Acetolactate synthases Mollv2 and Mollv6 are required for infection-related morphogenesis in *Magnaporthe oryzae*

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SUMMARY

Amino acids are important components in the metabolism of a variety of pathogens, plants and animals. Acetolactate synthase (ALS) catalyses the first common step in leucine, isoleucine and valine biosynthesis, and is the target of several classes of inhibitors. Here, Mollv2, an orthologue of the *Saccharomyces cerevisiae* ALS catalytic subunit Ilv2, and Mollv6, an orthologue of the *S. cerevisiae* ALS regulatory subunit Ilv6, were identified. To characterize Mollv2 and Mollv6 functions, we generated the deletion mutants Δ Mollv2 and Δ Mollv6. Phenotypic analysis showed that both mutants were auxotrophic for leucine, isoleucine and valine, and were defective in conidial morphogenesis, appressorial penetration and pathogenicity. Further studies suggested that Mollv2 and Mollv6 play a critical role in maintaining the balance of intracellular amino acid levels. Mollv2 and Mollv6 are both localized to the mitochondria and the signal peptide of Mollv6 is critical for its localization. In summary, our evidence indicates that Mollv2 plays a crucial role in isoleucine and valine biosynthesis, whereas Mollv6 contributes to isoleucine and leucine biosynthesis; both genes are required for fungal pathogenicity. This study indicates the potential of targeting branched-chain amino acid biosynthesis for anti-rice blast management.

INTRODUCTION

Magnaporthe oryzae is the causal agent of rice blast (Dean *et al.*, 2005; Wilson and Talbot, 2009). The conidia of *M. oryzae* play an important role in the disease cycle, whereas appressoria are critical in the initiation of disease symptoms. An understanding of the molecular mechanisms involved in conidiation and appressorium penetration-mediated plant infection is pivotal for the production of new strategies for disease management. Recent advances in genome sciences in plant pathogens, such as *M. oryzae*, have promoted our understanding of the molecular events leading to fungal pathogenicity, and of the design and implementation of new disease control strategies (Dean *et al.*, 2005; Howard and Valent, 1996; Wilson and Talbot, 2009).

The branched-chain amino acids (BCAAs) are synthesized by plants, algae, fungi, bacteria and archaea, but not by animals. Therefore, the enzymes of this pathway are potential target sites for the development of antifungal agents, antimicrobials and herbicides (Chipman *et al.*, 1998; McCourt and Duggleby, 2006). As acetolactate synthase (ALS) is the first enzyme in the BCAA biosynthetic pathway, most research has focused on this enzyme, which catalyses the first common step in the biosynthesis of valine, leucine and isoleucine in microorganisms and plants. Previously, many reports have demonstrated that ALS catalyses two parallel reactions: the condensation of two molecules of pyruvate to give rise to 2-acetolactate in the first step of the valine and leucine biosynthetic pathway, and the condensation of pyruvate and 2-ketobutyrate to yield 2-aceto-2-hydroxybutyrate in the second step of isoleucine biosynthesis (Duggleby and Pang, 2000; Leavitt and Umbarger, 1961).

The ALSs of the budding yeast *Saccharomyces cerevisiae* are involved in the synthesis of BCAAs, such as leucine, isoleucine and valine. ALS catalyses the first step in the biosynthesis of BCAAs. In fungi, the ALS enzyme contains a catalytic subunit of 60–70 kDa and a regulatory subunit that varies in size depending on the species and isoform (Pang and Duggleby, 1999, 2001). Both the catalytic and regulatory subunits are nuclear encoded and targeted to the mitochondria as a result of the presence of a mitochondrial signal peptide (Cassady *et al.*, 1972; Lee and Duggleby, 2006; Polaina, 1984; Sherman *et al.*, 1974). As no ALS has been identified in animals, inhibitors of ALS, aiming to inhibit the BCAA biosynthetic pathway, have been explored as potential antifungal drug targets (Chipman *et al.*, 1998; Goldstein and McCusker, 2001; McCourt and Duggleby, 2006). In *S. cerevisiae* and the human fungal pathogen *Cryptococcus neoformans*, mutation of the *ILV2* genes resulted in a loss of viability on nutrient-poor Yeast Nitrogen Base (YNB) medium and the *C. neoformans* Δ ilv2 mutant was also avirulent (Kingsbury *et al.*, 2004). The *Candida albicans* Δ ilv2 mutant displayed a dramatic decline in viability during isoleucine and/or valine starvation, and was also attenuated significantly in virulence (Kingsbury and McCusker, 2010). As amino acid biosynthesis and degradation are very complex processes and play a crucial role in the growth and development of the organisms, we examined the role of Mollv2- and Mollv6-mediated amino acid metabolism in the growth and pathogenicity of *M. oryzae*.

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RESULTS

Identification of Mollv6 and its functional partner Mollv2

Amino acids are important components in the metabolism of a variety of pathogens, plants and animals. MGG_01104, an orthologue of *S. cerevisiae* Ilv6p involved in valine, leucine and isoleucine biosynthesis, was identified by BLAST analysis and named 'Mollv6'. Mollv6, encoding a 316-amino-acid protein, is a regulatory subunit of ALS that possesses an N-terminal ACT (aspartate kinase, chorismate mutase and TyrA) domain and a C-terminal ALS domain. It is predicted to possess a signal peptide and a phosphorylation site (PRSP, Fig. 1A). Mollv6 showed 84%, 60%,

61%, 80% and 71% amino acid identity with Ilv6 proteins found in *Glomerella graminicola*, *S. cerevisiae*, *C. albicans*, *Fusarium oxysporum* and *Aspergillus fumigatus*, respectively. However, it has only 18% amino acid identity with Ilv6 of *Arabidopsis thaliana* (Fig. 1B).

