Characterisation of Four LIM Protein-Encoding Genes Involved in Infection-Related Development and Pathogenicity by the Rice Blast Fungus *Magnaporthe oryzae*

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**Abstract**

LIM domain proteins contain contiguous double-zinc finger domains and play important roles in cytoskeletal reorganisation and organ development in multi-cellular eukaryotes. Here, we report the characterization of four genes encoding LIM proteins in the rice blast fungus *Magnaporthe oryzae*. Targeted gene replacement of either the paxillin-encoding gene, *PAX1*, or *LRG1* resulted in a significant reduction in hyphal growth and loss of pathogenicity, while deletion of *RGA1* caused defects in conidiogenesis and appressorium development. A fourth LIM domain gene, *LDP1*, was not required for infection-associated developmental expression by *M. oryzae*. Live cell imaging revealed that *Lrg1*-GFP and *Rga1*-GFP both localize to septal pores, while *Pax1*-GFP is present in the cytoplasm. To explore the function of individual LIM domains, we carried out system wide deletion of each LIM domain, which revealed the importance of the Lrg1-LIM2 and Lrg1-RhoGAP domains for Lrg1 function and overlapping functions of the three LIM domains of *Pax1*. Interestingly, deletion of either *PAX1* or *LRG1* led to decreased sensitivity to cell wall-perturbing agents, such as Congo Red and SDS (sodium dodecyl sulfate). qRT-PCR analysis demonstrated the importance of both Lrg1 and Pax1 to regulation of genes associated with cell wall biogenesis. When considered together, our results indicate that LIM domain proteins are key regulators of infection-associated morphogenesis by the rice blast fungus.

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**Introduction**

The LIM domain is named after the three proteins (*Lin-11*, *Id-1* and *Mec-3*) and consists of two tandemly-repeated zinc fingers within a conserved domain of 50-60 amino acids with consensus sequence CX_2CX_16-23HX_2CX_2CX_16-21CX_2(C/H/D), where X denotes any amino acid; and/indicates alternatives [1–7]. However, unlike the DNA-binding function of many zinc fingers, the LIM domain does not bind DNA, but instead mediates specific protein-protein interactions, acting as a conserved scaffold to thereby regulate gene expression. Group 1 LIM proteins consists of LHX family proteins, which localize to the nucleus and act as transcription factors or co-factors to mediate protein-protein interactions and thereby regulate gene expression. Group 2 constitutes LMO proteins consisting of two or more LIM domains, but unlike nuclear LMOs, proteins in this group are present in the cytoplasm or nucleus or can shuttle between compartments to regulate gene expression. Group 3 contains paxillin, zyxin, testin and enigma, which possess additional functional domains such as LD (leucine-aspartate repeat), ATD (actin-target domain) and PDZ (first letters of three proteins *PSD95, Dlg1* and *Zo-1*). In addition to LIM domains, proteins in Group 4 contain mono-oxygenase or kinase motifs that distinguish them from Group 3 [15,16]. The conserved Group 3 paxillin proteins in animals consist of four characterized LIM domains at C-termini and an additional five LD motifs at the N-terminus [17,18]. Paxillin serves as an adapter protein, mediating signal transduction from the extracellular matrix to focal adhesions and the actin cytoskeleton [11,19]. Previous studies
showed that C-terminal LIM domains in paxillin are involved in binding the protein tyrosine phosphatase PTP-PEST to target the protein to focal adhesions, and also to bind α- and γ-tubulin to direct an interplay between actin filaments and microtubules [20–22]. Through its LD motifs at N-terminus, paxillin interacts with actopaxin (a member of the parvin family of focal-adhesion proteins), ILK (integrin-linked kinase), FAK (focal adhesion kinase), Pkl (paxillin kinase linker) and vinculin to regulate Rho GTPase signaling and focal adhesion turnover [20,21,23,24]. However, no LD motif has been discovered in the paxillin equivalent of yeasts and filamentous fungi, and only two or three LIM domains are present [25,26]. In *Staphylococcus aureus*, the paxillin homologue ScPxl1 coordinates Cdc42 and Rho1 function during polarized growth by directly binding to Rho1-GDP [27]. In *Schizosaccharomyces pombe*, SpPxl1 modulates Rho1 GTPase signaling and plays a role in formation and contraction of the actomyosin ring during cytokinesis by interaction with Rho1, Myo2 and Cdc15 [26,28]. In *Ashbya gossypii*, the paxillin-like protein AgPxl1 plays a role in apical branching in hyphae [29]. Together with Rho-GTPases and the formin protein AgRgL1, AgPxl1 also regulates spine length and spine wall integrity by directly interacting with AgRho1a and AgRho1b [30].

