

# Characterization of *MoLDB1* Required for Vegetative Growth, Infection-Related Morphogenesis, and Pathogenicity in the Rice Blast Fungus *Magnaporthe oryzae*

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**An insertional mutagenesis screen in the rice blast fungus, *Magnaporthe oryzae*, identified a novel mutant, A2-12-3, which is defective in infection-related morphogenesis and pathogenicity. Analysis of the mutation confirmed an insertion into *MoLDB1*, which putatively encodes an 806-amino-acid protein with a predicted LIM binding domain. Targeted gene deletion mutants of *MoLDB1* were unable to produce asexual or sexual spores and were significantly impaired in vegetative growth and fungal virulence. The  $\Delta$ *moldb1* mutants also showed reduced expression of genes coding hydrophobic proteins (e.g. *MPG1* and *MHP1*), resulting in an easily wettable phenotype in vegetative culture. Moreover, the expression of four genes encoding LIM proteins predicted from the *M. oryzae* genome was significantly downregulated by deletion of *MoLDB1*. Analysis of an *M. oryzae* strain expressing a *MoLdb1*-green fluorescent protein gene fusion was consistent with the protein being nuclear localized. When considered together, *MoLdb1* appears to be involved in regulation of cell wall proteins, including hydrophobins and LIM proteins, and is essential for conidiation, sexual development, appressorium formation, and pathogenicity in *M. oryzae*.**

*Magnaporthe oryzae* is the causal agent of rice blast, the most serious disease of cultivated rice throughout the world (Ou 1985; Howard and Valent 1996). In severe cases, the fungus can cause death of rice seedlings whereas, in old plants, it can prevent grain filling or destroy the grain-bearing structures of the plant (Howard and Valent 1996; Tucker and Talbot 2001; Talbot 2003). In the field, rice blast infections are mainly initiated by asexual spores (conidia) of the fungus. Conidia of the rice blast fungus adhere tightly to the hydrophobic leaf cuticle by means of an adhesive carried in the tip (Hamer et al. 1988). Release of the adhesive and attachment to the cuticle leads to

rapid germination and formation of a dome-shaped infection structure at the tip of a short germ tube, called an appressorium, which mechanically penetrates the rice cuticle by means of a narrow penetration peg (Howard et al. 1991). Soon after cuticle penetration, the fungus further differentiates into bulbous infection hyphae within infected host plant cells. Recently, cytological evidence has demonstrated that invasive hyphae of *M. oryzae* are surrounded by the invaginated plant plasma membrane and appear to move from cell to cell by means of plasmodesmata (Kankanala et al. 2007; Wilson and Talbot 2009). Necrotic disease lesions are developed within 4 to 5 days and release conidia to reinitiate successive rounds of plant infection. Each disease lesion from a susceptible host can give rise to more than 20,000 conidia over several days, serving as a source for secondary dispersal of the disease (Barksdale and Asai 1961; Ebbole 2007). Therefore, conidia of the fungus play a crucial role in fulfilling the disease cycle and causing epidemics in fields.

*M. oryzae* has been used as a model fungal pathogen for understanding the molecular basis of plant–fungus interactions due to its economic importance and genetic tractability (Valent 1990; Talbot 1995; Howard and Valent 1996; Talbot 2003; Ebbole 2007; Wilson and Talbot 2009). A detailed understanding of the molecular basis of plant infection by *M. oryzae* will benefit the development of new strategies to control the disease, and considerable progress has been made in understanding signaling pathways that regulate infection-related development by the fungus. It is now clear, for instance, that appressorium formation of *M. oryzae* requires the cAMP-response pathway which responds to inductive signals from the rice leaf, including surface hydrophobicity and wax monomers from the plant (Lee and Dean 1993; Mitchell and Dean 1995; Choi and Dean 1997; Xu et al. 1997; Adachi and Hamer 1998). A mitogen-activated protein kinase (MAPK) pathway, involving the PMK1 MAPK, is also required for the differentiation of appressoria (Xu and Hamer 1996; Zhao et al. 2005; Park et al. 2006; Zhao and Xu 2007). The *M. oryzae* appressorium is essential for rice blast disease and, therefore, many researchers have focused on investigating the molecular basis of appressorium development. Enormous appressorium turgor is used to generate physical force to breach the plant cuticle (De Jong et al. 1997; Wilson and Talbot 2009) and, consequently, melanin biosynthesis mutants which are unable to generate suf-

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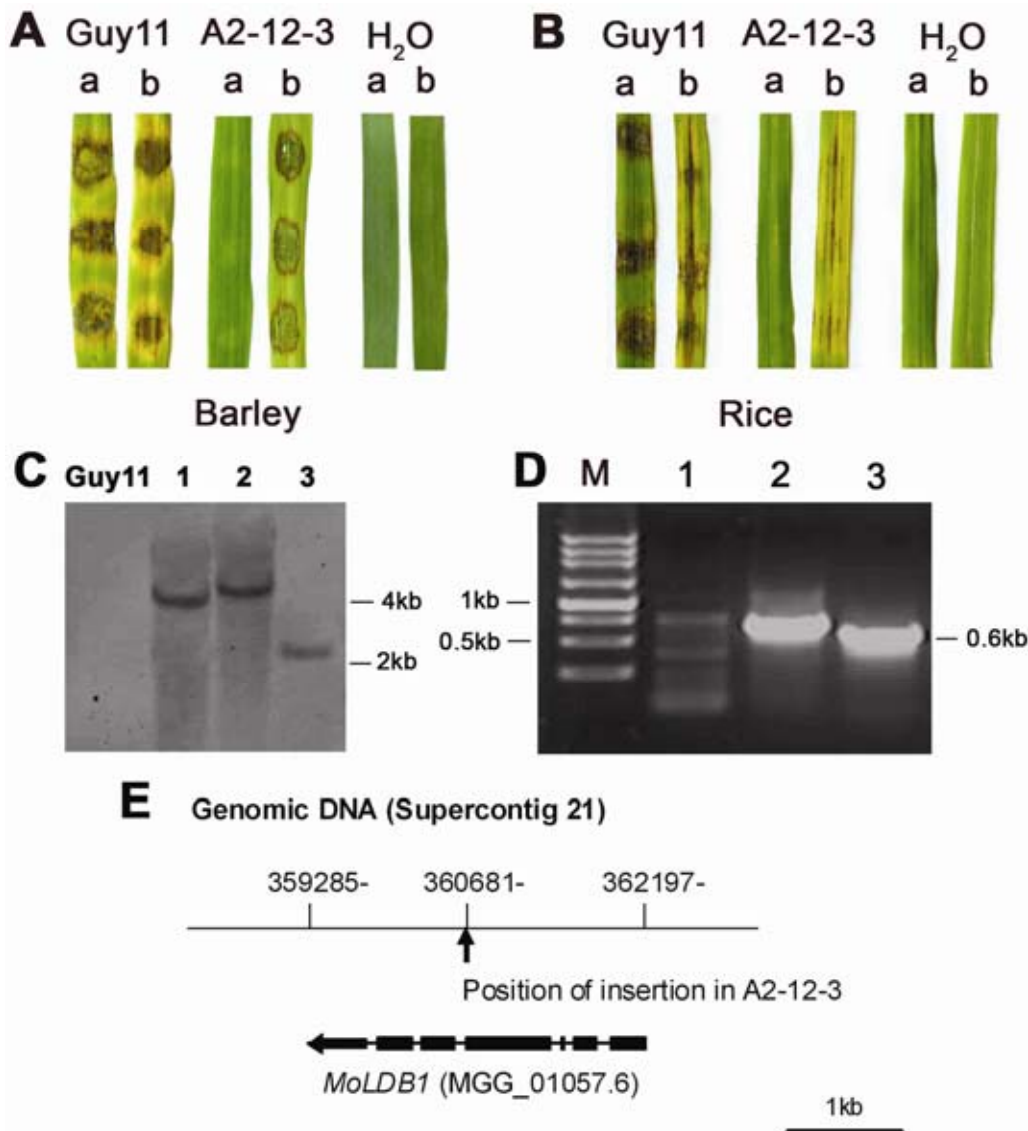
\*The e-Xtra logo stands for “electronic extra” and indicates that Figures 4 and 7 appear in color online.

ficient internal turgor are nonpathogenic (Chumley and Valent 1990). Hydrostatic turgor in appressoria is generated by the accumulation of glycerol (De Jong et al. 1997) derived from storage compounds in the spore, such as glycogen and lipids. Peroxisome biogenesis and fatty acid  $\beta$ -oxidation are essential for appressorium turgor generation (Ramos-Pamplona and Naqvi 2006; Wang et al. 2005, 2007). Cell wall differentiation of the appressorium involves an oxidative burst during the development of the infection cell. Deletion of two NADPH oxidase-encoding genes, *NOX1* and *NOX2*, independently affected appressorium function, preventing successful plant infection (Egan et al. 2007).

Recently, insertional mutagenesis has been used successfully to identify genes involved in pathogenicity (Shi and Leung 1995; Sweigard et al. 1998; Balhadère et al. 1999; Clergeot et al. 2001). The *PTH11* gene encoding a novel transmembrane

protein, for example, was first identified as an insertion mutant that did not form appressoria on inductive surfaces (Sweigard et al. 1998; DeZwaan et al. 1999), whereas *PDE1*, a P-type ATPase encoding gene, was also identified by restriction enzyme-mediated DNA integration mutagenesis (Balhadère and Talbot 2001). More recently, *Agrobacterium tumefaciens*-mediated transformation (ATMT) has been developed as a large-scale gene-tagging method in *M. oryzae* (Choi et al. 2007; Gupta and Chattoo 2007; Jeon et al. 2007; Meng et al. 2007).