Notably, the function of the ALS regulatory subunit Ilv6 depends on its catalytic partner, Ilv2. MGG_06868, an orthologue of *S. cerevisiae* Ilv2p (Kingsbury *et al.*, 2004), was also identified by BLAST analysis. Mollv2 encodes a 682-amino-acid protein and contains three PFAM domains: TPP_enzyme_N, TPP_enzyme_M and TPP_enzyme_C (Fig. 1A). Mollv2 exhibited 68%, 76%, 79% and 60% amino acid identity with Ilv2 homologues in *Aspergillus oryzae*, *Verticillium dahliae*, *G. graminicola* and *S. cerevisiae* S288c, respectively.

Fig. 1 Structure prediction and phylogenetic tree analysis of Mollv2 and Mollv6. (A) Schematic representation of domains identified in Mollv2 and Mollv6. TPP_enzyme_N, TPP_enzyme_M, TPP_enzyme_C represent Thiamine PyroPhosphate enzyme N-, M- and C-terminal TPP binding domains, respectively. SP, signal peptide. The archetypical ACT domain is found in the C-terminal regulatory domain of 3-phosphoglycerate dehydrogenase (3PGDH). ALS_ss_C is the C-terminal half of a family of proteins, which are the small subunits of acetolactate synthase. (B) Phylogenetic trees of Mollv2 (left) and Mollv6 (right) homologues from several other species were drawn by the divergence distance method using the CLUSTAL_W and MEGA 4 programs. Neighbour-joining tree with 1000 bootstrap replicates of phylogenetic relationships. Species names and GenBank accession numbers are as follows: left: XP_370371.1 (*Magnaporthe oryzae* Mollv2); EGU74990.1 (*Fusarium oxysporum*); EFQ31228.1 (*Glomerella graminicola*); XP_962652.2 (*Neurospora crassa*); XP_001819738.2 (*Aspergillus oryzae*); NP_013826.1 (*Saccharomyces cerevisiae*); XP_721692.1 (*Candida albicans*); XP_759386.1 (*Ustilago maydis*); AAK83371.1 (*Cryptococcus neoformans*); NP_190425.1 (*Arabidopsis thaliana*); right: XP_368140.1 (*M. oryzae* Mollv6); EFQ30072.1 (*Glomerella graminicola*); EGU76155.1 (*Fusarium oxysporum*); XP_956872.1 (*Neurospora crassa*); XP_001821356.1 (*Aspergillus oryzae*); NP_009918.1 (*Saccharomyces cerevisiae*); EEQ45326.1 (*Candida albicans*); XP_761168.1 (*Ustilago maydis*); XP_568613.1 (*Cryptococcus neoformans*); NP_850173.2 (*Arabidopsis thaliana*).

***MolLV2* and *MolLV6* are components of the multiple amino acid synthesis pathways**

In *S. cerevisiae* and *C. albicans*, the $\Delta Scilv2$ mutants exhibited a starvation-cidal phenotype (Kingsbury and McCusker, 2010). We therefore hypothesized that the disruption of *MolLV2* and *MolLV6* in *M. oryzae* would produce a similar result. The $\Delta Moilv2$ and $\Delta Moilv6$ mutants were obtained by targeted gene deletion via homologous recombination. When they were cultured on minimal medium (MM) agar plates, the $\Delta Moilv2$ mutant was almost unable to grow at day 5, whereas the growth of the $\Delta Moilv6$ mutant was noticeably slower than that of the wild-type. When isoleucine, leucine and valine were supplemented to MM, the growth defect of both mutants was mitigated to the level of the wild-type strain (Fig. 2A, B).

We further determined whether the growth defect of the mutants on MM was caused by a lack of one or several amino acids. When leucine, isoleucine and valine were added individually to MM, the results showed that supplementation of each single amino acid had no effect on mycelium growth of the $\Delta Moilv2$ mutant. We next assessed how the $\Delta Moilv2$ mutant reacted to the addition of two optional amino acids, and the results showed that $\Delta Moilv2$ responded only to the simultaneous addition of isoleucine and valine (Fig. 2C, D). We also assessed how the $\Delta Moilv6$ mutant reacted to supplementation of the amino acids. Compared with the wild-type Guy11, the mycelial growth of the $\Delta Moilv6$ mutant could be partly restored with only valine or by any two optional amino acids (Fig. 2E, F). We concluded that *MolLV2* was responsible for the synthesis of isoleucine and valine, whereas *MolLV6* contributed to valine, isoleucine and leucine biosynthesis. In addition, amino acid deficiency was the main reason for the growth defects seen in the $\Delta Moilv2$ and $\Delta Moilv6$ mutant strains.

***MolLV2* and *MolLV6* are involved in aerial hyphal growth, pigmentation, conidial morphogenesis and pathogenicity**

To gain an insight into the possible functions of *MolLV2* and *MolLV6* in the developmental stages of *M. oryzae*, we first examined the transcription levels of *MolLV2* and *MolLV6* during hyphal, conidial and infectious stages by quantitative reverse transcription-polymerase chain reaction (RT-PCR). Compared with the hyphal stage, the expression level of *MolLV2* was increased significantly in conidial (>six-fold) and infectious (>four-fold) stages. Similar to *MolLV2*, *MolLV6* was increased markedly in the conidial (>1.5-fold) stage, but decreased significantly in infectious (>three-fold) stages (Table S1, see Supporting Information). These results indicated that both *MolLV2* and *MolLV6* probably play crucial roles in conidiogenesis and infection of the host plant. To further evaluate the roles of *MolLV2*

and *MolLV6* in the growth and development of *M. oryzae*, the $\Delta Moilv2$ and $\Delta Moilv6$ mutants were generated and verified. Both mutants were first cultured on complete medium (CM), MM, V8 juice agar medium (V8), and straw decoction and corn (SDC) medium (Zhang *et al.*, 2010) to test the colony morphology and growth. The $\Delta Moilv2$ and $\Delta Moilv6$ mutants exhibited a significantly reduced colony growth rate and lacked aerial hyphae and pigmentation on MM, V8 and SDC media, in comparison with the wild-type Guy11. No significant difference was found in growth between the $\Delta Moilv2$ and $\Delta Moilv6$ mutants on CM, as both showed similarly reduced aerial hyphae and pigmentation at day 7 (Fig. 3A–C). No difference in mycelial dry mass was found when cultured in liquid CM (Fig. 3D). These results indicate that *MolLV2* and *MolLV6* play a role in the response to nutrition during vegetative growth, and both are necessary for aerial hyphal growth and colony pigmentation.