Two LIM proteins, Lrg1 and Rga1, which contain several LIM domains at the N-terminus and an extra RhoGAP domain at the C-terminus, have been identified in yeasts and the filamentous fungus *Neurospora crassa*. In *S. cerevisiae*, ScLrg1 is highly expressed in sporulating cells and may play a role during mating [31]. ScLrg1 has a specialized RhoGAP domain and negatively regulates 1, 3-β-glucan synthesis leading to an increase in 1, 3-β-glucan deposition in *A. niger* strains. It is therefore involved in the PKC1-mediated cell integrity pathway [32–34]. Disruption of *S. cerevisiae* ScLrg1 in haploid cells results in enhanced invasive growth and a strain-specific ‘clustered’ phenotype that is a consequence of failed infection-related morphogenesis and pathogenicity of the rice blast disease. To understand the function of genes encoding LIM proteins in *M. oryzae*, targeted gene deletion mutants of each LIM domain protein-encoding gene were generated and confirmed by Southern blot analysis (Fig. S1). Gene deletion mutants of *Rga1* and *Ldp1* were generated from the wild-type strain Guy11 [42]. However, we were unable to obtain *Lrg1* and *Pax1* gene deletion mutants from Guy11 after examining more than three hundred transformants from various independent transformation experiments. We therefore used the isogenic *M. oryzae* strains. It is therefore involved in the determination of the role of this signaling pathway in the fungus and to shed light on the wider role of the LIM domain in fungal development and pathogenicity. Our results reveal important roles for the LIM domain family of protein in rice blast disease.

**Results**

**Identification of four genes putatively encoding LIM proteins in *M. oryzae***

Bioinformatics analysis revealed four genes putatively encoding LIM domain-containing proteins in the *M. oryzae* genome as shown in Fig. 1A. These were named Pax1 (*M. oryzae* paxillin homolog), Ldp1 (*M. oryzae* LIM-domain-containing protein), Lrg1 (*M. oryzae* LIM and RhoGAP) and Rga1 (*M. oryzae* Rho GTPase activator) (termed *Lrg2* in Li et al. 2010a). Based on the phylogenetic analysis of LIM domain regions of LIM proteins from different fungal species, the four predicted LIM proteins in *M. oryzae* could be divided into four distinct clades as shown in Fig. 1B. The proteins were diverse in amino acid identity, but the LIM domain regions were more highly conserved. For instance, the Pax1 LIM domains were 83, 81, 80 and 79% identical to those in predicted paxillins from *Gaunannomyces graminis*, *Thielavia terrestris*, *Myceliophthora thermophila* and *N. crassa*, but only 17% identical to paxillin from *S. cerevisiae* (Fig. 1B).

**Targeted deletion of *PAX1*, *LRG1*, *RGA1* and *LDP1* in *M. oryzae***

To understand the function of genes encoding LIM proteins in *M. oryzae*, targeted gene deletion mutants of each LIM domain protein-encoding gene were generated and confirmed by Southern blot analysis (Fig. S1). Gene deletion mutants of *Rga1* and *Ldp1* were generated from the wild-type strain Guy11 [42]. However, we were unable to obtain *Lrg1* and *Pax1* gene deletion mutants from Guy11 after examining more than three hundred transformants from various independent transformation experiments. We therefore used the isogenic *A. niger* strains. *A. niger* mutants displayed a significant reduction in growth with the wild-type strain Guy11 as recipient strains for deletion of *Lrg1* and *Pax1* because they show high rates of homologous recombination [43,44]. Hereafter, both *A. niger* (Ku70) and *A. niger* (Ku80) are described as “wild type” strains due to their phenotypic similarity to Guy11 [43,44]. As listed in Table S1, we obtained three independently generated targeted gene replacement mutants for each of the four LIM protein genes, and selected LP55 (*Apax1*), LR80 (*Alrg1*), LG25 (*Arga1*) and LD17 (*Adlp1*) for detailed phenotypic analysis.

**Deletion of *PAX1* or *LRG1* significantly impaired vegetative growth of *M. oryzae***

To evaluate the role of *M. oryzae* LIM proteins in hyphal growth, strains were grown on solid complete medium (CM) for 10 days and colony diameters measured (Fig. 2A; Table 1). The *Apax1* and *Aalg1* mutants displayed a significant reduction in growth with diameter of (3.0±0.1) cm and (3.5±0.1) cm, respectively, compared with the wild type strains Ku80 of (6.9±0.1) cm and Ku70 of (6.9±0.1) cm (t-test, P<0.01) (Fig. 2A; Table 1). Similarly, when incubated in liquid CM medium for 48 h, the mutants grew slowly compared to the isogenic wild-type (Fig. 2B). Growth defects were complemented by re-introduction of the *PAX1* and *LRG1* genes into *Apax1* and *Aalg1* mutants, respectively (Fig. 2; Table 1). No obvious growth difference was observed in *Arga1* or *Adlp1* mutants as shown in Fig. 2 and Table 1. These results suggest that both *PAX1* and *LRG1* are involved in hyphal growth in *M. oryzae*. 
Figure 2. Vegetative growth of the \textit{Dpax1} and \textit{Dlrg1} mutants was significantly impaired in \textit{M. oryzae}. A. The vegetative growth of the \textit{Dpax1} and \textit{Dlrg1} mutants was significantly impaired. Colonies of the \textit{Dldp1} (LD17), \textit{Drga1} (LR80 and LR95) and \textit{Dpax1} (LP55 and LP62) mutants were formed on CM plates at 25°C for 10 d; B. The \textit{Dpax1} (LP55) and \textit{Dlrg1} (LR80) mutants grew slowly in liquid CM medium and formed small mycelium masses compared with those of wild-type strains. Wild-type strains: Guy11, Ku70 (\textit{Dku70}) and Ku80 (\textit{Dku80}). Gene complementation strains: GC22 (\textit{Drga1}:RGA1), RC38 (\textit{Dlrg1}:LRG1) and PC20 (\textit{Dpax1}:PAX1). Bar = 5 mm. doi:10.1371/journal.pone.0088246.g002
Table 1. Phenotypic analysis of LIM protein mutants in *M. oryzae*.