We have carried out a large-scale ATMT screen of *M. oryzae* to identify new genes necessary for infection-related morphogenesis and pathogenicity and have generated a mutant library containing more than 5,000 hygromycin-resistant transformants of *M. oryzae*. Very recently, we reported that MoRic8, a novel component of G-protein signaling, is required for regulating multiple stages of infection-related morphogenesis in *M. oryzae*



**Fig. 1.** Pathogenicity test of A2-12-3 mutant by cut-leaf assay and integration of exogenous T-DNA in the mutant. **A** and **B**, Barley and rice leaf segments were inoculated with the mycelium from Guy11 and A2-12-3; H<sub>2</sub>O was used as the control; a = unwounded leaf and b = abraded leaf. **C**, Southern blotting showing that a single T-DNA integration event occurred in the genome of A2-12-3. Lane 1, Guy11 genomic DNA was digested with *SalI*; lanes 2–4, A2-12-3 genomic DNA were digested with *SalI*, *EcoRV*, and *PstI*, respectively. The DNA gel was probed with a 1.4-kb hygromycin resistance gene cassette. **D**, Genomic DNA of A2-12-3 was digested with *EcoRI* and self-ligated; then, three rounds of polymerase chain reaction (PCR) amplifications were carried out with the primer pairs of LB1/RB1, LB2/RB2, and LB3/RB3. A 0.6-kb genomic DNA flanking the left site of the integration T-DNA was obtained. Lanes 1–3, PCR products amplified with the primer pairs LB1/RB1, LB2/RB2, and LB3/RB3, respectively; M, 250-bp DNA Ladder Marker (Takara). **E**, Position of the T-DNA insertion in A2-12-3 mutant and structure of the *MoLDB1* genomic locus. An arrow indicates the T-DNA insertion position in the *Magnaporthe* genome. Thick boxes represent the coding regions and the thin line joining these coding regions indicates the position of the introns.

(Li et al. 2010). Here, we present the identification and functional characterization of a novel pathogenicity-related gene of *M. oryzae*, *MoLDB1*, which encodes a predicted protein containing a LIM-binding domain. The name LIM is derived from the first three proteins in which the motif was found: Lin-11, Isl1, and Mec-3 (Freyd et al. 1990; Karlsson et al. 1990), which are transcriptional regulators of development (Agulnick et al. 1996). It has been suggested that LIM domain-binding proteins enhance transcriptional regulation by binding to LIM-domain proteins (Bach et al. 1997). Our experiments showed that deletion of *MoLDB1* results in complete inability to produce conidia and sexual spores. The *Δmolldb1* mutants were nonpathogenic in foliar infection assays and *MoLDB1* appears to play a regulatory role in cellular differentiation by the fungus to produce spores, sexual reproductive structures, and appressoria.

## RESULTS

### Identification of the T-DNA-tagged gene *MoLDB1* of *M. oryzae*.

Recently, we identified seven mutants impaired in the development of blast symptoms by T-DNA insertional mutagenesis (Li et al. 2010). One of the mutants, A2-12-3, was unable to sporulate and was also incapable of causing disease by inoculation of barley or rice leaf seedlings with hyphae (Fig. 1A and B). However, the mutant was still able to cause disease lesions

when the leaf surface was abraded before inoculation, although the disease severity was reduced compared with the isogenic wild-type strain, Guy11 (Fig. 1A and B). To investigate the T-DNA integration that had caused the mutation in A2-12-3, genomic DNA was digested with *Sall*, *EcoRV*, or *PstI*, and then probed with the 1.4-kb hygromycin-resistant gene cassette. DNA gel blot analysis showed that a single T-DNA integration event had occurred in the genome of A2-12-3 (Fig. 1C). To identify the T-DNA integration site in A2-12-3, genomic DNA was digested with *EcoRI*, and three rounds of polymerase chain reaction (PCR) amplification were carried out with the primer pairs LB1/RB1, LB2/RB2, and LB3/RB3 (Table 1) using self-ligated genomic DNA as template. A 0.6-kb genomic DNA flanking the left site of the integration event was obtained by PCR amplification and sequenced (Fig. 1D). The T-DNA insertion was found in the fourth exon of a predicted gene MGG\_01057.6 (GenBank XP\_368187) located on supercontig 21. The integration site was at position 360681-, which is 1,517 bp downstream of a predicted translational start site (Fig. 1E). To understand the T-DNA insertion event in detail, genomic DNA flanking the right border of the integrated T-DNA was also amplified and sequenced. Analysis of this sequence also demonstrated that the T-DNA was integrated in the same position of MGG\_01057.6 (Fig. 1E). We named the T-DNA tagged gene *MoLDB1* (for “*M. oryzae* LIM-domain-binding protein”), because it putatively encodes a predicted protein containing a LIM-binding domain.

To confirm the position and size of the introns of *MoLDB1*, the *MoLDB1* cDNA of the coding sequence was obtained by reverse-transcription (RT)-PCR with primers LDB-E-F/LDB-S-R (Table 1) and sequenced. Comparison of the cDNA and sequenced genomic DNA confirmed that *MoLDB1* has an open reading frame of 2,913 bp interrupted by six introns (81, 63, 94, 73, 76, and 105 bp, respectively) and putatively encodes an 806-amino-acid protein, which is identical to the protein sequence predicted by automated annotation of the *M. oryzae* genome sequence (ID: MGG\_01057.6; Broad Institute) (Fig. 1E). Putative homologues of *MoLDB1* were found in the genomes of related fungal species. *MoLdb1* showed 46.39 and 32.07% amino acid identity with *Neurospora crassa* NCU01543 and *Sclerotinia sclerotiorum* SS1G\_08183 (Fig. 2A), respectively. Phylogenetic analysis of the putative homologues of *MoLdb1* from some fungal species indicated that *MoLdb1* is most closely related to *N. crassa* NCU01543 and *S. sclerotiorum* SS1G\_08183 but is genetically distant to other homologues (Fig. 2B). Interestingly, *MoLdb1* seems to define a gene family found only in fungi. Moreover, homologues of the *MoLdb1*-encoding gene are present in the genomes of *Schizosaccharomyces pombe* and *S. japonicus* but not in *Saccharomyces cerevisiae*.

### *MoLDB1* is required for *M. oryzae* to cause rice blast disease.

To verify the nonpathogenic phenotype of A2-12-3 caused by the insertion mutation, we performed a targeted gene deletion of *MoLDB1* using the gene-replacement vector pLDB-LS-KO (Fig. 3A). Gene replacement events were analyzed by PCR amplification with primers 3LF and 3LR (Table 1) and Southern blot analysis performed (Fig. 3B). The resulting *Δmolldb1* null mutants, AK58 and AK66 (Table 2), were selected based on absence of hybridization to the deleted fragment probe (a PCR product amplified with the primers 3LF and 3LR using genomic DNA of the wild-type strain Guy11 as the template) and the presence of a single copy of the *HPH* cassette (Fig. 3B). One of the transformants resulting from ectopically integrated pLDB-LS-KO, ECT1, was used as a control strain (Fig. 3B).