Conidia and appressoria are very important infection-associated structures of *M. oryzae*. To examine sporulation in the $\Delta Moilv2$ and $\Delta Moilv6$ mutants, we observed conidia formation on SDC medium at 24 h post-conidial induction and again at day 7. Strikingly, no conidia were found in either mutant (Fig. 4A). When stained with lactophenol aniline for conidiophore formation (Zhou *et al.*, 2009), no conidiophore stalks were found in either mutant strain (Fig. 4B). Consistent with the essential role of *MolLV2* and *MolLV6* in conidial morphogenesis, the expression of several conidiation-associated genes, including *MoCOS1*, *MoCON2*, *MoCON7*, *MoCOM1*, *MoHOX2* and *MoSTUA*, was down-regulated ($P < 0.01$) in the $\Delta Moilv2$ and $\Delta Moilv6$ mutants (Fig. 4C). As no conidia were produced by either mutant strain, mycelium was used to inoculate the detached barley leaves and wounded and nonwounded rice leaves for pathogenicity assessment. After 5 days, no disease symptoms were found on surfaces, in contrast with the wild-type Guy11 and the complemented strains (Fig. 5A, B), indicating that *MolLV2* and *MolLV6* are essential for the pathogenicity of *M. oryzae*.

Because appressoria are important infectious structures in the infection of rice plants by *M. oryzae*, we assessed whether the $\Delta Moilv2$ and $\Delta Moilv6$ mutants still develop appressoria (Kim *et al.*, 2009; Liu *et al.*, 2010). The mycelia were harvested from liquid CM culture (48 h of growth) and inoculated on barley leaves for appressorium formation. Interestingly, $\Delta Moilv2$ and $\Delta Moilv6$ mutants both developed appressoria; however, they could not extend on barley tissues at 24 and 48 h post-infection (Fig. 5C). We also further examined the pathogenicity of $\Delta Moilv2$ and $\Delta Moilv6$ mutants on rice roots. Similar to the results on barley and rice leaves, the $\Delta Moilv2$ mutant showed no virulence 9 days after inoculation. In contrast, the $\Delta Moilv6$ mutant caused typical rice blast lesions under the same conditions, similar to the wild-type and complemented strains (Fig. 5D).

Fig. 2 Growth of $\Delta Mollv2$ and $\Delta Mollv6$ mutants on minimal medium (MM) supplemented with different amino acids. (A) Wild-type Guy11, mutants and complemented strains were inoculated on MM or MM plates supplemented with amino acid (MM+); day 5 after inoculation at 28 °C. The concentrations of amino acids were as follows: leucine, 0.46 mM; isoleucine, 0.23 mM; valine, 1.28 mM. (B) The dry weights of mycelia from wild-type Guy11, $\Delta Mollv2$ mutant, $\Delta Mollv6$ mutant and complemented strains. Error bars represent standard deviation from the mean. Experiments were repeated three times with similar results. (C, E) Recovery of $\Delta Mollv2$ mutant and $\Delta Mollv6$ mutant on MM medium supplemented with amino acids 5 days after inoculation at 28 °C. (D, F) The colony radius of wild-type Guy11, mutants and complemented strains 5 days after inoculation. Ile, isoleucine; Leu, leucine; Val, valine. Each experiment was repeated three times with similar results, and asterisks represent significant difference ($P < 0.01$).

Fig. 3 *MoilV2* and *MoilV6* are required for aerial hyphal growth, but mycelium dry weights are not affected in wild-type Guy11 and mutants. (A) Colony morphology was observed on complete medium (CM), minimal medium (MM), V8 juice agar medium (V8) and straw decoction and corn (SDC) medium for 7 days at 28°C. (B) The colony diameters were measured and subjected to statistical analysis. The experiment was performed in triplicate. Error bars represent standard deviation, and asterisks represent significant differences ($P < 0.01$). (C) Aerial hyphal growth is reduced in $\Delta Moilv2$ and $\Delta Moilv6$ mutants. Strains were inoculated at 28 °C in the dark for 7 days. (D) The dry weights of mycelia from Guy11, $\Delta Moilv2$ and $\Delta Moilv6$ mutants and the complemented strains. Error bars represent standard deviation from the mean. The experiment was repeated three times with similar results.

Exogenous amino acids partly restore conidial formation, appressorium formation and pathogenicity to the $\Delta Moilv2$ and $\Delta Moilv6$ mutants

To determine whether exogenous amino acids could restore the conidial defect of the mutants, the medium was supplemented with three amino acids (leucine, isoleucine and valine) at 1× (1AA) or 5× (5AA) concentration. When 1AA amino acid was

added to SDC medium, sparse aerial hyphae and few conidia were observed in the $\Delta Moilv2$ and $\Delta Moilv6$ mutants. In contrast, many more aerial hyphae and asexual spores were observed in the mutants at high concentrations of amino acid (5AA) (Fig. 6A, B). The statistics of sporulation quantity yielded consistent results (Fig. 6C).