| Strain | Growth (cm) | Conidiation (×10⁶ plate⁻¹) | Appresorium (%) | Penetration (%) |
|--------|-------------|-----------------------------|-----------------|-----------------|
|         | GB          | OE                          |                  |                 |
| Guy11   | 6.8±0.1a    | 99.7±6.0a                   | 97.0±3.6a       | 96.7±3.5a       |
| Drga1   | 6.8±0.1a    | 95.7±10.7a                  | 13.3±3.8b       | 95.7±2.5a       |
| Drga:RGA1 | 6.8±0.1a   | 98.7±8.3a                   | 96.7±4.2a       | 95.0±3.0a       |
| dldp1   | 6.9±0.1a    | 93.3±7.6a                   | 95.7±2.6a       | 94.5±4.5a       |
| Ku80    | 6.9±0.1a    | 93.3±1.5a                   | 95.7±3.5a       | 93.7±2.1a       |
| Dpax1   | 3.0±0.1d    | 0                           | 0               | 0               |
| Dpax1:PAX1 | 5.9±0.1b  | 62.3±3.1b                   | 96.3±1.5a       | 91.7±4.4a       |
| Ku70    | 6.8±0.1a    | 95.7±1.5a                   | 94.7±4.0a       | 92.3±4.5a       |
| Dlrg1   | 3.5±0.1c    | 3.3±1.5c                    | 0               | 0               |
| Dlrg1:LRG1 | 5.9±0.1b  | 65.3±4.5b                   | 94.7±5.1a       | 93.7±3.5a       |

a. The diameter of colonies grown on CM plates at 25°C for 10 d.

b. The conidia washed from the 15d-old CM cultures.

c. Percentage of appressorium formation on GB (GelBond) surfaces incubated for 24 h or OE (onion epidermis) surfaces incubated for 48 h at 25°C.

d. Percentage of invasive hyphae formation from appressoria incubated on OE for 24 h at 25°C. More than 300 spores or appressoria were counted for each strain.

e. Different letters after mean values indicated significant difference at P-value of 0.05. Data were calculated from three independent experiments conducted in triplicates. Strains: Guy11, LG25 (*Drga1*), GC22 (*Drga:RGA1*), LD17 (*dldp1*), Ku80, LP55 (*Dpax1*), PC20 (*Dpax1:PAX1*), Ku70, LR80 (*Dlrg1*) and RC38 (*Dlrg1:LRG1*).

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Figure 3. The *Dpax1* mutants were unable to produce conidia and deletion of *LRG1* resulted in significant reduction in conidiation in *M. oryzae*. A. Microscopic observation of conidia and conidiophores of the cultures on solid CM medium. Bar = 0.0031 mm; B. Bar chart showed conidial production of different strains on solid CM medium. Conidia per plate were carefully harvested from 15d-old cultures. Data were calculated from three independent experiments conducted in triplicates. Different small letters indicated significant difference at P-value of 0.05. The strains used for the analysis were Guy11, Ku80, Ku70, LP55 (*Dpax1*), LR80 (*Dlrg1*), LD17 (*dldp1*), LG25 (*Dlrg1*), PC20 (*Dpax1:PAX1*) and RC38 (*Dlrg1:LRG1*).

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mutants (Fig. 3A). Conidia produced by the revealed a reduction in aerial conidiophores in (LR80) with 0, 1 or 2 septa. All data in whereas LR80 (three independent experiments conducted in triplicates. Different conidial size in length and width of different strains. LG25 (D PAX1 of M. oryzae septation in and (Fig. 4A and B), while the D lrg1 mutant was unable to produce any conidia (Table 1; Fig. 3A and B), while D lrg1 6 lrg1 104 spores/plate (Table 1; Fig. 3B). Re-introduction into D lrg1 lrg1 1.5) generated smaller conidia both in length and D lrg1 D lrg1 104 spores/plate, which was only 3% of D lrg1 D lrg1 mutant were significantly smaller with average length and width of (11.66±1.47) μm and (6.01±0.43) μm, respectively, compared to the wild type Ku70 with a length of (21.97±0.35) μm and a width of 9.10±0.27 μm (t-test, P<0.01) (Fig. 4A and B). By contrast, the D aegl mutant produced elongated conidia with (25.51±0.34) μm in length compared to the wild type Guy11 with a length of (21.90±0.35) μm (Fig. 4A and B). The D aegl mutant also produced conidia with 0, 1 or 2 septa (Fig. 4A). The proportion of 0, 1 and 2 septal conidia of the D aegl mutant was 44.6%, 45.3% and 9.8%, respectively. These effects were all complemented by re-introduction of wild type alleles of each gene as shown in Fig. 4. No effect on sexual reproduction was observed by loss of LIM domain genes (Fig. S2), suggesting that these proteins are not required for sexual sporulation in M. oryzae.