**Table 1.** Primers used in this study

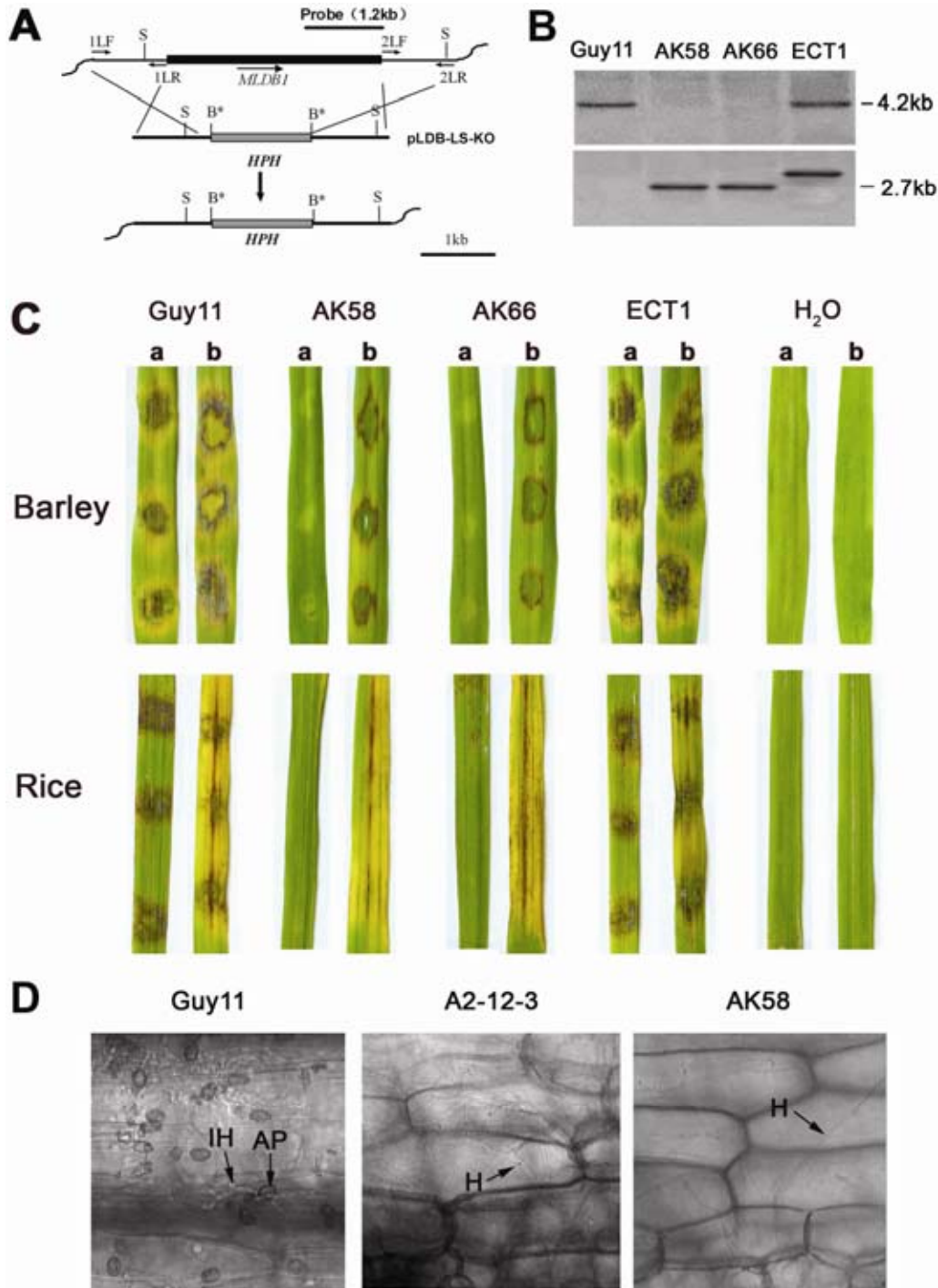
Name	Sequence (5'→3') <sup>a</sup>
LB1	TGCAA GGCGA TTAAG TTGGG TA
RB1	CCCTCCCAACAGTTGCGCAGCCT
LB2	GCCAG GGTTC TCCA GTCAC
RB2	GGCGAATGCTAGAGCAGCTT
LB3	CGTTG TAAAA CGTCG GCCAG
RB3	GCTTG GATCA GATTG TCGTT TCC
LDB-E-F	<u>GAATTC</u> ATGAGCATGGGACCTTCG
LDB-S-R	<u>GTCGACT</u> CATGACTGAGCTTGGG
1LF	GTGTCCGACAGCGACTACA
1LR	GAGGATCCATTGATTGGCGTGTCGATG
2LF	<u>CCGGATCC</u> TCATGATACGGTGAGGTCAG
2LR	CACAGGATCAAAGTAGCTGG
3LF	GGCCGTGTTTAGGTTTGTGTC
3LR	TCATGACTGAGCTTGGGGCC
HPH-BF	<u>CTGGATCC</u> TATTGAAGGAGCATTTTTGGGC
HPH-BR	<u>CTGGATCC</u> GCTCTTGTTCGGTCGGCATCTA
CF1	<u>GATCTAGACT</u> TGCCAGCTGTCCAGGCCGA
CR1	<u>ATAAGCTT</u> TGACTGAGCTTGGGGCCCGG
GF1	<u>ATAAGCTT</u> ATGTTGAGCAAGGGCGAGGAGC
GR1	<u>TGAAGCTT</u> GTGGAGATGTGGAGTGGGCGCTT
MPG-RT-F	GGTCTTCCGTCTCCATG
MPG-RT-R	TGTTGATCGGGATGTTCTTG
MHP-RT-F	CACCATCATCGCCACCATC
MHP-RT-R	CAGCACTGAGCAGAGCCGTA
10105-RT-F	CGGCAGCGGAGACTATGA
10105-RT-R	CGCAAATGTCGGTGAAGC
9134-RT-F	GCAGCGGAGCCTACAACAA
9134-RT-R	TCCAAGAACAGGGAGCAGACA
MoLDB-RT-F	GGCCGTGTTTAGGTTTGTGTC
MoLDB-RT-R	GCTGGCTTGGTGTCTCA
4377-RT-F	TTTGTCCGTTGAGGGTGT
4377-RT-R	TTGTCCGCATCTGTATCTT
4186-RT-F	CCACCGAACGCCATACCT
4186-RT-R	TTCTTGCCTTGCTGCTC
5738-RT-F	ATCAACGGCAGGAAAGAGTG
5738-RT-R	TTGTGGCAGGTGAAGAAGC
6198-RT-F	ATGCCTTCGCTCCAGAT
6198-RT-R	TACGCAGAGGTGCTTCCA
Tub-F	ACCCTCGAACGGAAAG
Tub-R	GACGAAGTGGATGCTACGC

<sup>a</sup> Introduced restriction sites are underlined. *EcoRI* = GAATCC, *Sall* = GTC GAC, *BamHI* = GGATCC, *XbaI* = TCTAGA, and *HindIII* = AAGCTT.



To assess the ability of *Δmoldb1* mutants to cause rice blast disease, mycelium of *Δmoldb1* null mutants AK58 and AK66 prepared in liquid complete medium (CM) culture was used to inoculate leaves of the susceptible barley cv. Golden Promise and rice cv. CO-39, respectively. Consistent with the original

analysis of A2-12-3, *Δmoldb1* mutants were nonpathogenic on both susceptible barley and rice leaves but were still able to cause some disease symptoms when leaf surfaces were abraded (Fig. 3C). Because of the lack of sporulation, we were unable to inoculate the hosts with a conidial suspension.



**Fig. 3.** Targeted gene replacement of *MoLDB1* and pathogenicity test of *Δmoldb1* mutants. **A**, Construction of the *MoLDB1* gene-replacement vector pLDB-LS-KO and targeted gene replacement of *MoLDB1*. S = *SalI* and B = *BamHI*; \* represents introduced restriction sites. **B**, *Δmoldb1* Mutants confirmed by Southern blot analysis. Genomic DNAs from the wild-type strain Guy11 and three transformants were digested with *SalI* and probed with a 1.2-kb fragment (top) amplified with the primers 3LF and 3LR and a 1.4-kb HPH cassette (bottom), respectively. Lane 1, Guy11; lanes 2 and 3, *Δmoldb1* mutants AK58 and AK66; lane 4, ectopic transformant ECT1. **C**, Pathogenicity test of *Δmoldb1* mutants. Barley and rice segments were inoculated with the mycelium from the wild-type strain Guy11, AK58 (*Δmoldb1*), AK66 (*Δmoldb1*), and ECT1 (ectopic transformant). H<sub>2</sub>O was used as the control; a = unwounded leaf and b = abraded leaf. The *Δmoldb1* mutants were nonpathogenic to susceptible barley and rice; however, they were partially pathogenic to abraded leaves. **D**, Appressorium formation of the wild-type strain Guy11 on barley leaf surface. Mycelium of the wild-type strain Guy11, A2-12-3, and AK58 was harvested from 48-h liquid complete medium cultures and inoculated on barley leaves. Appressorium formation was observed after 48 h of incubation at 25°C. Numerous appressoria were produced by Guy11; however, no appressoria were observed for the inoculation of A2-12-3 and AK58. AP = appressorium, IH = infectious hypha, and H = hypha.

Therefore, these results demonstrated that the nonpathogenic phenotype of the A2-12-3 mutant was caused by T-DNA integration and that *MoLDB1* is necessary for production of spores by *M. oryzae*.

To investigate whether the nonpathogenic phenotype of *Amoldb1* mutants on intact host leaves was caused by the defect in appressorium formation, we harvested mycelium of the *Amoldb1* mutant AK58 from liquid CM culture and placed it on barley leaf surfaces to induce appressorium formation. No appressorium-mediated penetration events were observed at 24 or even 48 h postinoculation with the *Amoldb1* mutant AK58. However, numerous appressoria were formed and penetration events occurred normally in the isogenic wild-type strain Guy11 (Fig. 3D). These results indicated that *Amoldb1* mutants are impaired in cuticle penetration due to an inability to form appressoria and that *MoLDB1* is necessary for production of infection structures required for plant infection by *M. oryzae*. The experiments also showed that mycelial fragments of the wild-type strain have the capacity to form appressoria on host leaves and that cuticle penetration can be mediated by appressoria that differentiate from hyphae. These are consistent with previous reported observations of hypha-driven appressorium formation and cuticle penetration by the fungus (Kim et al. 2009; Liu et al. 2010).

#### ***MoLDB1* is required for infection-related morphogenesis.**

Deletion of *MoLDB1* caused defects in vegetative growth, conidiogenesis, and fertility in *M. oryzae* (Fig. 4). Compared with the wild-type strain Guy11, *Amoldb1* mutants formed mycelium that was not well pigmented and generated fewer aerial hyphae (Fig. 4A). The *Amoldb1* mutant AK58 and A2-12-3 were also reduced in vegetative growth, forming colonies with diameters of  $4.33 \pm 0.06$  and  $4.37 \pm 0.15$  cm, respectively, after a 10-day incubation on CM at 25°C compared with a colony diameter of  $6.63 \pm 0.12$  cm for wild-type strain Guy11 ( $P < 0.01$ ) (Fig. 4B). The ability to form spores was evaluated by carefully washing the surface of 10-day-old cultures on CM plates. A2-12-3, AK58, and AK66 were unable to form conidia, whereas the wild-type strain Guy11 produced numerous conidia with  $20.73 \pm 2.71 \times 10^6$  spores/plate (Fig. 4C and D). In order to induce sporulation, *Amoldb1* mutants (AK58 and AK66) and A2-12-3 were also grown on different growth media, including oatmeal agar (OMA), OMA with tomato juice, sterilized barley grains, and sterilized rice leaves. However, no spores were obtained from cultures of the *Amoldb1* mutants and A2-12-3 on any tested growth media (data not shown). These results showed that asexual sporulation was completely blocked by the deletion or disruption of *MoLDB1*, indicating that *MoLDB1* is essential for conidiation in *M. oryzae*. To understand whether mutants could form conidiophores, we observed the cultures of *Amoldb1* (AK58) and Guy11 after incubation on CM at 25°C for 10 days by light and scanning electron microscopy. Surprisingly, no conidiophores were observed from cultures of AK58, whereas Guy11 formed normal conidiophores and conidia (Fig. 5). Simi-

larly, we did not observe conidiophores in the *Amoldb1* mutant (AK66) or A2-12-3 (data not shown). These results suggest that the defect in conidiation of the *Amoldb1* mutants may be caused by the lack of aerial conidiophore formation.