To further examine the pathogenic ability of the recovered conidia, assays were conducted on barley and rice seedlings. Even

Fig. 4 Disruption of *Mollv2* and *Mollv6* affects conidiophore formation and conidial production. (A) Development of conidia on conidiophores is affected by *Mollv2* and *Mollv6* deletion. Light microscopy was performed on strains grown on straw decoction and corn (SDC) medium for 7 days. Bars equal 100 μm . (B) Aerial structures stained with lactophenol aniline blue. Conidia and aerial hyphae stained blue, and conidiophores stained grey. Bars equal 30 μm . (C) Reduced expression patterns are found in six encoded conidiation-associated genes in $\Delta Mollv2$ and $\Delta Mollv6$ mutants. RNA was extracted from mycelia grown in liquid complete medium (CM) for 2 days. Error bars represent the standard deviation, and asterisks represent significant difference among the strains tested ($P < 0.01$) according to Duncan's multiple range test.

with the added high concentrations of amino acids, the conidia produced by the $\Delta Mollv2$ mutant were not sufficient for application as a spray. Hence, the conidia were dropped directly onto barley leaves. The results demonstrated that the pathogenicity defect of the $\Delta Mollv2$ mutant could be recovered slightly by the spores collected from plates with SDC medium and 1AA. Accordingly, the lesions were similar to those of the wild-type when inoculated with the spores induced by 5AA (Fig. 7A). Similarly, rice seedling assays were performed by applying the $\Delta Mollv6$ spores

recovered under 1AA or 5AA. The results demonstrated that, at 1AA, the mutant formed far fewer lesions than the wild-type Guy11. At 5AA, it was fully pathogenic (Fig. 7B).

To address whether exogenous amino acids have an effect on conidia morphology and appressorium formation, we observed conidia and appressorium formation on inductive surfaces. The results demonstrated that the $\Delta Mollv2$ and $\Delta Mollv6$ mutants produced one- or two-celled conidia, as well as three-celled normal conidia, under 1AA treatment, but produced only normal conidia under 5AA

Fig. 5 $\Delta Moilv2$ and $\Delta Moilv6$ mutants are completely nonpathogenic. (A) Mycelial plugs from wild-type strain Guy11, $\Delta Moilv2$ mutant, $\Delta Moilv6$ mutant and complemented strains were inoculated on barley segments. (B) Mycelial plugs from wild-type strain Guy11, $\Delta Moilv2$ mutant, $\Delta Moilv6$ mutant and complemented strains were inoculated on unwounded (left) and wounded (right) rice leaves. The mutants were nonpathogenic to unwounded and wounded susceptible rice. CK, inoculation with agar blocks. (C) Appressorium formation of wild-type Guy11, mutants and complemented strains on the barley leaf surface. Mycelia from wild-type Guy11, mutants and the complemented strains were cultured in liquid complete medium (CM) and inoculated on the barley leaf surface. Appressorium formation was observed by light microscopy 48 h after inoculation at 28 °C. AP, appressorium; H, hypha; IH, infectious hypha; PP, penetration peg. (D) Blast symptoms on rice roots. Arrows show necrotic lesions.

treatment. Further observations determined that these recovered conidia were able to form melanized appressoria at 24 h, and the appressorium formation rate was much higher when formed by 5AA-recovered conidia than by 1AA-recovered conidia (Fig. 7C, D).

Successful penetration of host tissue and the development of invasive hyphae require sufficient hydrostatic pressure within the appressorium. Appressorial turgor pressure was assessed using glycerol assays (Howard *et al.*, 1991; Wang *et al.*, 2005), which

Fig. 6 Δ Mollv2 and Δ Mollv6 mutants partly recovered their conidial defects with the addition of different concentrations of exogenous amino acids. (A) Development of conidia on conidiophores. Light microscopic observation was performed on strains grown on straw decoction and corn (SDC) medium supplemented with 1AA and 5AA for 7 days. Bars, 100 μm . (B) Recovery of Δ Mollv2 and Δ Mollv6 mutants on SDC medium supplemented with 1AA and 5AA. CK, no amino acids were added. (C) Conidial recovery of mutants compared with the control. Conidia produced by wild-type Guy11, mutants and complemented strains were harvested and quantified. 1AA, leucine (0.46 mM), isoleucine (0.23 mM) and valine (1.28 mM); 5AA, leucine (2.3 mM), isoleucine (1.15 mM) and valine (6.4 mM). Values indicate standard deviations from the means. Asterisks represent significant difference ($P < 0.01$).

indicated that the turgor pressure of the mutants was much lower than that of the wild-type under 1AA at the same glycerol concentration. In contrast, the turgor pressure increased to a similar level to the wild-type at 5AA under the same conditions (Fig. 7E), indicating that leucine, isoleucine and valine play a role in appressorium development. Together with the pathogenicity results above, we conclude that deletion of *MoILV2* and *MoILV6* blocks the synthesis of leucine, isoleucine and valine, which are essential for the infection-related morphogenesis of *M. oryzae*.

Functional characterization of different domains of Mollv2 and Mollv6

Amino acid sequence analysis revealed that Mollv2 contains the TPPN, TPPM and TPPC domains (Fig. 1A), which are highly conserved with Ilv2 proteins found in *S. cerevisiae* and other fungi. To further determine the individual role of these domains, we generated the domain deletion constructs *MoILV2* $^{\Delta\text{TPPN}}$, *MoILV2* $^{\Delta\text{TPPM}}$ and *MoILV2* $^{\Delta\text{TPPC}}$ -GFP and transformed them into the Δ Mollv2 knockout mutant. The resulting green fluorescent protein (GFP) transformants were screened and analysed. Phenotype analysis revealed that all three transformants had similar phenotypes to the original Δ Mollv2 mutant, with fewer aerial hyphae and less colony pigmentation, and were defective in conidiation (Fig. 8A).

Similarly, all of the defects shown by these transformants could be fully restored when exogenous amino acids were added.

The Mollv6 protein has been predicted to begin with a signal peptide targeting ALS to the mitochondria. Sequence analysis revealed that Mollv6 contains a signal peptide. In addition, Mollv6 harbours an ACT domain, an ALS domain and the PRSP phosphorylation site (Fig. 1A). The transformants expressing the *MoILV6* $^{\Delta\text{SP}}$ -GFP fusion construct were defective in the formation of aerial hyphae and in conidiation, and the transformants expressing the *MoILV6* $^{\Delta\text{ACT}}$, *MoILV6* $^{\Delta\text{ALS}}$ and *MoILV6* $^{\Delta\text{PRSP}}$ -GFP constructs were all similar in phenotype to the Δ Mollv6 mutant when cultured on CM (Fig. 8B). We also quantified the number of conidia produced by each transformant, and found that all of the transformants showed significantly decreased conidiation (Table 1).