**Δlrg1 and Δpax1 mutants are unable to form appressoria**

To understand the roles of the LIM proteins in appressorium-mediated plant infection by the blast fungus, conidial suspensions of D aegl, D aegl and D lrp1 mutants were incubated on hydrophobic GelBond to induce appressorium formation, and on onion epidermis to observe tissue penetration. The wild-type strains Guy11, Ku70, Ku80 and the complementation strains D aegl:RGAl, D aegl:LRG1, Δpax1:PAx1 produced normal melanized appressoria on both plastic and onion epidermis surfaces and formed invasive hyphae on onion epidermal cells (Table 1; Fig. 5A). By contrast, the D aegl mutant failed to form appressoria or invasive hyphae (Table 1; Fig. 5A). In D aegl mutants only 13.3% of conidia formed appressoria on plastic hydrophobic surface, but D aegl mutants produced appressoria and penetrated plant cells normally (Table 1; Fig. 5A), indicating that RGA1 may be involved in surface recognition and sensing in M. oryzae, but in a manner that does not affect morphogenesis on the plant surface. Δlrg1 mutants formed appressoria and penetrated normally. Given that Δlrg1 mutants are unable to produce conidia, we prepared hyphal suspensions to induce appressorium formation on plastic hydrophobic surface. We did not observe any appressorium formation, whereas Ku80 and the complemented strain (Δpax1:PAx1) produced normal appressorium when prepared in the same way (Fig. 5B). We conclude that LRG1 and PAx1 are essential for appressorium formation and penetration in M. oryzae.

**Δpax1 and Δlrg1 mutants are unable to cause rice blast disease**

To determine whether LIM proteins are involved in pathogenicity of M. oryzae, we performed plant infection assays. In a cut-barley-leaf assay inoculated with mycelial fragments, Δpax1 and Δaegl mutants failed to produce blast disease symptoms, whereas Δaegl and Δlrg1 mutants and all complemented strains caused obvious disease (Fig. 6A). Similarly, when 2-week-old rice seedlings were spray-inoculated with conidial suspensions of different strains (or inoculated by mycelial suspension for Δaegl due to its lack of conidiation), Δpax1 and Δaegl mutants were non-pathogenic (Fig. 6B). We also performed rice root infection assays [45] and found that Δpax1 and Δaegl mutants were unable to cause disease (Fig. 6C).

**Δlrg1 and Δrga1 localize to septal pores in M. oryzae**

To analyze the localization of each LIM protein in asexual sporulation, we quantitatively measured conidial production by harvesting conidia from 15-day-old cultures of M. oryzae. The Δpax1 mutant was unable to produce any conidia (Table 1; Fig. 3A and B), while the Δaegl mutant produced significantly reduced numbers of conidia with (3.3±1.5) ×10^4 spores/plate, which was only 3% of the number of spores generated by the wild-type strain Ku70 (95.7±1.5) ×10^4 spores/plate (Table 1; Fig. 3B). Re-introduction of D PAx1 and D LRG1 into Δpax1 and Δaegl mutants complemented this phenotype, respectively (see Table 1; Fig. 3). Microscopy revealed a reduction in aerial conidiophores in Δpax1 and Δaegl mutants (Fig. 3A). Conidia produced by the Δaegl mutant were

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**Figure 4. The Δlrg1 and Δrga1 mutants produced morphologically abnormal conidia.** A. Microscopic observation of conidia from different strains: Guy11, Ku70, LR80 (Δlrg1), RC38 (Δlrg1:LRG1), LG25 (Δrga1) and GC22 (Δrga1:RGa1). Bar = 5 μm; B. Bar chart showed conidial size in length and width of different strains. LG25 (Δrga1) produced significantly longer conidia than the wild-type strain Guy11, whereas LR80 (Δlrg1) generated smaller conidia both in length and width compared to Ku70; C. Proportions of conidia of the Δlrg1 mutant (LR80) with 0, 1 or 2 septa. All data in B and C were calculated from three independent experiments conducted in triplicates. Different letters indicated significant difference at P-value of 0.05.

