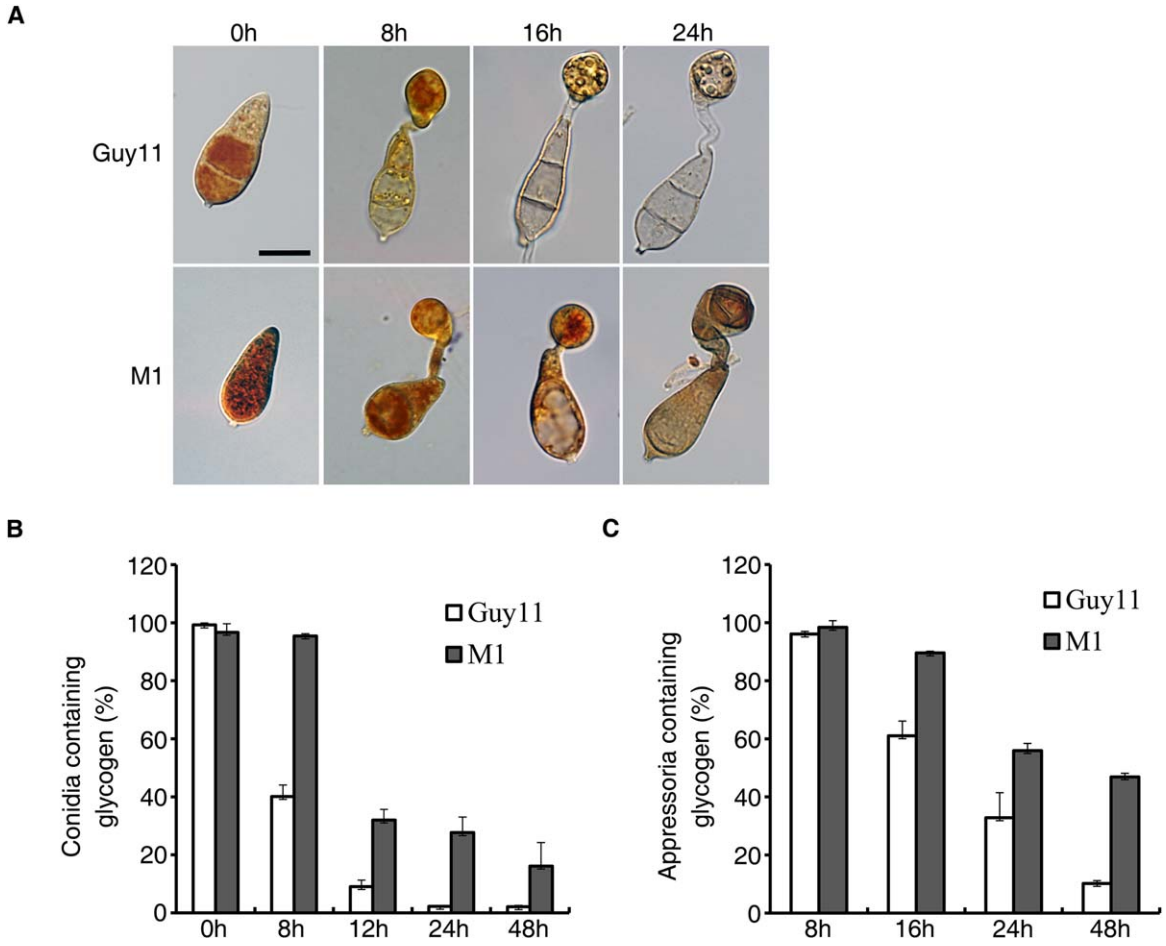

Fig. 8 Mobilization and degradation of glycogens during appressorium morphogenesis. (A) Conidia of Guy11 and *Mocdc14* mutant M1 were incubated on hydrophobic surfaces for the indicated time and examined for glycogens (yellowish-brown deposits) after staining with iodine. Bar, 10 μm. (B) Percentage of conidia containing glycogen at each time point. (C) Percentage of appressoria containing glycogen at each time point. The *Mocdc14* mutant was defective in the mobilization of glycogen from conidia to developing appressoria and glycogen degradation in appressoria.

In *S. cerevisiae*, temporal control of actin ring assembly by CDK and Cdc14 may help to ensure that cytokinesis onset occurs after nuclear division is complete (Miller *et al.*, 2015). In *S. pombe*, Clp1 regulates entry into mitosis and septum formation (Trautmann and McCollum, 2005) and interacts with the contractile ring (CR) scaffold protein Mid1 for cytokinesis (Clifford *et al.*, 2008). In *C. albicans*, deletion of *CaCDC14* does not interfere with cytokinesis, but results in defects in cell separation (Clemente-Blanco *et al.*, 2006). Unlike the unicellular ascomycete species, septation in *M. oryzae* does not result in the cleavage of adjacent cells in vegetative hyphae and conidia. Deletion of *MoCDC14* does not abolish septum formation and mitosis, although nuclear distribution and septation become irregular. The *Mocdc14* mutant still produces normal hyphae with unevenly distributed septa, but fails to produce normal three-celled, uni-nucleate conidia with three nuclei. These results indicate that MoCdc14 may have slightly different functions in septation during hyphal growth and conidiogenesis. In *M. oryzae*, nuclear division mainly occurs in the hyphal tip compartment, but the formation of three-celled conidia requires two asymmetrical mitotic divisions followed by cytokinesis. The defects of the *Mocdc14* mutant in the completion of septation after the first asymmetrical mitotic division may affect the distinct identity of the two nuclei in developing conidia, which may block the second mitotic division of the nucleus in the tip compartment.