The domains are crucial to the function of Mollv2 and Mollv6 during pathogenicity

To test whether deletion of the domains affects the function of Mollv2 and Mollv6 during pathogenicity, conidial suspensions or mycelial plugs of each transformant were inoculated onto susceptible barley or rice leaves. Because the transformants expressing the *MoILV2* $^{\Delta\text{TPPN}}$ -GFP, *MoILV2* $^{\Delta\text{TPPM}}$ -GFP and *MoILV2* $^{\Delta\text{TPPC}}$ -GFP constructs were defective in conidiation, the mycelial plugs were

Fig. 7 Pathogenicity assays of recovered conidia, appressorium development and appressorium turgor pressure measurement. (A) Conidial suspensions (5×10^4 conidia/mL) from wild-type strain Guy11 and ΔMoiLv2 mutant were dripped on barley segments. (B) Conidial suspensions (5×10^4 conidia/mL) from wild-type strain Guy11 and ΔMoiLv6 mutant were sprayed on rice leaves. The experiments were repeated three times and showed the same results. Asterisks indicate a significant difference between wild-type, ΔMoiLv2 and ΔMoiLv6 mutants at $P < 0.01$ according to Duncan's range test. (C) Appressorium development of recovered conidia on hydrophobic GelBond surfaces at the interval times indicated. Conidia were collected from straw decoction and corn (SDC) medium with different concentrations of amino acids at 28°C for 10 days. (D) Percentage of appressorium formation on the hydrophobic surfaces. (E) Statistical analysis of collapsed appressoria after 24 h of incubation in 1, 2, 3, 4 and 5 M glycerol solution for 10 min.

inoculated onto wounded or nonwounded barley leaves. The results showed that all transformants were nonpathogenic and exhibited similar results to those recorded for the ΔMoiLv2 mutant (Fig. 9A). The conidial suspensions recovered from SDC medium supplemented with amino acids were inoculated, and pathogenicity could be partly restored under 5AA conditions (Fig. 9B). These results indicate that each of the TPPN, TPPM and TPPC domains is essential for the function of *MoiLv2* during conidiation, pathogenicity and amino acid synthesis pathways in *M. oryzae*.

We further tested the pathogenicity of the transformants expressing *MoiLv6*^{ASP}-GFP, *MoiLv6*^{ACT}-GFP, *MoiLv6*^{ALS}-GFP and *MoiLv6*^{PRSP}-GFP constructs. The *MoiLv6*^{ASP}-GFP transformants were unable to produce conidia, and so the mycelial plugs were

inoculated onto wounded or nonwounded rice leaves. The transformants were completely incapable of causing blast disease on rice leaves, even on wounded leaves (Fig. 9A), but the conidia recovered under 5AA conditions caused typical lesions (Fig. 9D). For the *MoiLv6*^{ALS}-GFP transformant, fewer conidia were collected from SDC medium, and so the conidial suspension was dropped onto detached barley leaves. This transformant was still able to cause disease lesions, but the lesion size was much smaller than those produced by the wild-type (Fig. 9C). Moreover, the conidial suspension recovered with 5AA supplementation restored the pathogenicity to the same level as the wild-type on rice leaves (Fig. 9D). The same assays were conducted on the *MoiLv6*^{ACT}-GFP and *MoiLv6*^{PRSP}-GFP transformants. Compared with the wild-type,

Fig. 8 Functional characterization of different domains of Mollv2 and Mollv6. (A) Colony morphology was observed on complete medium (CM) agar plates in the dark for 5 days at 28 °C, and then photographed. (B) Phenotypes of various mutant strains were observed on straw decoction and corn (SDC) medium supplemented with different concentrations of amino acids at 28 °C for 10 days, and then photographed. CK, no amino acids were added.

Table 1 Characterization of MollV2 and MollV6 domain-deleted mutants in *Magnaporthe oryzae*.

Strains	Conidial size (μm)*		Conidiation (10 ³ /cm ²)†		
	Length	Width	CK	+1AA	+5AA
Guy11	26.9 ± 1.7A‡	10.6 ± 1.1A	28.0 ± 0.8A	24.0 ± 0.6A	45 ± 0.8A
Δ Mollv2	—	—	—	0.19 ± 0.05E	1.1 ± 0.1E
MollV2 Δ TPPN	—	—	—	0.2 ± 0.06E	0.8 ± 0.1E
MollV2 Δ TPPM	—	—	—	0.08 ± 0.05E	1.7 ± 0.2E
MollV2 Δ TPPC	—	—	—	0.05 ± 0.02E	0.5 ± 0.04E
Δ Mollv6	—	—	—	3.2 ± 0.4D	7.7 ± 0.4CD
MollV6 Δ SP	—	—	—	7.0 ± 1.0C	12.0 ± 1.4C
MollV6 Δ ACT	20.2 ± 1.5C	10.5 ± 1.3A	16.3 ± 0.9B	13.4 ± 0.9B	21.3 ± 1.03B
MollV6 Δ ALS	22.9 ± 1.3B	10.5 ± 1.1A	0.1 ± 0.02C	1.9 ± 0.2DE	4.1 ± 0.6DE
MollV6 Δ PRSP	20.5 ± 1.1B	9.4 ± 1.0B	3.4 ± 0.6C	15.6 ± 1.0B	19.9 ± 0.6B

*Sizes of conidia were determined from three independent experiments with over 100 conidia in each repeat.

†Conidiation was measured by counting the number of conidia collected with sterilized distilled water from 10-day-old plates.

‡Duncan's range test was used to determine significance at $P < 0.01$. The same letters in a column denote no significant difference.

—, not determined; CK, no amino acids were added.