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**PAX1 and LRG1 are involved in conidiogenesis and septation in M. oryzae**

To analyze the roles of each LIM protein in assexual sporulation, we quantitatively measured conidial production by harvesting conidia from 15-day-old cultures of M. oryzae. The Δapax1 mutant was unable to produce any conidia (Table 1; Fig. 3A and B), while the Δaegl mutant produced significantly reduced numbers of conidia with (3.3±1.5) ×10^4 spores/plate, which was only 3% of the number of spores generated by the wild-type strain Ku70 (95.7±1.5) ×10^4 spores/plate (Table 1; Fig. 3B). Re-introduction of D PAx1 and D LRG1 into Δapax1 and Δaegl mutants complemented this phenotype, respectively (see Table 1; Fig. 3). Microscopy revealed a reduction in aerial conidiophores in Δapax1 and Δaegl mutants (Fig. 3A). Conidia produced by the Δaegl mutant were significantly smaller with average length and width of (11.66±1.47) μm and (6.01±0.43) μm, respectively, compared to the wild type Ku70 with a length of (21.97±0.35) μm and a width of 9.10±0.27 μm (t-test, P<0.01) (Fig. 4A and B). By contrast, the Δaegl mutant produced elongated conidia with (25.51±0.34) μm in length compared to the wild type Guy11 with a length of (21.90±0.35) μm (Fig. 4A and B). The Δaegl mutant also produced conidia with 0, 1 or 2 septa (Fig. 4A). The proportion of 0, 1 and 2 septal conidia of the Δaegl mutant was 44.6%, 45.3% and 9.8%, respectively. These effects were all complemented by re-introduction of wild type alleles of each gene as shown in Fig. 4.
localization of both Lrg1- and Rga1-GFP fusions (Fig. 7A and B), whereas Pax1-GFP was observed throughout the cytoplasm (Fig. 7C). To visualize LRG1 and PAX1 expression during appressorium development, conidia of the strains RC38 (Δlrg1:LRG1) and PC20 (Δpax1:PAX1) were allowed to germinate on hydrophobic GelBond. Lrg1-GFP and Pax1-GFP expression was observed during initial stages of germination and appressorium formation but decreased during appressorium maturation (Fig. S3 and Fig. S4).

Functional characterization of individual LIM domains of Lrg1 and Pax1
Lrg1 and Pax1 contain multiple LIM domains and Lrg1 contains an additional Rho-GAP domain (Fig. 1A). To determine the function of these individual domains we constructed alleles in which each domain was individually deleted and introduced these into the Δlrg1 and Δpax1 mutants. Deletion of either Lrg1-LIM2 or the Lrg1-RhoGAP domain resulted in an inability to overcome the defects of the Δlrg1 mutant in conidiation, appressorium formation and pathogenicity. Furthermore, the septal pore localization of Lrg1-GFP was not observed (Fig. 8A), suggesting that both domains are necessary for correct localization and function of Lrg1. Deletion of the Lrg1-LIM1 domain affected localization but not the function of Lrg1, while deletion of the third LIM domain, Lrg1-LIM3 had no observable effect (Fig. 8A). Taken together, these results suggest that the LIM2 domain is the most critical for function of Lrg1.

We next investigated the function of each LIM domain of Pax1 (Fig. 8B). Deletion of any LIM domain singly had no discernable effect, whereas deletion of all three LIM domains impaired...
function of Pax1 completely (Fig. 8B). This suggests that the three LIM domains of Pax1 may have redundant or overlapping functions in infection-related morphogenesis.

Deletion of PAX1 or LRG1 led to up-regulation of genes involved in cell wall biosynthesis and remodeling.

To determine whether LIM proteins are associated with regulation of cell wall integrity in M. oryzae, given their roles in sporulation and appressorium development, we exposed each mutant to exogenous hyperosmotic concentrations of NaCl and sorbitol, or to agents associated with cell wall stress, Congo Red (CR), sodium dodecyl sulfate (SDS) and H₂O₂ (Table S3). Dpax1 and Dlrg1 mutants were more tolerant to 1 M NaCl, but showed no significant difference to 1.2 M sorbitol compared with the wild type strains Ku80 and Ku70, respectively (Table S3). On CM plates with 100 mg/ml Congo Red, a low inhibition rate of (1.2 ± 0.5)% and (10.7 ± 4.4)% was observed in Dpax1 and Dlrg1 mutants, respectively, comparing with (27.8 ± 1.9)% in Ku80 and (26.9 ± 5.5)% in Ku70, respectively (Table S3). Similarly, in CM plates with 0.05% SDS, inhibition rates of (45.0 ± 2.5)% and (52.3 ± 1.9)% for Dpax1 and Dlrg1 mutants were observed, respectively, which was significantly lower than (61.5 ± 2.2)% of Ku80 and (64.3 ± 4.6)% of Ku70 (Table S3), suggesting that deletion of either PAX1 or LRG1 led to a decreased sensitivity to these cell wall-perturbing agents.

Drga1 and Dldp1 mutants had similar sensitivity to Guy11 and all mutants showed normal sensitivity to H₂O₂ (data not shown). Pax1 and Lrg1 are therefore likely to play roles in the cellular response to osmotic and cell wall integrity. To test this idea, we conducted qRT-PCR to determine the expression levels of genes associated with cell wall synthesis including chitin and glucan synthases. This revealed that the...
majority of cell wall biosynthesis-related genes (CHS1 to CHS6 and GLS1) were up-regulated in the absence of PAX1 and LRG1 (Fig. 9). We conclude that both LIM domain proteins are involved in regulation of cell wall integrity in M. oryzae.