To determine the role of *MoLDB1* in sexual reproduction, the wild-type Guy11 (*MAT1-2*), A2-12-3, and AK58 were crossed with a standard tester strain TH3 (*MAT1-1*) of *M. oryzae* to allow perithecial production. After 3 weeks, the junctions between mated individuals were examined for the presence of perithecia. We observed numerous perithecia at the junctions of the wild-type strains Guy11 and TH3 but no perithecia were produced by crossing A2-12-3 with TH3 or AK58 with TH3, indicating that *MoLDB1* is also essential for fertility and development of fruiting bodies by *M. oryzae* (Fig. 4E). Taken together, we concluded that *MoLDB1* is required for efficient vegetative growth and for cellular differentiation from hyphal growth, which occurs during conidiogenesis, appressorium formation, and perithecial development.

#### ***MoLdb1* is localized to the nucleus.**

In order to investigate the temporal and spatial pattern of *MoLDB1* expression during infection-related development, a 1.58-kb promoter fragment upstream of the gene and the entire *MoLDB1* protein-coding sequence were fused in-frame to the green fluorescent protein (GFP)-encoding gene, *GFP* (*sGFP*), and introduced into A2-12-3 and the *Amoldb1* mutant AK58, respectively. Transformants carrying a single integration of the plasmid were selected by DNA gel blot analysis (data not shown). An independent single p*MoLDB1*-GFP insertion event occurred in four transformants: LS-5, LS-11, AC26, and AC38 (Table 2). Punctate green fluorescence was observed in all these transformants. AC26 was used to investigate the spatial localization of the MoLdb1 protein in detail. In this analysis, GFP fluorescence was observed in both mycelium and conidia of AC26, and each cell contained one fluorescence punctum (Fig. 6A), suggesting that MoLdb1 may localize to the nucleus of each cell. To test this idea, mycelium and conidia of AC26 were stained with 4'-6-diamidino-2-phenylindole (DAPI) to stain nuclei specifically. The merged image of GFP and DAPI staining showed that MoLdb1-GFP localizes to the nucleus and that each cell contains a single nucleus (Fig. 6A).

To observe *MoLDB1* expression and nuclear division patterns during appressorium development in *M. oryzae*, conidia of the strain AC26 were allowed to germinate on hydrophobic Gel-Bond film surfaces. During conidium germination, the nucleus in the germinating cell entered mitosis approximately 4 to 6 h postinoculation (hpi) (Fig. 6B). After 8 hpi, one of the daughter nuclei migrated to the incipient appressorium and three nuclei that remained in the conidium degenerated and could no longer be seen after 12 to 18 hpi. This is consistent with previous reported observations (Veneault-Fourrey et al. 2006). The pattern was identical in the other transformants (LS-5, LS-11, or AC38) (Table 2). By 24 hpi, the only surviving nucleus was contained within the mature appressorium (Fig. 6B).

**Table 2.** Strains of *Magnaporthe oryzae* used in this study

Strain	Brief description	Reference
Guy11	Wild-type, MAT1-2	Leung et al. 1988
A2-12-3	T-DNA insertional mutant	This study
AK58	<i>Amoldb1</i> mutant of Guy11	This study
AK66	<i>Amoldb1</i> mutant of Guy11	This study
ECT1	Ectopic transformant of Guy11 with integration of pLDB-LS-KO	This study
ECT3	Ectopic transformant of Guy11 with integration of pLDB-LS-KO	This study
LS5	A2-12-3 transformed with p <i>MoLDB1</i> -green fluorescent protein (GFP)	This study
LS11	A2-12-3 transformed with p <i>MoLDB1</i> -GFP	This study
AC26	AK58 transformed with p <i>MoLDB1</i> -GFP	This study
AC38	AK58 transformed with p <i>MoLDB1</i> -GFP	This study

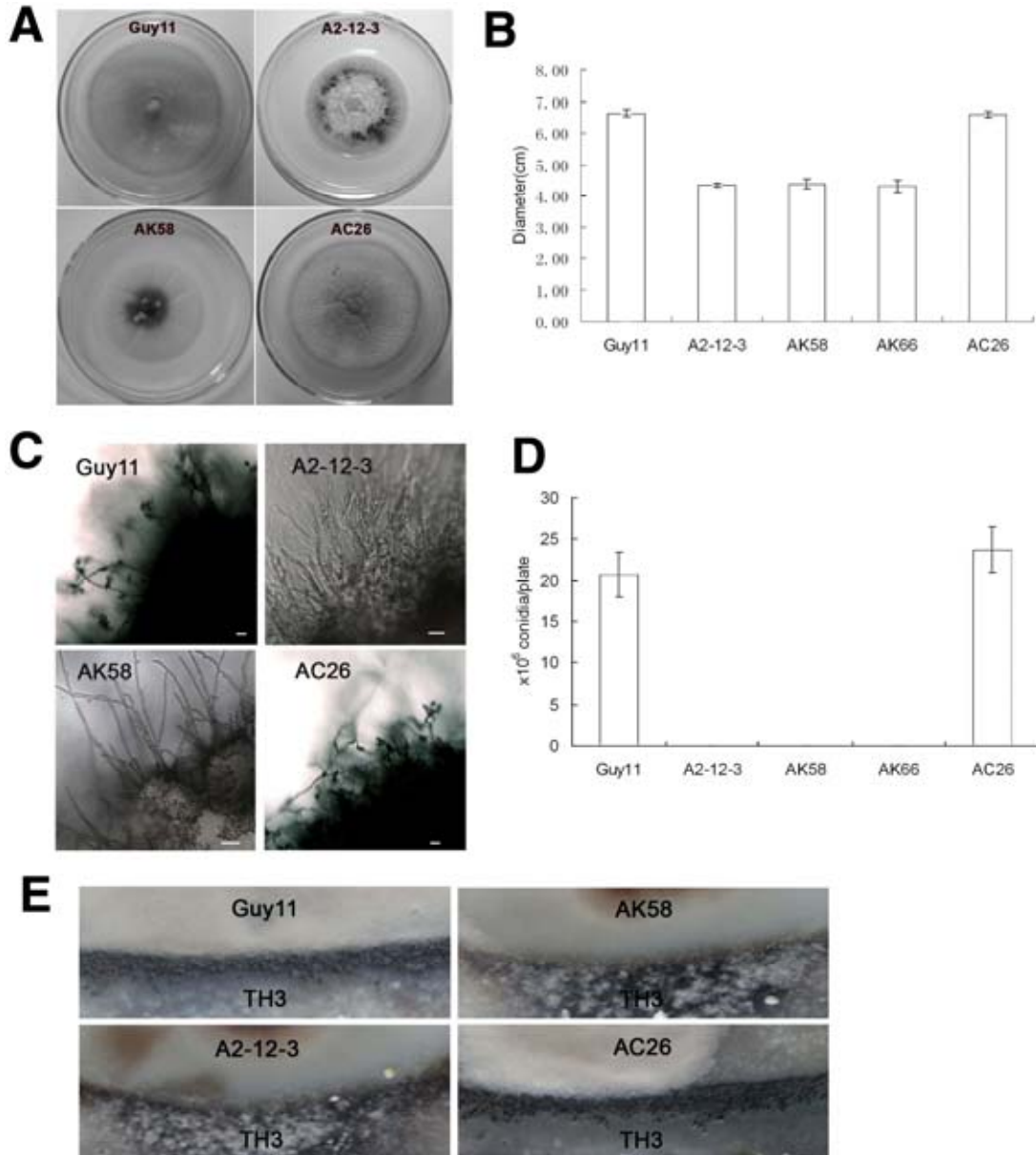
**Reintroduction of *MoLDB1* restores the ability to cause rice blast disease.**

To ensure that the phenotypes observed in A2-12-3 or *Δmoldb1* mutants were associated with the gene disruption or replacement event, we carried out phenotypic analysis of complemented transformants LS-5, LS-11, AC26, and AC38 (Table 2). All four of the GFP-expressing transformants exhibited full virulence to barley and rice by cut-leaf assay using mycelium inoculations (Fig. 7A) or by seedling assays with conidial spray inoculation (data not shown). The phenotypes of A2-12-3 and the *Δmoldb1* mutants, including vegetative growth, conidiation, and fertility, were fully complemented by reintroduction of the gene (Fig. 4). The complemented transformants were also restored in their ability to form melanized appressoria on

hydrophobic GelBond surfaces (Fig. 7B). We conclude that *MoLDB1* is essential for multiple steps of plant infection-related morphogenesis development and pathogenicity in *M. oryzae*.

***Δmoldb1* Mutants show an easily wettable phenotype.**

Colonies of A2-12-3 and the *Δmoldb1* mutants AK58 and AK66 were distinct from the wild-type strain Guy11 and lacked production of aerial hyphae and conidiophores (Figs. 4A and 5). Therefore, we tested the surface hydrophobicity of these strains. Water (10 μl), 0.2% gelatin, Tween20 at 250 μg ml<sup>-1</sup>, or detergent solution containing 0.2% sodium dodecyl sulfate (SDS) and 50 mM EDTA were each placed on the surface of 10-day-old colonies of Guy11 and AK58, respectively. Drops of water and 0.2% gelatin remained on the cultures of



**Fig. 4.** Phenotypic analysis of *Δmoldb1* mutants of *Magnaporthe oryzae*. **A**, Colonies of the wild-type strain Guy11, A2-12-3, *Δmoldb1* mutant AK58, and the complementation strain AC26. Photographs were taken after incubating on complete medium (CM) (Talbot et al.1993) at 25°C for 10 days. **B**, Bar chart showing the colony diameters of the strains: Guy11, A2-12-3, *Δmoldb1* mutants AK58 and AK66, and complementation strain AC26. Error bars represent the standard deviation. **C**, Both T-DNA insertional mutant A2-12-3 and *Δmoldb1* mutant AK58 are unable to produce any conidia on CM at 25°C for 10 days, whereas the wild-type strain Guy11 and AC26 form numerous conidia on media. Bar = 10 μm. **D**, Bar chart showing the conidial production of the strains Guy11, A2-12-3, *Δmoldb1* mutants AK58 and AK66, and AC26. Error bars represent the standard deviation. **E**, Fertility assay. Guy11 × TH3 and AC26 × TH3 form numerous perithecia at the junctions between mated individuals on oatmeal medium; no perithecia were observed for the crosses of A2-12-3 × TH3 and AK58 × TH3.