Fig. 9 Pathogenicity test of *Mollv2* and *Mollv6* domain-deleted mutants. (A) Mycelial plugs were inoculated on rice leaves, including unwounded (a) and wounded (b) treatment. (B) Recovered conidia from *MoILV2*^{ΔTPPN}, *MoILV2*^{ΔTPPM} and *MoILV2*^{ΔTPPC} mutants were dripped on barley leaves and photographed after 7 days. (C) Recovered conidia from *Moilv6*^{ΔALS} mutant were dripped on barley leaves and photographed after 5 days. CK, inoculation with distilled water. (D) Conidial suspensions from wild-type strain Guy11 and *Moilv6*^{ΔSP}, *Moilv6*^{ΔACT} and *Moilv6*^{ΔPRSP} mutants were sprayed on rice leaves and photographed after 7 days. Conidia were collected from straw decoction and corn (SDC) medium with different concentrations of amino acids at 28 °C for 10 days, and conidia were counted with a haemocytometer at a concentration of 5×10^4 conidial/mL. The experiments were repeated three times and showed the same results.

fewer lesions were formed in both transformants, and the pathogenicity of the mutants could be restored when different concentrations of amino acids were added (Fig. 9D). Taken together, the signal peptide, ACT, ALS domains and PRSP site are all essential for the function of *Mollv6*, including pathogenicity, in *M. oryzae*.

Co-localization of different functional domains of *Mollv2* and *Mollv6*

To evaluate the subcellular localization of *Mollv2* and *Mollv6* in *M. oryzae*, a GFP was fused with the C-terminus of the *MoILV2*

and *MoILV6* coding sequences and introduced into the mutant strains. Green fluorescence was observed in all the transformants, indicating that *Mollv2*-GFP and *Mollv6*-GFP fusion proteins might be located in the mitochondria. Subsequently, the fluorescence signals were detected by the specific fluorescent dye of mitochondria under a confocal laser scanning microscope. The observation of co-localization confirmed that *Mollv2* and *Mollv6* were both localized in the mitochondria (Fig. 10A).

To explore the role of the domains of the *Mollv2* protein, we generated *MoILV2* TPPN, TPPM and TPPC domain-specific mutant alleles and transformed them into the Δ *Moilv2* mutant.

Fig. 10 Co-localization of different functional domains of Mollv2 and Mollv6. (A) Co-localization of conidia and growing hyphae was detected in Δ Mollv2 mutants. (B) Co-localization of conidia and growing hyphae was observed in Δ Mollv6 mutants. Bars, 10 μ m. In the merged image, the original green fluorescent protein (GFP) is green, red is MitoTracker Red CMXRos stain and yellow denotes the co-localized spots. Mycelia were incubated with 100 nM MitoTracker Red CMXRos for 10 min at room temperature; the conidia were collected and then stained for 25 min. Mycelia or conidia were then washed twice with phosphate-buffered saline (PBS) and fixed in 10% formalin buffered in PBS at room temperature. Bars, 10 μ m. DIC, differential interference contrast image.

Co-localization of the fluorescence signals in the mitochondria of these strains indicated that the protein localization was not affected by deletion of the individual domains (Fig. 10A). The same method was used to determine the role of the predicted signal peptide in Mollv6, which indicated that *Mollv6*^{ASP}-GFP was localized to the cytoplasm instead of the mitochondria (Fig. 10B). Similarly, strains with the *Mollv6*^{ΔACT}-GFP, *Mollv6*^{ΔALS}-GFP and *Mollv6*^{ΔPRSP}-GFP domain-specific mutant alleles all indicated that the ACT domain, the ALS domain and the phosphorylation site PRSP were not crucial for the mitochondrial localization of Mollv6 (Fig. 10B). Taken together, we have demonstrated that Mollv6 exists in mitochondria for normal functions, and all domains must be present for infection-related morphogenesis in *M. oryzae*.

DISCUSSION

In previous reports, *S. cerevisiae* and *C. neoformans* Δ ilv2 mutants have been shown to be starvation-cidal and unable to survive *in vivo* and/or avirulent (Kingsbury *et al.*, 2004). Subsequently, other studies have demonstrated that *C. albicans* Δ ilv2 mutants die at profoundly rapid levels following isoleucine and valine starvation, and are also highly attenuated in virulence (Kingsbury and McCusker, 2010). Despite these studies, an Ilv2 homologue has not been functionally characterized in any phytopathogenic fungus. Here, we identified the ALS catalytic subunit Mollv2 and also the regulatory subunit Mollv6 in *M. oryzae*, and found that Mollv2 and Mollv6 are also involved in amino acid biosynthesis. Moreover, we also found that defects in the biosynthesis of leucine, isoleucine and valine as a result of *Mollv2* or *Mollv6*

mutation had a profound influence on growth and fungal pathogenicity.

In this study, targeted deletion of *Mollv2* or *Mollv6* caused defects in infection-related morphogenesis and virulence. Our results indicated that Mollv2 and Mollv6 affect hyphal growth, colony pigmentation and conidiophore formation, but not hyphal-driven appressoria development. In many fungi, the aerial hypha are very important to conidiophore differentiation and asexual spore production (Adams *et al.*, 1998; Kikuma *et al.*, 2007). The Δ Mollv2 and Δ Mollv6 mutants exhibited autolysis of the aerial hypha on SDC medium, but the aerial hypha of the mutants was recovered when supplemented with amino acids, indicating that the lost ability of conidiophore formation may be caused by autolysis of the aerial hypha, which may be attributable to the defect in the biosynthesis of certain amino acids. One surprise result was that the Δ Mollv6 mutant caused rice blast on rice roots, but not on leaves. As the infectious mechanism of *M. oryzae* on rice roots has been well clarified (Tucker *et al.*, 2010), we conclude that the pathogenic difference in Δ Mollv6 on the two organs is a result of organ-specific, infectious-related development mechanisms.