In the rice blast fungus, appressorium morphogenesis is regulated by the cell cycle (Saunders *et al.*, 2010a; Wilson and Talbot, 2009). In the wild-type, after one mitosis has occurred in the germ tube, one daughter nucleus migrates to the developing appressoria, but the other returns to the germinating conidium. The nucleus which moves into the developing appressoria is assumed to be arrested in G1 and mature appressoria have a single nucleus. In the *Mocdc14* mutant, 10.4 ± 3.0% of 24-h appressoria had two or more nuclei. One possibility is that the nucleus

entering the developing appressorium may continue to divide without cytokinesis as a result of defects in mitotic exit associated with *MoCDC14* deletion. Because the *Mocdc14* mutant is defective in the formation of the septum that delimits the appressoria from the rest of the germ tubes, it is also possible that more than one nucleus migrates into the developing appressoria. Furthermore, when mature appressoria are melanized, conidia and germ tubes undergo autophagic cell death in the wild-type (Veneault-Fourrey *et al.*, 2006). However, conidial compartments often still contain nuclei after appressoria have been melanized in the *Mocdc14* mutant when appressorium-delimiting septa are not properly formed. Complete septation of appressoria from the rest of the germ tubes by the appressorium-delimiting septum may be a prerequisite for the triggering of autophagic cell death.

In *M. oryzae*, appressorium melanization is important for appressorium turgor generation (deJong *et al.*, 1997). The *Mocdc14* mutant still forms melanized appressoria on germ tubes, but parts of the germ tubes and sometimes conidia are also melanized. Similar defects in melanization have been observed in the *Mocdc14* mutant when assayed for the formation of appressorium-like structures at hyphal tips. Fragments of hyphae are often melanized together with appressorium-like structures, depending on the position of the septum that delimits the appressorium-like structures. These results indicate that the formation of these special septum-delimiting appressoria or appressorium-like structures from the rest of the germ tubes or hyphae plays a critical role in defining the boundary of melanin deposition. Enzymes involved in melanin biosynthesis may be only expressed or active after the completion of the appressorium-delimiting septum. In *M. oryzae*, deletion of the *MoAND1* gene, an orthologue of *A. nidulans* ApsA, results in septation defects in hyphae, but has no effect on the formation of appressoria and the appressorium-delimiting septum (Jeon *et al.*, 2014). An earlier study in *M. oryzae* with hydroxyl urea treatment and NimA mutations has also shown that the differentiation of appressoria requires a cytokinetic event that is distinct from cell divisions within hyphae (Saunders *et al.*, 2010a). Our studies show that MoCdc14 is involved in deciding the occurrence and position of this special septum during appressorium formation.

In infection assays, the *Mocdc14* mutant is almost non-pathogenic. The defects of the *Mocdc14* mutant in plant infection can be related directly to its defects in growth and appressorium morphogenesis. In addition, the *Mocdc14* mutant is defective in glycogen mobilization and degradation, which is important for appressorium turgor generation and penetration. However, mutants with deletion of *MoCDC14* are also defective in infection through wounding, suggesting a critical role of MoCdc14 during invasive growth. In rare plant cells penetrated by the *Mocdc14* mutant, only limited growth of invasive hyphae is seen and the mutant fails to spread into neighbouring cells. Rare invasive hyphae formed by the mutant inside plant cells are less branching than those of the wild-type. In *M. oryzae*, bulbous invasive hyphae are considered to show pseudohyphal-like growth in plant cells (Kankanala *et al.*, 2007; Yi and Valent, 2013). It is possible that MoCdc14 is important for constriction or septum formation in invasive hyphae with pseudohyphal growth.

EXPERIMENTAL PROCEDURES

Strains and culture conditions

The *M. oryzae* wild-type strain Guy11 and mutants used in this study (Table 1) were cultured on OTA or complete medium (CM) plates at 25°C as described previously (Xu and Hamer, 1996; Zhou *et al.*, 2011b). For fungal transformation, protoplast preparation and polyethylene glycol (PEG)-mediated transformation of *M. oryzae* were performed as described previously (Park *et al.*, 2006). Hygromycin B (Calbiochem, La Jolla, CA, USA) and geneticin (MP Biochemicals, Santa Ana, CA, USA) were added to final concentrations of 300 and 500 μg/mL, respectively, for transformant selection. For DNA isolation, vegetative hyphae were harvested from 2-day-old liquid CM cultures (Zhao *et al.*, 2005). Measurements of growth rate and conidiation were performed as described previously (Li *et al.*, 2004; Park *et al.*, 2004).

Generation of the *Mocdc14* deletion mutants

To delete the *MoCDC14* gene, the double-joint PCR method (Yu *et al.*, 2004) was used to generate the *CDC14* gene replacement construct (Fig. S1A). The 1080-bp upstream and 992-bp downstream flanking sequences of *MoCDC14* were amplified with the primer pairs C1F/C2R and C3F/C4R (Table S1, see Supporting Information), respectively, and ligated with the *hph* cassette amplified with primers HYG/F and HYG/R from pCB1003 (Carroll *et al.*, 1994). The products of double-joint PCR were amplified with primers CCF and CCR (Table S1) and transformed into protoplasts of Guy11. Hygromycin-resistant transformants were screened by PCR and putative gene replacement mutants were confirmed by Southern blot analysis.

Generation of the *MoCDC14*-GFP fusion construct

To generate the *MoCDC14*-GFP fusion construct, the entire *MoCDC14* gene, including its promoter region, was amplified with primers 14GFP-F and 14GFP-R (Table S1) and cloned into *Xho*I-digested pFL2 (Zhou *et al.*, 2011b) by the yeast gap repair approach (Zhou *et al.*, 2011a). The *MoCDC14*-GFP fusion construct recovered from yeast Trp+ transformants was confirmed by sequencing analysis and transformed into the *Mocdc14* deletion mutant M1 (Table 1). Geneticin-resistant transformants expressing the *MoCDC14*-GFP construct were verified by PCR and examined for GFP signals.

Appressorium formation, penetration and plant infection assays

Conidia were harvested from 10-day-old OTA cultures and resuspended to a concentration of 5×10^4 conidia/mL (Zhou *et al.*, 2012). Appressorium formation by germ tubes on artificial surfaces was assayed as described

previously (Wang *et al.*, 2015; Zhou *et al.*, 2011b). Appressorial penetration and invasive hyphal development were assayed with barley and onion epidermal cells (Chi *et al.*, 2009; Kong *et al.*, 2013). For spray infection assays, conidia were adjusted to 1×10^5 conidia/mL in 0.25% gelatin and used for inoculation of 14-day-old seedlings of rice cultivar CO-39 (Kong *et al.*, 2013).

Assays for the formation and penetration of appressorium-like structures

Assays for appressorium-like structures at hyphal tips were performed as described previously (Liu *et al.*, 2010). For infection assays with culture blocks, the second leaves of 8-day-old seedlings of barley cultivar Golden Promise were inoculated with 1–2-mm^2 blocks of 10-day-old OTA cultures as described previously (Liu *et al.*, 2010). Penetration, invasive growth and lesion development were examined as described previously (Liu *et al.*, 2010; Yang *et al.*, 2010).

Cell wall and glycogen staining

The cell wall was stained with 10 μg/mL CFW (Sigma-Aldrich, St. Louis, MO, USA), as described previously (Zhou *et al.*, 2011b), to visualize septa in hyphae and during appressorium formation. Conidia incubated at room temperature on hydrophobic surfaces were stained for glycogens with 60 mg of KI and 10 mg of I_2 (Thines *et al.*, 2000; Zhang *et al.*, 2014). Glycogen mobilization and degradation during appressorium formation were examined with an Olympus BX51 epifluorescence microscope (Olympus Corporation, Tokyo, Japan).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis

Hyphae harvested from 7-day-old OTA cultures of the wild-type strain Guy11 and *Mocdc14* mutant M1 were used for RNA isolation with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After treatment with the DNA-free kit (Promega, Madison, WI, USA), purified RNA samples were used for cDNA synthesis with the Fermentas first cDNA synthesis kit (Hanover, MD, USA). The resulting first cDNA was used for qRT-PCR assays as described previously (Ding *et al.*, 2010) with the primers listed in Table S1. Data from three biological replicates were used to estimate the relative expression levels of CON1, CON2, CON7, COM1 and HTF1 with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The *M. oryzae* actin gene MGG_03982.6 was used as the endogenous control for normalization.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 The *MoCDC14* gene replacement construct and deletion mutants. (A) The *MoCDC14* locus and gene replacement construct. The *MoCDC14* and *hph* genes are marked with white and black arrows, respectively. H, *Hind*III. (B) Southern blot analysis with the wild-type (Guy11) and *Mocdc14* transformants (M1, M49, M51 and M69). All the DNA samples were digested with *Hind*III. The blots were hybridized with probe A (left) amplified with primers C5F and C6R and probe B (right) amplified with H852 and H850.

Fig. S2 Assays of the expression levels of *CON1*, *CON2*, *CON7*, *COM1* and *HTF1* by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). For each gene, the relative expression level in Guy11 was set to unity. The mean and standard deviation were calculated using data from three independent replicates.

Table S1 Polymerase chain reaction (PCR) primers used in this study.