Guy11 after 24 h of incubation, even for 48 h of incubation, and drops of Tween20 at 250  $\mu\text{g ml}^{-1}$  could stay on colonies of Guy11 for approximately 30 min before soaking into the mycelium, while the detergent solution (0.2% SDS and 50 mM EDTA) immediately soaked into colonies of Guy11 (Fig. 8A). By contrast, in the mutant AK58, drops of water and 0.2% gelatin remained on the surface of mature mycelium at the center of the colony for 1 to 2 h but drops of water and 0.2% gelatin immediately soaked into young mycelium. However, Tween20 at 250  $\mu\text{g ml}^{-1}$  or the detergent solution immediately soaked into mycelium of the fungus regardless of its maturity (Fig. 8A). Similar results were observed for the mutants of A2-12-3 and AK66. The results indicate that deletion of *MoLDB1* causes an easily wettable phenotype in *M. oryzae*. An easily wettable phenotype means that the fungal culture become easily waterlogged, due to a loss of surface hydrophobicity brought about by the absence of the rodlet layer associated with aerial hyphae and conidiospores (Talbot et al. 1993). Because of the wettable phenotype of the  $\Delta\text{moldb1}$  mutants and A2-12-3, we reasoned that *M. oryzae* hydrophobin genes might be downregulated in the mutants. Hydrophobins are morphogenetic proteins required for aerial hypha development and formation of the hydrophobic rodlet layer on the surface of spores (Kershaw et al. 1998). To test this idea, we investigated *M. oryzae* hydrophobin-encoding genes, including *MPG1*, *MHP1*, and two *MHP1* homologues (MGG\_09134.6 and MGG\_10105.6), by quantitative RT-PCR (qRT-PCR). *MPG1* encodes a class I hydrophobin required for efficient appressorium development and pathogenicity (Talbot et al. 1993). *MHP1* encodes a class II hydrophobin required for pathogenicity (Kim et al. 2005). qRT-PCR analysis showed that expression of hydrophobin-encoding genes was significantly ( $P < 0.01$ ) downregulated in the  $\Delta\text{moldb1}$  mutant AK58 (Fig. 8B). Expression of *MPG1* was downregulated 130-fold in AK58 compared with wild-type Guy11. These results demonstrated that deletion of *MoLDB1* leads to low expression of hydrophobin genes, resulting in the wettable phenotype of colonies of the  $\Delta\text{moldb1}$  mutants and their inability to differentiate conidia and appressoria.

#### Downregulation of the genes encoding LIM proteins in $\Delta\text{moldb1}$ mutants.

Bioinformatic and genomic analysis showed that *M. oryzae* MoLdb1 contains a putative LIM-binding domain and that there are four predicted genes encoding LIM-domain-containing proteins in *M. oryzae* genome (Dean et al. 2005) (Table 3). All were predicted to be nuclear localized by WoLF PSORT (Table 3). *MoLRG1* (MGG\_04377.6) and *MoPAX1* (MGG\_05738.6) contain three LIM domains while *MoLRG2* (MGG\_04186.6) and *MoLDP1* (MGG\_06198.6) contain two LIM domains (Table 3). Additionally, both *MoLRG1* and *MoLRG2* have RhoGAP domains (GTPase activator proteins toward Rho/Rac/Cdc42-like small GTPases) (Table 3). In order to investigate the expression pattern of these genes, we carried out qRT-PCR in the  $\Delta\text{moldb1}$  mutant AK58. The results showed that expression of all the predicted genes containing LIM domains were significantly ( $P < 0.01$ ) downregulated in AK58 (Fig. 9), indicating that gene deletion of *MoLDB1* may affect the function of LIM proteins in *M. oryzae*.

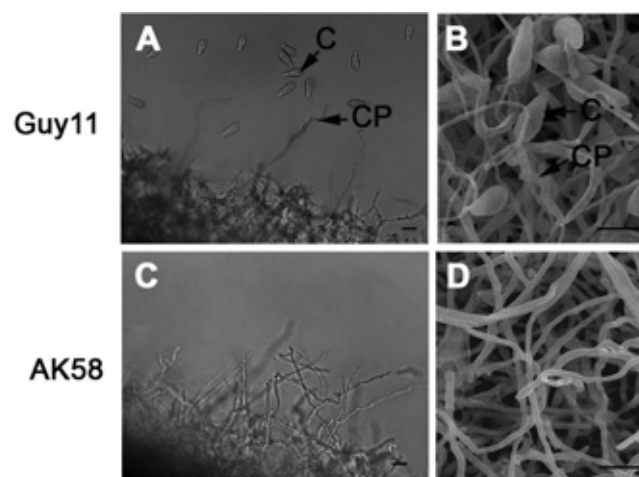
## DISCUSSION

Insertional mutagenesis is an effective way to identify novel genes involved in plant infection-related morphogenesis and pathogenicity of fungal pathogens, including *M. oryzae* (Wilson and Talbot 2009). Recently, we have reported that seven mutants impaired in pathogenicity could be identified from 5,000 transformants obtained by T-DNA insertional mutagenesis (Li et al.

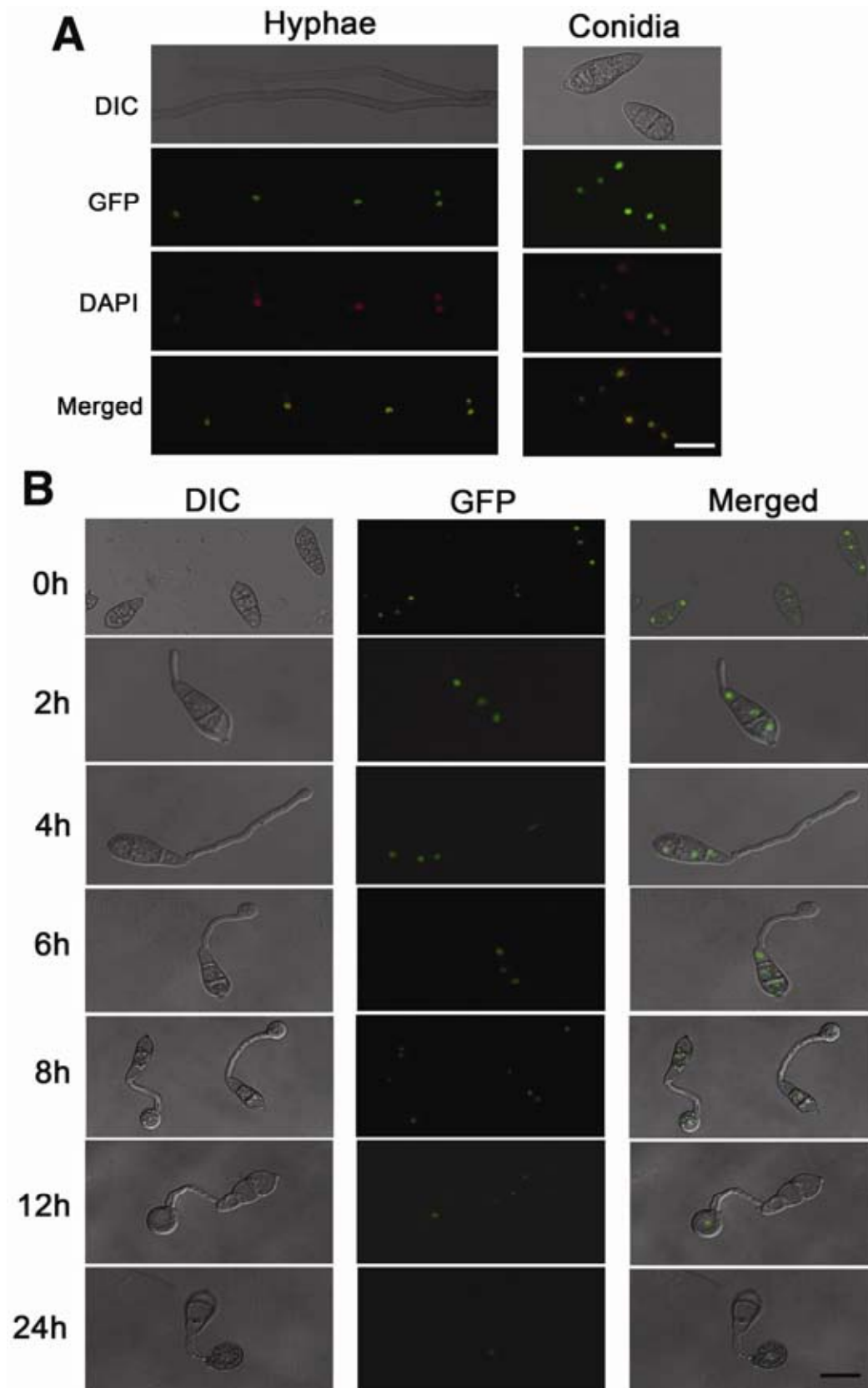
2010). In this report, we characterized one of these mutants, A2-12-3, which was defective in conidiation and pathogenicity. Nested inverse PCR revealed that the integrated T-DNA in the mutant disrupted a genomic region corresponding to a locus, putatively encoding a LIM-domain-binding protein, MoLdb1. Our results showed that *MoLDB1* was required for efficient fungal vegetative growth, and was necessary for conidiation and pathogenicity. BLAST searches show MoLdb1 to be homologous to PtaB (AAG49358.1) from *Aspergillus nidulans*, a protein of unknown function with a LIM-binding domain (Conlon et al. 2001). To our knowledge, homologues of *MoLDB1* from other fungi have not yet been functionally characterized.