Discussion

LIM proteins play key roles in cytoskeleton organization, organ development and cell fate determination in eukaryotes [7]. To date, a large number of LIM proteins have been characterized in animals, but very few have been reported in fungi, with reports in S. cerevisiae [25,27,31,33,34,36], Schizosaccharomyces pombe [26,28], A. gossypii [29,30], and N. crassa [37]. In this study, we characterized four genes, PAX1, LRG1, RGA1 and LDP1 in the rice blast fungus M. oryzae. Targeted gene replacement analysis revealed that Apax1 mutants showed significantly reduced in vegetative growth and were unable to produce conidia and appressoria (Fig. 2, 3 and 5B). Apax1 mutants were also incapable of causing blast disease on susceptible hosts (Fig. 6). Atlg1 mutants were also impaired in vegetative growth and unable to form appressoria or cause disease (Fig. 2, 5 and 6). These results therefore suggest that both Pax1 and Lrg1 are involved in regulating vegetative growth, conidiation, appressorium formation and pathogenicity in M. oryzae. Deletion of RGA1 resulted in only minor changes to conidial morphology and appressorium formation (Fig. 4 and 5), while deletion of LDP1 did not lead to any developmental effects. To our knowledge, this is the first study to describe LIM proteins as pathogenicity determinants in fungi.

Pax1 encodes a paxillin-like protein in M. oryzae. Previously, fungal paxillin-related proteins have been functionally identified in S. cerevisiae, where ScPxl1 localizes to sites of polarized growth and is required for selection and maintenance of polarized growth sites [25,27]. In S. pombe, SpPxl1 is a component of the fission yeast actomyosin ring, which localizes to the division area and plays a role in formation and contraction of the actomyosin ring during cytokinesis [36,28]. LIM domains are necessary for its function [28]. In A. gossypii, the paxillin-like protein AgPxl1 is necessary for apical branching and hyphal growth and localizes to emergence sites of new branches [29]. In addition, AgPxl1 also localizes to septa during cross wall formation but only temporarily in mature septa [29]. Recently, the conserved C-terminal LIM domains of AgPxl1 have been shown to be necessary for protein function and to contribute to tip localization [30]. We found that M. oryzae Pax1 is also crucial for hyphal growth (Fig. 2), which is similar to observation in the above fungal species. Moreover, the three LIM domains of Pax1 were essential for both its function and localization, but only when they were all present in the Pax1 protein (Fig. 8B).
Previously, co-immunoprecipitation (Co-IP) experiments have shown that SpPxl1 interacts directly with Rho1 in S. pombe and with Rho1-GDP (the inactive form of Rho1) in S. cerevisiae and A. gossypii [27,28,30]. However, we failed to observe any interactions between M. oryzae Pax1 and Rho GTPases in Y2H assays (Fig. S5). It is therefore not clear whether Pax1 can physically interact with the inactive form of Rho GTPases. It has been reported that the C-terminal LIM domains of animal paxillin were able to bind the tyrosine phosphatase PTP-PEST to target the protein to focal adhesions [46]. However, we did not find any interaction between M. oryzae Pax1 and PTP-PESTs in Y2H assays (Fig. S5). Therefore, to further understand the roles of Pax1 during fungal morphogenesis it will be necessary to carry out detailed in vivo interaction studies using co-immunoprecipitation to identify interacting partners.

M. oryzae Lrg1 belongs to a class of LIM proteins containing both RhoGAP and LIM domains. To date, only S. cerevisiae and N. crassa Lrg1 proteins have been functionally characterized in fungi. ScLrg1 shows a peak of expression during sporulation and plays a role during mating [31]. Disruption of ScLrg1 resulted in reduction of cell fusion, diploid formation and inhibition of mother-daughter separation [34,35]. In N. crassa, NcLrg1 is essential for apical tip extension and for restricting excessive branch formation in sub-apical regions of hyphae and is also involved in determining the size of the hyphal compartments [37]. Like NcLrg1, we demonstrated that M. oryzae Lrg1 is also required for cell compartmentalization by regulating conidial shape and septation, and is necessary for normal growth, appressorium formation and pathogenicity.

Previously, domain functional analysis by site-directed mutagenesis has provided evidence that the three LIM domains and RhoGAP domain of Neurospora Lrg1 were both essential for its function of growth and septation, but only the LIM domains were crucial for localization of NcLrg1 protein to hyphal tips and septal pore [37]. Consistently, we found that Lrg1 expressed specifically at septal pores in hyphae and conidia, both Lrg1-LIM2 (the 2nd LIM) and Lrg1-RhoGAP affected function and localization of Lrg1 (Fig. 7 and 8). In addition, S. cerevisiae Lrg1 has been reported to specifically interact with the active form of Rho1 in Y2H analyses [32]. Another report also revealed that the RhoGAP domain of the ScLrg1 protein (containing its putative GAP domain and some flanking sequences) can interact with the mutant form of Rho1 (Rho1LQ60H/T206S), a hyperactive derivative which mimics the GTP-bound form [33]. However, in this study, we did not detect any interactions between M. oryzae Lrg1 and Rho GTPases in Y2H assays (data not shown). Whether Lrg1 interacts with the active form of Rho1 and other Rho GTPases will be explored in the near future.