One of the most important findings was that Mollv2 and Mollv6 are known to encode ALS, which is involved in the leucine, isoleucine and valine synthesis pathways. In *C. neoformans*, the Δ ilv2 mutant could be recovered from SD medium by the addition of isoleucine and valine, and when proline was used as the nitrogen source instead of ammonium (Kingsbury *et al.*, 2004). In this study, we found that the growth defect of the Δ Mollv2 and Δ Mollv6 mutants could be restored by a single or multiple amino acids. The growth defect of the Δ Mollv2 mutant was consistent with that of isoleucine and valine deprivation in *S. cerevisiae* and

C. albicans (Nisbet and Payne, 1979; Payne *et al.*, 1991). In addition, our results demonstrated that the intracellular amino acid should be maintained at an appropriate level for the normal growth and development of *M. oryzae*. Although Mollv2 and Mollv6 are the catalytic and regulatory subunits of ALS, respectively, other regulators might also exist to produce their unexpected differential roles. Mollv2 and Mollv6 might possess different downstream targets involved in amino acid metabolism. Mollv2 has been predicted to possess three functional domains (TPP_N, TPP_M and TPP_C), which are well conserved among fungal organisms. Deletion of the respective domains caused a similar phenotype to the Δ Mollv2 mutant to be exhibited. We conclude that all domains are required for fungal morphogenesis and pathogenicity. In a previous study, ALS in the biosynthesis of BCAAs was associated with mitochondrial function (Ryan and Kohlhaw, 1974). In addition, the Ilv2 protein is synthesized in the cytosol as a precursor protein with an N-terminal mitochondrial targeting sequence (MTS), which is imported directly into the mitochondria (Dasari and Kölling, 2011; Falco *et al.*, 1985). Consistent with these reports, our data revealed that the Mollv2 protein was localized to the mitochondria of growing hyphae and conidia. Localization was also observed in the mitochondria of growing hyphae in functionally deleted mutants. Hence, these domains are not essential for the localization of the Mollv2 protein, but are required for the function of Mollv2.

Mollv6 harbours ACT, an ALS domain and a phosphorylation site PRSP. Our results also demonstrate that Mollv6 is localized to the mitochondria and that the conserved domains are not necessary for this localization. Previous studies of fungal Ilv6 proteins have indicated that the precursor proteins maintain a signal peptide targeting ALS to the mitochondria (Cassady *et al.*, 1972; Lee and Duggleby, 2006). The signal peptide is required for mitochondrial localization, and for conidial morphology and infection-related development. Studies of Mollv6 have indicated the highly conserved nature of Ilv6 proteins.

In summary, we have demonstrated that Mollv2 and Mollv6 are involved in amino acid biosynthesis and are essential for mycelial growth, asexual development and the pathogenicity of *M. oryzae*. This is the first report to characterize the functions of Mollv2 and Mollv6 in filamentous fungi. The findings provide evidence for the importance of amino acid metabolism in the development of a filamentous phytopathogen. As ALSs synthesized by Mollv2 and Mollv6 are conserved in fungi, these proteins may be suitable for future exploitation as a novel antifungal target.

EXPERIMENTAL PROCEDURES

Fungal strains and growth conditions

Magnaporthe oryzae strain Guy11 was used as the wild-type strain and the mutants were generated from Guy11 in this study (Table S2, see

Supporting Information). All strains were cultured at 28 °C under dark conditions on CM (Talbot *et al.*, 1993; Zhang *et al.*, 2011b). Protoplast preparation and transformation were performed as described. Transformants were selected on medium with 250 µg/mL hygromycin B or 200 µg/mL zeocin (Invitrogen, Carlsbad, CA, USA). Standard yeast culture media, including yeast extract, peptone and dextrose (YPD) and synthetic dextrose (SD), were prepared as described previously (Qi *et al.*, 2012). *Saccharomyces cerevisiae* strains were maintained at 30 °C for 2–3 days. *Escherichia coli* JM109 was cultured at 37 °C and used for normal bacterial transformations in this study. Long-term storage for fungus strains was performed as described previously (Talbot *et al.*, 1993).

Disruption and complementation of target genes in *M. oryzae*

For the construction of the *Mollv2* gene disruption vector, a 1.0-kb upstream flanking sequence fragment and a 1.0-kb downstream flanking fragment were amplified from *M. oryzae* genomic DNA with primers FL7387/FL7548 and FL7549/FL7390, respectively. Similarly, the *Mollv6* disruption vector was constructed with primers FL7918 (F)/FL7919 (R) and FL7920/FL7921, respectively (Fig. S1A, B, see Supporting Information). By overlap PCR, a 2-kb fragment was amplified and cloned into a pMD19-T vector (TaKaRa Co., Dalian, China). The hygromycin resistance gene cassette was amplified by Pfu (TaKaRa Co.) with primers FL1111/FL1112 using pCB1003 as a template, and then inserted into the pMD19-T vector, which had been digested with *Pme*I. The resulting plasmid was prepared for the transformation of *M. oryzae* Guy11 protoplasts, as described previously (Talbot *et al.*, 1993). To confirm the disruption gene, the genomic DNA of wild-type Guy11 and the Δ Mollv2 mutant was digested with *Eco*RV, and Southern hybridization analysis was performed with a 742-bp fragment (probe 1), which was amplified with the primer pairs FL7804/FL8737. Similarly, the genomic DNA of wild-type Guy11 and the Δ Mollv6 mutant was digested with *Eco*RI, and analysed by Southern blot with a 690-bp fragment (probe 3) from *M. oryzae* genomic DNA. A 1.4-kb *HPH* fragment (probe 2) was used to verify the copy number of *HPH* in the Δ Mollv2 and Δ Mollv6 mutants (Fig. S1C). RT-PCR was also performed to confirm the mutants; no transcription products were detected in the Δ Mollv2 and Δ Mollv6 mutants (Fig. S1D).

For complementation, the fragments containing the full-length coding region and a 1.5-kb native promoter regions of the genes, were amplified using primers FL9241/FL9242 and FL11921/FL11922, and then inserted into pYF11 (bleomycin resistance) to generate pYF11-*Mollv2* and pYF11-*Mollv6*, and transformed into the mutant strains, respectively. We then generated *Mollv2*-GFP and *Mollv6*-GFP fusion constructs. The constructs were then reintroduced into the protoplasts of the Δ Mollv2 and Δ Mollv6 mutants, the complemented strains were screened by bleomycin resistance and GFP was observed by fluorescence microscopy. All primers used in this study are listed in Table S3 (see Supporting Information).