LIM-domain-binding proteins were originally identified as cofactors for LIM-homeodomain and LIM-only proteins which have fundamental roles in development, and have been shown themselves to have essential functions in diverse biological processes in different organisms (Agulnick et al. 1996; Jurata et al. 1996; Bach et al. 1997; Visvader et al. 1997; Matthews and Visvader 2003; Matthews et al. 2008). All known Ldb proteins carry a conserved nuclear localization sequence and are found predominantly in the nucleus (Jurata et al. 1996). Consistent with these, the GFP-tagging experiments confirmed that the *M. oryzae* MoLdb1 nucleus localizes to the nucleus (Fig. 6). Bioinformatic analysis showed that putative homologues of MoLdb1 were also found in the genomes of other fungal species (Fig. 2B). However, MoLdb1 contrasts with Ldbs from human and animals. Alignment analysis showed that *M. oryzae* MoLdb1 shares very low identity with Ldb1a (NP\_003884), Ldb1b (BAE95402), Ldb2a (AAC28342), and Ldb2b (AAC28343) from human. Similarly, MoLdb1 is highly dissimilar to the Ldbs from animals, including mouse, chicken, xenopus, and zebrafish.

MoLdb1 is a morphogenetic regulator. Conidiogenesis is a complex process that involves a cascade of morphological events (Kim et al. 2009). *M. oryzae* produces three-celled conidia on a conidiophore, a specialized structure elongated through apical extension of an aerial hypha (Kim et al. 2009). Asexual reproduction and infection-related development play key roles in the disease cycle in *M. oryzae*. In the field, dissemination of the fungus during an epidemic occurs by aerial dispersal of conidia produced on conidiophores emerging from



**Fig. 5.** Mutant  $\Delta\text{moldb1}$  is unable to form conidiophores. **A** and **B**, Wild-type strain Guy11 formed normal conidiophores and conidia; **C** and **D**, AK58 ( $\Delta\text{moldb1}$ ) was unable to form conidiophores. Light microscope photographs (**A** and **C**) and scanning electron microscope (**B** and **D**) photographs were taken from cultures after incubating on complete medium (Talbot et al. 1993) at 25°C for 10 days. CP = conidiophore and C = conidium; bar = 10  $\mu\text{m}$ .

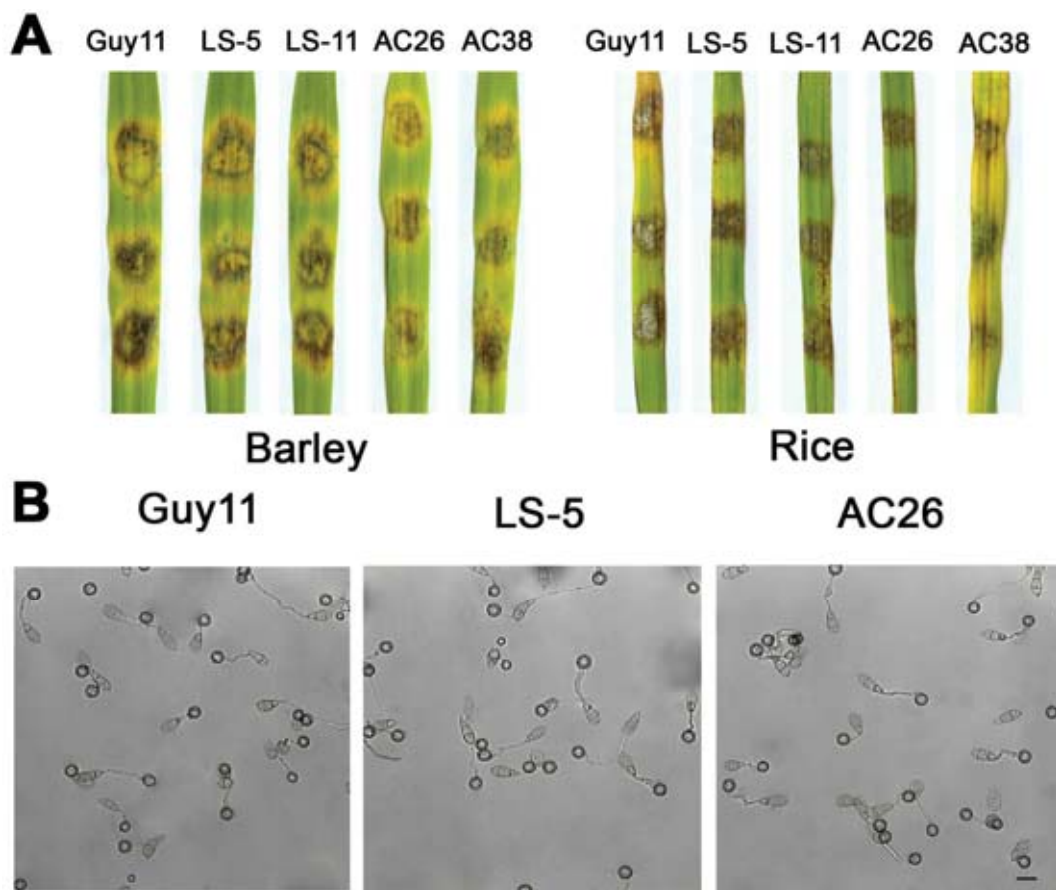


**Fig. 6.** Intracellular localization of MoLdb1-green fluorescent protein (GFP). **A**, Expression of *MoLDB1* in conidia and mycelium of *Magnaporthe oryzae* AC26. MoLdb1 C-terminal GFP fusion strategy was conducted to generate GFP expression transformants. The strain AC26 carries a single GFP-carboxy translational fusion of *MoLDB1*. GFP fluorescence was observed in both mycelium and conidia of AC26, and each cell contained one fluorescence punctum. Mycelium and conidia of AC26 were stained by 4'-6-diamidino-2-phenylindole (DAPI), which is a nuclear counterstain for use in multicolor fluorescent techniques and stains nuclei specifically. The merged image of GFP and DAPI staining showed that MoLdb1-GFP localizes to the nucleus and that each cell contains a single nucleus. DIC = differential interference contrast. Bar = 10  $\mu$ m. **B**, Patterns of *MoLDB1* expression and nuclear division during appressorium development in *M. oryzae*. Conidia of the strain AC26 was allowed to germinate on hydrophobic GelBond film surfaces. Bar = 10  $\mu$ m.

disease lesions. Production and dissemination of conidia from disease lesions of rice blast is a regulated process (Ebbole 2007). Molecular genetic analysis of conidiation reveals several conidiation-associated genes that have distinct effects on control of conidiation and conidial morphology (Shi and Leung 1994, 1995). The *con7* mutant, for instance, produces a mixture of normal and aberrantly shaped conidia unable to form appressorium, and is nonpathogenic (Odenbach et al. 2007). Recently, a novel gene, *COM1*, coding a putative transcription regulator has been characterized (Yang et al. 2010). Disruption or deletion mutants of *COM1* produce slender conidia and are defective in appressorium turgor generation, penetration, and infectious growth (Yang et al. 2010). However, very few mutants have been identified that have completely lost the ability to form conidia in *M. oryzae*. Very recently, eight genes (*MoHOX1* to *MoHOX8*) encoding putative homeobox transcription factors have been characterized in *M. oryzae* (Kim et al. 2009; Liu et al. 2010). Deletion mutants of *MoHOX2* completely abolished asexual sporulation. However, the mutants were still pathogenic through hypha-driven appressoria in a manner similar to that of the wild type (Kim et al. 2009; Liu et al. 2010). One of the most interesting findings we report here is that *Δmoldb1* mutants have completely lost the ability to form differentiation spores, including meiotically generated ascospores (Fig. 4C and E). To induce mutants to sporulate, several media were used in our experiments but no conidia were ever observed, indicating that *MoLDB1* is essential for sporulation. Furthermore, we did not observe conidiophores in cultures of AK58 (Fig. 5), indicating that the defect in conidiation of the *Δmoldb1* mutants is associated with lack of conidiophore formation.

For pathogenicity tests, the conventional method is to harvest conidia from *M. oryzae* cultures, then to spray them onto susceptible plant surfaces. Here, we used mycelium from liquid cultures as the inoculum due to the defect of conidium production of the *Δmoldb1* mutants or A2-12-3. Using this alternative inoculation method, the wild-type strain Guy11 was still able to form appressoria efficiently from mycelium and could penetrate host leaf surfaces and cause blast disease (Fig. 3D). However, gene replacement mutants of *MoLDB1* were unable to cause blast disease on susceptible rice or barley leaves. Therefore, the nonpathogenic phenotype of the mutants is caused by their inability to form appressoria, because they did cause infectious growth on abraded leaves (Figs. 1A and B and 3C). These results showed that *MoLdb1* is essential for conidiation and appressorium formation.