Rga1 also contains a RhoGAP domain, but deletion of RGA1 and LRG1 led to distinct phenotypic changes, although the two proteins displayed the same cellular localization to septal pores. In S. cerevisiae, ScRga1 interacts with Cdc42 and activates the pheromone-response pathway [36]. However, no interaction was detected between Rga1 and Cdc42 in M. oryzae (data not shown). Whether Rga1 is able to interact with the constitutively active Cdc42 GTPase remains unknown.

Determining the precise function of each LIM domain protein will require specific identification of their target proteins and interacting partners, which is currently underway.

**Materials and Methods**

**Strains, culture conditions and molecular manipulations**

Wild-type and recombinant strains of M. oryzae used in this study are listed in Table S1. Standard growth and storage procedures for fungal strains were performed, as described previously [47]. To prepare mycelial suspensions, mycelium was harvested from 48 h liquid CM cultures. *Escherichia coli* strain DH-5α was used for routine bacterial transformations and maintenance of all plasmids used in this study. Southern blot analysis was performed by the digoxigenin (DIG) high prime DNA labeling and detection starter Kit I (Roche, Mannheim, Germany). General procedures for nucleic acid analysis followed standard protocols [48].

**Construction of gene knockout vectors and generation of gene deletion mutants**

Primers for constructing gene deletion vectors are listed in Table S2. Approximate 1 kb up- and down-stream region of each targeted gene and 1.5 kb *byC-HPH* cassette were amplified from *M. oryzae* genome and cloned into pCB1003. Using a similar construction strategy, as described previously [49], targeted gene deletion vectors of the four LIM protein genes were constructed. *M. oryzae* protoplasts were prepared by digesting mycelium with Glucanex (Novozyme Switzerland AG) and harvested protoplasts diluted to 10^6 cells/ml in STC buffer (0.6 M sorbitol, 10 mM Tris-HCl pH7.5, 10 mM CaCl2) for fungal transformation. Hygromycin resistant transformants were selected and gene deletion events analyzed by PCR amplification and confirmed by Southern blot.

**Gene complementation assays and functional analysis of various domains**

To construct complementation vectors of *PAX1, LRG1 and RGA1*, full length gene-coding sequence of each gene and promoter region (~1.5 kb) was amplified and cloned into pGEM-T (Promega). A 1.4 kb GFP allele was amplified and ligated in-frame to the C-terminus of each gene. Finally, each resulting fragment containing promoter-ORF-GFP was cloned into pCB1532 [50] to generate the complementation vector. The resulting vectors were transformed into *Apax1*, *Alrg1* and *Arg1* mutants, respectively. Transformants were screened for sulfonylurea resistance on BDCM.

Based on corresponding gene complementation vectors, LIM domain deletion vectors were constructed by overlapping extension PCR, as described previously [51]. Resulting vectors were transformed into *Apax1* and *Alrg1* mutants, respectively. Transformants were screened for sulfonylurea resistance on BDCM.

**Fungal growth, sporulation, appressorium development assays and genetic crosses**

Vegetative growth was assessed by measurement of colony diameters in plate cultures of *M. oryzae* grown on CM medium at 25°C for 10 d. Osmotic, oxidative and cell wall integrity assays were carried out in CM agar supplemented with 1 M NaCl, 1.2 M sorbitol (Amresco), 50 μg/ml Congo Red (Sigma), 0.05% sodium dodecyl sulfate (SDS) and 5 mM H2O2 (Sigma), respectively. Inhibition rates (%) were calculated as follows: (D0−D1)/D0×100, D0 and D1 represent diameters of the cultures on CM medium at 25°C for 10 d with and without exogenous treatment, respectively. Conidiogenesis was analyzed by harvesting conidia from the surface of 15-day-old plate cultures and determining the concentration of resulting conidial suspension using a haemocytometer.
Conidial shape and size were observed and measured by optical microscopy (Olympus, CX21). For appressorium formation and penetration assays, 20 μl conidial suspensions of $1 \times 10^5$ spores/ml were dropped onto hydrophobic GelBond film and onion epidermis surfaces and cultured at 25°C for 24 h or 48 h. The percentage of conidia forming appressoria was determined by microscopic examination of at least 300 conidia or appressoria.

Fertility assays were carried out by pairing Guy11 (MAT1-2, Ku70, Ku80 and isogenic mutants with the standard tester strain MAT1-1) on oatmeal agar (OMA) plates, as described previously [52,53]. Junctions between mated individuals were repeated at least three times.