Phenotype assays

To assess the growth and colony characteristics, the strains were cultured on CM, MM, V8 (100 mL V8 juice, 0.2 g CaCO₃, 15 g agar in 1 L distilled water) and SDC (100 g straw, 40 g corn powder, 15 g agar in 1 L distilled water) medium for 7 days (Song *et al.*, 2010). The colony diameter was

also measured in these conditions. Mycelia were collected from 2-day-old liquid culture for the measurement of biomass, and then washed by distilled water and lyophilized. Fungal biomass was determined by mycelial dry weight (Zhang *et al.*, 2011c). All the experiments were repeated three times, each with three replicates.

SDC medium was used for sporulation; strains were maintained at 28 °C for 7 days, followed by constant fluorescent light for another 3 days (Zhang *et al.*, 2011a). Conidia were collected by collecting with distilled water, followed by filtration through Miracloth (Calbiochem, San Diego, CA, USA). Appressorium formation was assessed on hydrophobic GelBond film by incubation the conidial droplets in a humid environment at 28 °C (Guo *et al.*, 2010, 2011). All growth assays were performed in duplicate, and were repeated three times.

Amino acid assays

To determine the effect of leucine, isoleucine and valine auxotrophy on growth and conidiation, media were supplemented with one-fold amino acid (1AA), including isoleucine (0.23 mM), valine (1.28 mM) and leucine (0.46 mM), and five-fold amino acid (5AA), including isoleucine (1.15 mM), valine (6.4 mM) and leucine (2.3 mM), at 28°C. Experiments were performed in triplicate.

Plant infection assays

For pathogenicity tests, mycelia were cultured on CM at 28 °C for 5 days, and then inoculated on wounded or nonwounded leaves of the susceptible rice variety CO39. To observe appressorium penetration and infection hyphal growth, mycelia were cultured in liquid CM for 2 days, washed twice with distilled water and then inoculated onto barley leaves (Dou *et al.*, 2011). Root infection assays were carried out as described previously (Dufresne and Osbourn, 2001). Lesion formation was examined at 9 days post-inoculation. These experiments were all repeated three times.

Co-localization observation of conidia or mycelia in *M. oryzae*

MolLV2 and *MolLV6* were cloned in frame at the C-terminal end with the GFP gene in the pYF11 vector and transformed into mutant strains. Transformants were selected and observed. MitoTracker Red CMXRos (Invitrogen, Cat. M7512) is a red-fluorescent dye that stains mitochondria in live cells. Mycelia were incubated with 100 nM MitoTracker Red CMXRos for 5–10 min at room temperature, the conidia were collected and then stained for 20–25 min. Subsequently, mycelia or conidia were washed twice with phosphate-buffered saline (PBS) and fixed in 10% formalin buffered in PBS at room temperature.

Quantitative RT-PCR

For quantitative real-time RT-PCR, total RNA was extracted and purified by the EZNA Total RNA Kit (Omega Bio-tek, Norcross, GA, USA). Five micrograms of total RNA were reverse transcribed into first-strand cDNA using the oligo(dT) primer and M-MLV Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Quantitative real-time

RT-PCR was run on an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Reactions were performed in a 20-μL volume containing 10 μL of SYBR premix Ex Taq (2×, SYBR Prime Script RT-PCR kit; TaKaRa Co.), 0.4 μL of ROX reference dye (50×, SYBR Prime Script RT-PCR kit; TaKaRa Co.), 2 μL of cDNA template (50 ng), 0.4 μL of each primer (10 μM) and 6.8 μL of sterile distilled water. Transcriptions of genes were analysed and the *ACT1N* gene was used as an endogenous control. Fold changes were calculated as $2^{-\Delta\Delta C_t}$ to analyse the relative abundance of transcripts. Quantitative real-time RT-PCR was repeated in triplicate with three independent biological experiments, and the primer pairs (FL16539/FL16540, FL16541/FL16542) used in this section are listed in Table S3.

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SUPPORTING INFORMATION

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

Fig. S1 Targeted gene replacement and confirmation of *MoILV2* and *MoILV6*. (A) Construction of the *MoILV2* targeted gene replacement vector. A 2.5-kb fragment of the *MoILV2* coding region was replaced with a 1.4-kb hygromycin B resistance gene cassette (*HPH*) fragment. (B) Construction of the *MoILV6* targeted gene replacement vector. A 1.0-kb fragment of the *MoILV6* coding region was replaced with a 1.4-kb *HPH* fragment. (C) The $\Delta Moilv2$ mutant was confirmed by Southern blot analysis. Genomic DNA from Guy11 and the $\Delta Moilv2$ mutant was digested with *EV*. The DNA was hybridized with probe 1, a 742-bp polymerase chain reaction (PCR) fragment amplified from the genomic DNA of wild-type strain Guy11 using primers FL7804(F)/FL8737(R). Similarly, genomic DNA from wild-type Guy11 and the $\Delta Moilv6$ mutant was digested with *EI*. The mutant was validated by probe 3, a 690-bp fragment from genomic DNA amplified with the primers FL8498(F)/FL8500(R). Probe 2, a 1.4-kb *HPH* fragment, was used to verify the copy number of *HPH* in the $\Delta Moilv2$ and $\Delta Moilv6$ mutants. *EV*, *EcoRV*; *EI*, *EcoRI*. (D) Reverse transcription-polymerase chain reaction (RT-PCR) analysis of *MoILV2* and *MoILV6* in Guy11 and the mutants, using *ACTIN* as control. Gene disruption resulted in the loss of the transcriptional products in the $\Delta Moilv2$ and $\Delta Moilv6$ mutants.

Table S1 Real-time reverse transcription-polymerase chain reaction (RT-PCR) quantification of *MoILV* gene expression in *Magnaporthe oryzae*.

Table S2 Wild-type, mutants and recombinant strains of *Magnaporthe oryzae* used in this study.

Table S3 Primers used in this study.