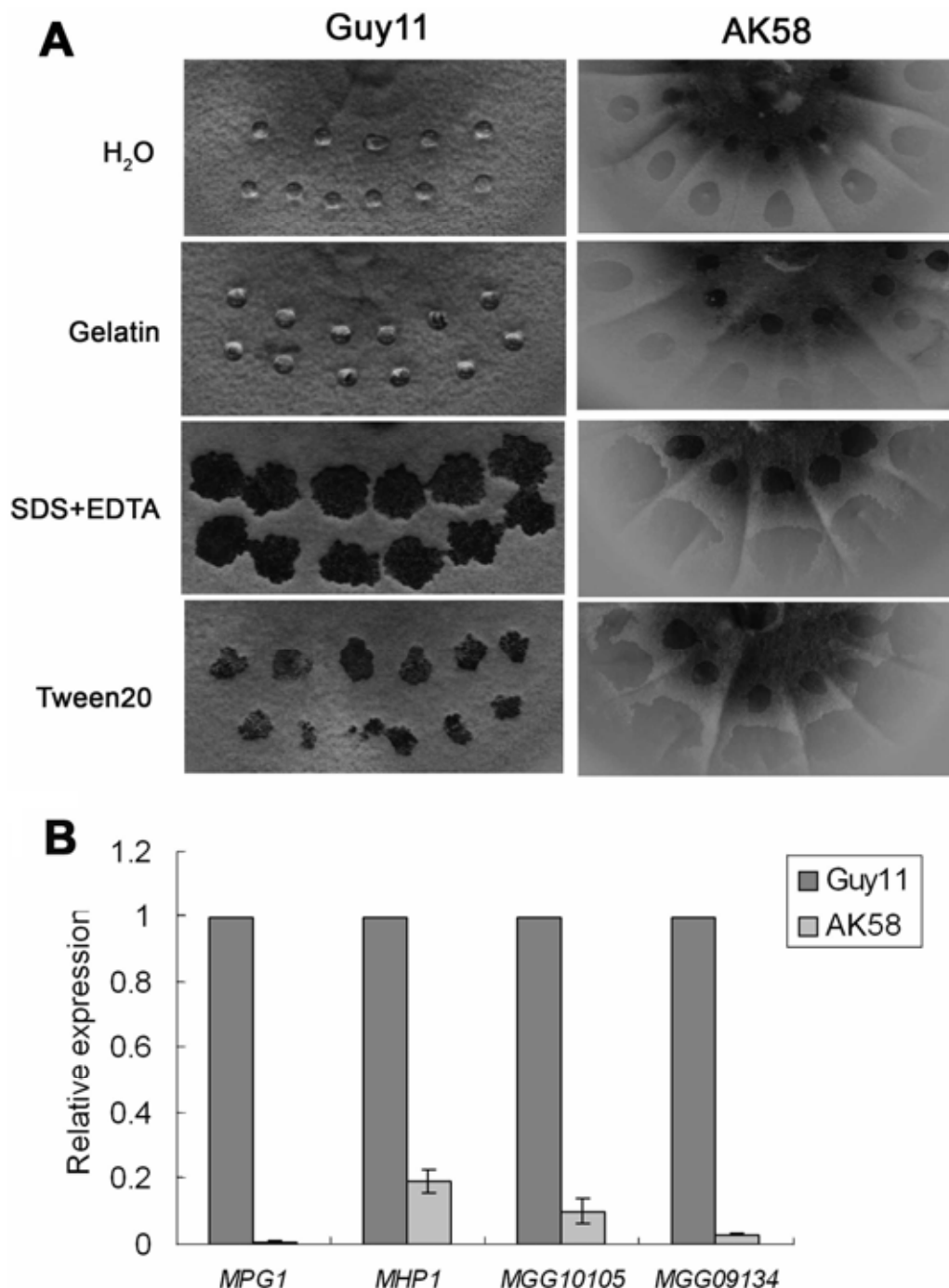
Fungal hydrophobins are secreted morphogenetic proteins that form amphipathic films by self-assembly of protein monomers at the interface between fungal cell walls and the air or solid hydrophobic surface. Mpg1 is an *M. oryzae* class I hydrophobin that is required for efficient appressorium development and pathogenicity (Talbot et al. 1993). Deletion of *MPG1* in *M. oryzae* produced mutants lacking a conidial rodlet layer formation displaying an easily wettable phenotype (Talbot et al. 1996; Kershaw et al. 1998). *MHP1* encodes a class II hydrophobin in *M. oryzae*, which is required for conidial development, viability, and pathogenicity (Kim et al. 2005). The *Δmhp1* mutants do not show a water-wettable phenotype but are detergent wettable (Kim et al. 2005). Deletion of *MoLDB1* significantly affected hydrophobicity of *M. oryzae* mycelium (Fig. 8A). Consistent with this, qRT-PCR showed that genes encoding hydrophobins,



**Fig. 7.** Complementation of *Δmoldb1* mutants of *Magnaporthe oryzae* by reintroduction of *MoLDB1*. **A**, Pathogenic analysis of complemented transformants LS-5, LS-11, AC26, and AC38. Barley and rice leaf segments were inoculated with the mycelium of the wild-type strain Guy11, LS-5, LS-11, AC26, and AC38, respectively. All the transformants exhibited full virulence to barley and rice. **B**, Appressorium formation of Guy11, LS-5, and AC26 on hydrophobic GelBond surfaces. The two transformants formed melanized appressoria.

including *MPG1*, *MHP1*, and two homologues of *MHP1*, were dramatically downregulated in the  $\Delta$ *molldb1* mutant AK58 (Fig. 8B). Thus, the wettable phenotype was associated with low levels of genes encoding hydrophobins. However,  $\Delta$ *molldb1* mutants completely lost the ability to produce asexual and sexual spores and were unable to infect unwounded host leaves. Therefore, it is possible that *MoLDB1* regulates other sporulation-related or pathogenicity-related genes.

The LIM domain is a zinc finger structure that is present in several types of proteins, including homeodomain transcription factors, kinases, and proteins that consist of several LIM domains (Dawid et al. 1998; Jurata and Gill 1998; Bach 2000). Proteins containing one, two, or multiple LIM domains have been shown to play important roles in a variety of fundamental biological processes (Bach 2000). The LIM domain has been demonstrated to be a protein–protein interaction motif that is



**Fig. 8.** Deletion of *MoLDB1* causes the defect in cell surface hydrophobicity. **A**, Surface hydrophobicity of the wild-type strain Guy11 and the  $\Delta$ *molldb1* mutant AK58 was assessed by placing a 10- $\mu$ l drop of water, 0.2% gelatin, 0.2% sodium dodecyl sulfate (SDS) + 50 mM EDTA, and Tween20 at 250  $\mu$ g ml<sup>-1</sup> on the cultures. Drops of water and 0.2% gelatin remained on the cultures of Guy11, while the others were soaked into colonies. The photographs were taken after 3 h. **B**, Expression of the genes coding hydrophobins in the  $\Delta$ *molldb1* mutant AK58 measured by quantitative reverse-transcription polymerase chain reaction. The abundance of the gene transcripts was calculated relative to endogenous control ( $\beta$ -tubulin gene) using the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen 2001). The level of gene expression in Guy11 was taken as 1 and the relative expression in the mutant AK58 was normalized based on 1. Error bars represent the standard deviation. Expression of the genes coding hydrophobins (*Mpg1*, *Mhp1*, *MGG\_09134*, and *MGG\_10105*) was significantly affected by the deletion of *MoLDB1* ( $P < 0.01$ ).

critically involved in these processes. Bioinformatic analysis showed that the *M. oryzae* genome contains four LIM-domain proteins, which were predicted to be nuclear localized by WoLF PSORT (Table 3). It will be necessary to confirm the nuclear localization pattern of the *Magnaporthe* LIM proteins by an independent experimental method in future, because the current analysis, based solely on WoLFPSORT analysis, is simply a computational prediction. Recently, it has been reported that *N. crassa* Lrg1 (NCU02689), a LIM-domain-containing protein, which is a likely homologue of *M. oryzae* MoLrg1 (MGG\_04377.6), localizes to hyphal tips and sites of septation via its three LIM domains (Vogt and Seiler 2008). Interestingly, *Neurospora* Lrg1 is essential for apical tip extension and to restrict excessive branch formation in subapical regions of the hypha and is also involved in determining the size of the hyphal compartments (Vogt and Seiler 2008). In *S. cerevisiae*, four LIM-domain-containing proteins have been characterized: Lrg1 (Lorberg et al. 2001), Rga1 (Stevenson et al. 1995), Rga2 (Smith et al. 2002), and Pxl1 (Gao et al. 2004; Mackin et al. 2004). Our results showed that deletion of *MoLDB1* significantly downregulated the genes coding for the four LIM-domain proteins found in *M. oryzae* (Fig. 9). However, we did not detect direct interactions between MoLdb1 and these LIM domain proteins in Y2H assays (data not shown). Also, no physical interactions between the LIM-binding domain of MoLdb1 and LIM domains of the four LIM proteins in *M. oryzae* were detected in Y2H (data not shown). Recently, functional characterization of the four LIM-domain proteins in *M. oryzae* has revealed that they also were essential

for plant infection morphogenesis or pathogenicity in *M. oryzae* (*unpublished data*). When considered together, our results suggest that MoLdb1 downregulates genes encoding hydrophobins and LIM-domain proteins and is required for vegetative growth, conidiation, mating, and pathogenicity.

## MATERIALS AND METHODS

### Strains, culture conditions, and DNA analysis.

All mutants described in the present study were generated from the *M. oryzae* wild-type strain Guy11 (Table 2). Standard growth and storage procedures for fungal strains were performed, as described previously (Talbot et al. 1993). *Escherichia coli* DH-5 $\alpha$  was used for routine bacterial transformations and maintenance of various plasmids in this study. Southern blot analysis was performed by the digoxigenin high prime DNA labeling and detection starter Kit I (Roche, Mannheim, Germany). General procedures for nucleic acid analysis followed standard protocols (Sambrook et al. 1989).