Pathogenicity assays

For barley infection assay, mycelium was prepared and used to inoculate 1-week-old cut barley leaves of cultivar Golden Promise. Mycelium was placed onto barley leaves and incubated in a humid chamber at 25°C. Disease lesions were examined at 7 d after inoculation. For rice infection assays, conidial suspensions were diluted in 0.2% gelatin to $1 \times 10^5$ conidia/ml and 5 ml of each conidial suspension spray-inoculated onto 2-week-old rice seedlings. Conidia of the strain PC20 (pax1:pax1 dIII) were crossed with TH3 (MAT1-1) on oatmeal agar (OMA) plates, as described previously [52,53]. Junctions between mated individuals were repeated at least three times.

Quantitative RT-PCR analysis

Quantitative RT-PCR (qRT-PCR) was performed consistent with the guidelines for minimum information for publication of quantitative Real-Time PCR experiments (MIQE) [54]. Total RNA was isolated from mycelium using RNAiso Plus reagent (TaKaRa) and used to synthesize first-strand cDNA using PrimeScript<sup>®</sup> RT (TaKaRa). RT-PCR was performed with SYBR<sup>®</sup> Premix Ex Taq™ Kit (TaKaRa) using the BIO-RAD CFX96™ Real-Time System. Primers used for qRT-PCR assays are listed in Table S2. The relative expression level of each gene was calculated as the 2$^{-\Delta\Delta Ct}$ method [55] with the histone gene MGG_01160.6 as reference. Mean and standard deviation were determined with data from three replicates.

Yeast two-hybrid (Y2H) assay

Y2H assay was performed according to the BD Matchmaker Library Construction & Screening Kits instructions (Clontech, PaloAlto, CA, USA). Full-length cDNAs of each candidate gene were amplified with primers listed in Table S2. The Pax1 cDNA was cloned into bait plasmid pGBK and cDNAs of Roh1-5, Cdc42, Rac1 and PTP-PEST1-3 were respectively cloned into prey plasmid pGAD. The resulting bait vector and each prey vector were co-transformed into yeast strain AH109. Growth of yeast transformants was determined on SD-Trp-Leu-His-Ade medium.

Supporting Information

Figure S1 Gene deletion of LIM protein genes and confirmation. A, LDP1 deletion strategy (left) and confirmation by Southern blot (right). Genomic DNA was digested with XbaI and probed with upstream flanking sequence of LDP1. Lane 1, wild type strain; Lane 2 and 3, LDP1<sup>-</sup>; lane 4 and 5, ectopic transformants. X, XbaI; P, PstI; SpeI, SpeI; E, EcoRI; N, NdeI. B, PAX1 deletion strategy (left) and confirmation by Southern blot (right). Genomic DNA was digested with HindIII and probed with upstream flanking sequence of PAX1. Lane 1, wild type strain; Lane 2 to 4, PAX1<sup>-</sup>; lane 5, ectopic transformant. H, HindIII; P, PstI; SpeI, SpeI; E, EcoRI. C, RGA1 deletion strategy (left) and confirmation by Southern blot (right). Genomic DNA was digested with SacI and probed with upstream flanking sequence of RGA1. Lane 1, wild type strain; Lane 2 to 4, RGA1<sup>-</sup>; lane 5, ectopic transformant. Sac, SacI; P, PstI; SpeI, SpeI; E, EcoRI. D, LRG1 deletion strategy (left) and confirmation by Southern blot (right). Genomic DNA was double-digested with SalI and KpnI and probed with downstream flanking sequence of LRG1. Lane 1, wild type strain; Lane 2 and 3, LRG1<sup>-</sup>; lane 4, ectopic transformant. K, KpnI; Sal, SalI; P, PstI; Spe, SpeI; E, EcoRI. Asterisk represents restriction sites introduced or derived from vectors.

Figure S2 Fertility assay of LIM protein mutants. The four LIM protein mutants, Aldp1 (LD17), Apax1 (LP53), Alg1 (LR80) and Anga1 (LG25) were crossed with TH3 strain, respectively. Numerous black perithecia were observed at the junction of different crosses, indicating that these LIM proteins are not required for sexual reproduction by M. oryzae.

Figure S3 Patterns of LRG1 expression during appressorium development. Conidia of the strain RC38 (lrp1:Lrg1) was allowed to germinate on hydrophobic GelBond film surfaces. Photographs were taken at various time intervals. BF = bright field. Scale bar = 10 μm.

Figure S4 Patterns of PAX1 expression during appressorium development. Conidia of the strain PC20 (Apax1:PAX1) was allowed to germinate on hydrophobic GelBond film surfaces. Photographs were taken at various time intervals. BF = bright field. Scale bar = 10 μm.

Table S1 Wild-type and recombinant strains of M. oryzae used in this study.

Table S2 PCR primers used in this study.

Table S3 Inhibitory effects of various chemicals on vegetative growth of Apax1 and Anga1 mutants.

Author Contributions

Conceived and designed the experiments: YL X. Yue YQ X. Yan ZM ZW. Performed the experiments: YL X. Yue YQ X. Yan ZM ZW. Analyzed the data: YL NJT ZW. Wrote the paper: YL NJT ZW.
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