### Pathogenicity assays.

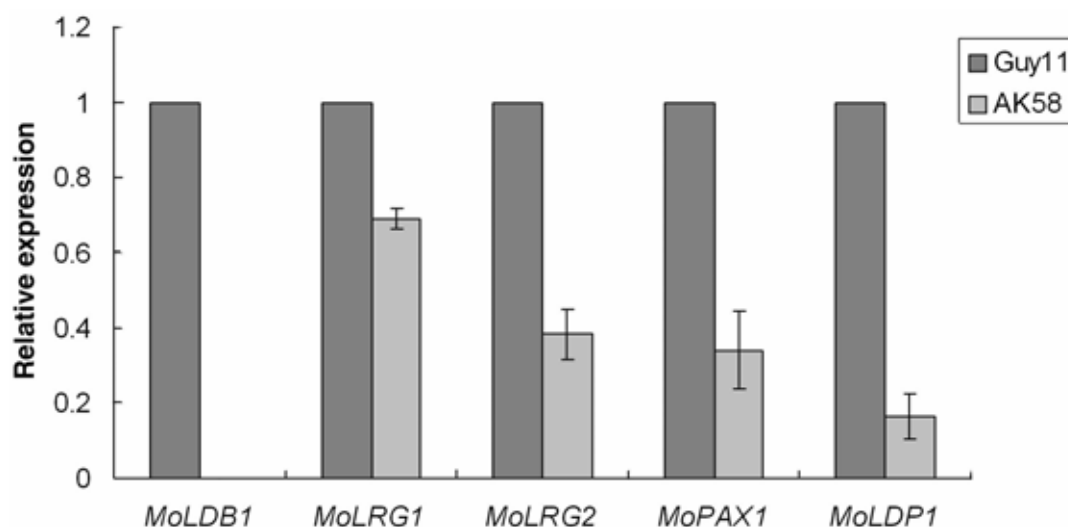
Plant infection assays were carried out using cut-leaf assays. Fragments were cut from the leaves of 10-day-old barley cv. Golden Promise and 14-day-old rice cv. CO-39 seedlings, both highly susceptible toward *M. oryzae*, and placed in plastic plates containing wetted filters. Mycelium from 2-day-old liquid CM cultures at 25°C were placed onto leaf sections and the plates were incubated in a cycle of 12 h of light and 12 h of darkness at 25°C. Wounded rice leaves were prepared by removing the

**Table 3.** Predicted proteins in the *Magnaporthe oryzae* genome that contain LIM or LIM-binding domains

Gene	Predicted ORF	Pfam domain <sup>a</sup>	Predicted location <sup>b</sup>
<i>MoLDB1</i>	MGG_01057.6	LIM-binding domain (PF01803): 431 to 568 (7e-04)	Nuclear
<i>MoLRG1</i>	MGG_04377.6	Three LIM domains (PF00412): 97 to 156 (2.7e-09), 161 to 217 (2.9e-12), 467 to 521 (3.2e-06); RhoGAP domain (PF00620): 849 to 1001 (5.2e-37)	Nuclear
<i>MoLRG2</i>	MGG_04186.6	Two LIM domains (PF00412): 17 to 71 (2.5e-09), 74 to 127 (0.00015); RhoGAP domain (PF00620): 1010 to 1160 (4.6e-45)	Nuclear
<i>MoPAX1</i>	MGG_05738.6	Three LIM domains (PF00412): 704 to 740 (4.8e-08), 800 to 852 (3e-08), 863 to 918 (5.2e-09)	Nuclear
<i>MoLDP1</i>	MGG_06198.6	Two LIM domains (PF00412): 512 to 570 (1.2e-12), 575 to 628 (9.8e-12)	Nuclear

<sup>a</sup> Domain search at the web site of the Sanger Institute and the National Center for Biotechnology Information.

<sup>b</sup> Protein localization predicted with WoLF PSORT.



**Fig. 9.** Predicted genes coding LIM-domain proteins were significantly downregulated by the deletion of *MoLDB1*. Expression of the predicted genes coding LIM-domain proteins in the *Δmoldb1* mutant AK58 measured by quantitative reverse-transcription polymerase chain reaction. The abundance of the gene transcripts was calculated relative to endogenous control ( $\beta$ -tubulin gene) using the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen 2001). The level of gene expression in Guy11 was taken as 1 and the relative expression in the mutant AK58 was normalized based on 1. Error bars represent the standard deviation. Expression of the predicted genes coding LIM-domain proteins, MoLrg1 (MGG\_04377), MoLrg2 (MGG\_04186), MoPax1 (MGG\_05738), and MoLdp1 (MGG\_06198), was significantly affected by the deletion of *MoLDB1* ( $P < 0.01$ ).

surface cuticle by abrasion with an emery board as described previously (Wang et al. 2007). Disease lesions were examined and photographed after 5 days of incubation.

#### Analysis of fungal growth, sporulation, and genetic crosses.

Vegetative growth was assessed by measurement of colony diameter on plate cultures of *M. oryzae* grown on CM. Conidial development was assessed by harvesting conidia from the surface of 10-day-old plate cultures and by determining the concentration of the resulting conidial suspension using a hemacytometer. Each test was repeated three times. Fertility assays were carried out by pairing Guy11 (*MAT1-2*), A2-12-3, or *Amoldb1* mutants with standard tester strain TH3 (*MAT1-1*) on OMA plates. The plates were incubated at 25°C with a 12-h photo phase until the colonies contacted each other, then placed under continuous white fluorescent light at 18°C for 3 to 4 weeks. The junctions between the mated individuals were examined for the capacity to form perithecia. This mating test was repeated three times.

#### Construction of vectors and fungal transformation.

For the construction of the *MoLDB1* gene replacement vector, a 1.4-kb *HPH* gene cassette, which encodes hygromycin phosphotransferase under control of the *A. nidulans* TrpC promoter (Carroll et al. 1994), was amplified with primers HPH-BF and HPH-BR (Table 1) using pCB1003 as a template. Then, 1-kb flanking sequences on either border of the *MoLDB1* gene locus were amplified using primer pairs of 1LF/1LR and 2LF/2LR and cloned sequentially into pGEM-T easy vectors (Promega Corp., Madison, WI, U.S.A.) to generate pGEM-L and pGEM-R, respectively. pGEM-R was digested with *Bam*HI and *Pst*I and the releasing fragment was inserted into pGEM-L with the correspondent ends, leaving a single *Bam*HI site for insertion of the *HPH* cassette with *Bam*HI ends. The resulting construct vector, pLDB-LS-KO, was linearized and transformed into *M. oryzae* Guy11 for generating homologous recombinants, as previously described (Talbot et al. 1993).

The *MoLDB1* C-terminal GFP-tagging vector, pMoLDB1-GFP, was constructed by amplification of a 4.5-kb fragment, including a 2.9-kb *MoLDB1* gene-coding sequence and a 1.6-kb promoter region using primers CF1 and CR1 (Table 1) and by amplification of a 1.5-kb fragment GFP allele (Chiu et al. 1996) carrying the *A. nidulans* trpC terminator using primers GF1 and GR1 (Table 1). Construction of pMoLDB1-GFP was carried out by cloning the 4.5-kb PCR product into pGEM-T easy vector to generate pGM-LDB. The 1.5-kb GFP allele was then cloned to pGEM-T easy vector and digested with *Hind*III to release the GFP allele with *Hind*III ends, which was inserted into the *Hind*III site of pGM-LDB to create pGM-LDB-GFP. The pGM-LDB-GFP vector was checked by DNA sequencing to confirm the correct orientation of GFP insertion and in-frame fusion. Finally, pGM-LDB-GFP was digested with *Not*I to release the *MoLDB1* C-terminal GFP-tagging fragment, which was inserted into the *Not*I site of pCB1532, which contains the *ILV1* allele conferring resistance to sulfonylurea (Sweigard et al. 1997) to give pMoLDB1-GFP. The resulting plasmid, pMoLDB1-GFP, was transformed into *Amoldb1* mutant AK58 and A2-12-3, respectively. Transformants carrying a single insertion were selected and complementation of the *Amoldb1* mutant phenotypes checked. GFP fluorescence was observed using a Leica TCS SP5 inverted confocal laser scanning microscope (Leica, Wetzlar, Germany).

#### DAPI nuclear staining.

Mycelium, conidia, and appressoria of complementation transformants AC26 and AC38, were stained by DAPI (236276; Roche) at 0.8 mg ml<sup>-1</sup>, a popular nuclear counterstain for use

in multicolor fluorescent techniques which stains nuclei specifically, and observed by confocal microscopy at an excitation wavelength of 350 nm.

#### qRT-PCR.

Total RNA was extracted from mycelium of *M. oryzae* Guy11 and AK58 (Table 2) using the SV Total RNA Isolation System (Z3100; Promega Corp.) according to the manufacturer's instructions. Total RNA was utilized for synthesis of the first-strand cDNA using the PrimeScript 1st Strand cDNA synthesis kit (D6110A; TaKaRa, Tokyo). The resultant cDNA was used as a template for qRT-PCR. qRT-PCR was performed with a SYBR Green realtime PCR master mix kit (QPK-201; TOYOBO, Osaka, Japan) using an iCycler iQ multicolor realtime PCR detection system (Bio-Rad, Munich, Germany). All qRT-PCR reactions were conducted in duplicate for each cDNA sample and were repeated three times. The primer pairs MPG-RT-F/MPG-RT-R, MHP-RT-F/MHP-RT-R, 10105-RT-F/10105-RT-R, and 9134-RT-F/9134-RT-R (Table 1) were used to determine the relative expression of *MPG1* (MGG\_10315.6), *MHP1* (MGG\_01173.6), MGG\_09134.6, and MGG\_10105.6, respectively. The primer pairs MoLDB-RT-F/MoLDB-RT-R, 4377-RT-F/4377-RT-R, 4186-RT-E/4186-RT-R, 5738-RT-F/5738-RT-R, and 6198-RT-F/6198-RT-R (Table 1) were used to determine the relative expression of *MoLDB1*, *MoLRG1*, *MoLRG2*, *MoPAX1*, and *MoLDPI* (Table 3), respectively. The *M. oryzae*  $\beta$ -tubulin gene (MGG\_00604.6) amplified with the primer pairs of Tub-F/Tub-R was used as an endogenous control. The abundance of the gene transcripts was calculated relative to this control using the 2<sup>- $\Delta$ ACT</sup> method (Livak and Schmittgen 2001).

#### ACKNOWLEDGMENTS

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## AUTHOR-RECOMMENDED INTERNET RESOURCES

- WoLF PSORT database: [wolfpsort.org](http://wolfpsort.org)  
 Sanger Institute Pfam database: [pfam.sanger.ac.uk/search](http://pfam.sanger.ac.uk/search)  
 National Center for Biotechnology Information conserved domains database: [www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi](http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi)