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Characterization of Lipid Phosphate Phosphatase Genes and *in Planta* Expressed Orphan Genes in the Rice Blast Fungus

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ABSTRACT

Considering the implication of diacylglycerol (DAG) in both metabolism and signaling pathways, maintaining proper levels of DAG is critical to cellular homeostasis and development in fungi. Except the PIP_2-PLC mediated pathway, metabolic pathways leading to generation of DAG converge on dephosphorylation of phosphatidic acid catalyzed by lipid phosphate phosphatases (LPP). To understand the role of LPPs in fungal development and pathogenicity, five genes encoding putative LPPs (*MoLPP1* to *MoLPP5*) were identified and analyzed in a model plant pathogenic fungus, *Magnaporthe oryzae*. Expression analysis using qRT-PCR showed the

expression of five genes during plant infection. Targeted disruption of genes except *MoLPP4* showed that *MoLPP3* and *MoLPP5* are required for normal progression of infection-specific development and proliferation within host plants, whereas *MoLPP1* and *MoLPP2* are dispensable for fungal pathogenicity. Furthermore, exogenous addition of saturated DAG not only restored defect in appressorium formation but also complemented reduced virulence in both mutants (*ΔMolpp3* and *ΔMolpp5)*. These results indicate the importance of *MoLPP*-mediated DAG for fungal development and pathogenesis. To further understand the role of *in planta* expressed genes in fungal pathogenicity, seven *in planta* expressed and *M. oryzae* specific genes were identified (*MoSPC1* to *MoSPC7*) for functional analysis. Although the origin of such "orphan" genes remains unknown, they are considered to be involved in species-specific adaptive processes. Expression analysis using qRT-PCR confirmed the expression of four among seven genes during plant infection. However, individual deletion mutant of four genes (*MoSPC1*, *MoSPC2*, *MoSPC3* and *MoSPC7*) did not show significant phenotypes compared to the wild-type strain in infection-related fungal development and pathogenicity. The length, GC contents, codon adaptation index and expression during mycelial growth of the four genes suggest that these genes are likely to new born genes in evolutionary history of *M. oryzae*. Synteny

analyses of these genes using closely related fungal species corroborated that these genes have evolved *de novo* in the *M. oryzae* genome. Based on the data, we conjecture that four orphan genes may be products of *de novo* gene birth process and that their adaptive potential is in the course of being tested for retention or extinction by natural selection.

KEY WORDS: *Magnaporthe oryzae*, diacylglycerol, lipid phosphate phosphatase, orphan gene, fungal development and pathogenicity.

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CHAPTER 1

Regulation of Cellular Diacylglycerol Through Lipid Phosphate Phosphatases Is Required for Pathogenesis of the Rice Blast Fungus, *Magnaporthe oryzae*

ABSTRACT

Considering implication of diacylglycerol in both metabolism and signaling pathways, maintaining proper levels of diacylglycerol (DAG) is critical to cellular homeostasis and development. Except the PIP_2-PLC mediated pathway, metabolic pathways leading to generation of DAG converge on dephosphorylation of phosphatidic acid catalyzed by lipid phosphate phosphatases. Here we report the role of such enzymes in a model plant pathogenic fungus, *Magnaporthe oryzae*. We identified five genes encoding putative lipid phosphate phosphatases (*MoLPP1* to *MoLPP5*). Targeted disruption of four genes (except *MoLPP4*) showed that *MoLPP3* and *MoLPP5* are required for normal progression of infection-specific development and proliferation within host plants, whereas *MoLPP1* and *MoLPP2* are indispensable for fungal pathogenicity. Reintroduction of *MoLPP3* and *MoLPP5* into individual deletion mutants restored all the defects. Furthermore, exogenous addition of saturated DAG not only restored defect in appressorium formation but also complemented reduced virulence in both mutants. Taken together, our data indicate differential roles of lipid phosphate phosphatase genes and requirement of proper regulation of cellular DAGs for fungal development and pathogenesis.

INTRODUCTION

Diacylglycerol (DAG) plays crucial roles in cells as a second messenger in lipid-mediated signaling pathway and as an intermediate in lipid metabolism (Bell and Coleman, 1980; Nishizuka, 1984). DAGs is not a single molecular species but a pool of molecules varying with acyl chain length and saturation level (Deacon et al., 2002; Pettitt et al., 1997). Mammalian cells produce more than 50 different types of DAGs including polyunsaturated, di-unsaturated, monounsaturated or saturated forms (Pessin and Raben, 1989). Different DAGs interact with a diverse array of proteins with C1 domain(s) having different specificities and affinities for DAG, leading to remarkable complexity in DAG-dependent cellular processes (Wakelam, 1998).

Yeast and mammals have two *de novo* pathways for production of DAG (Athenstaedt and Daum, 1999). In one pathway, DAG is synthesized from glycerol-3-phosphate and in another pathway, DAG is generated from dihydroxyaceton phosphate. These two precursors produce lysophosphatidic acid (LPA) and phosphatidic acid (PA) through two acylation steps and finally PA is transformed to DAG by the action of lipid phosphate phosphatase (LPP) (Nanjundan and Possmayer, 2003) (Figure 1). In addition to *de novo* pathways, DAGs can be produced in a manner that is highly dependent on extracellular

stimulation. Polyunsaturated DAG is generated from phosphatidyl inositol-4-5-bisphosphate (PIP_2) by the activity of phospholipase C (PLC) through a single step reaction (Nishizuka, 1992). Alternatively, monounsaturated/saturated DAGs can be generated in a two-step reaction. In the first step, monounsaturated/saturated phosphatidic acid (PA) is produced from phospholipids through the activity of PLD and in the second step, dephosphorylation of PA by the members of LPP family enzyme results in monounsaturated/saturated DAG (Toke et al., 1998a). Yeast has two different types of the enzymes, lipid phosphate phosphatase (LPP) and diacylglycerol pyrophosphate phosphatase (DPP) (Toke et al., 1998a) to dephosphorylate PA, whereas mammals lack DPP. Both the LPP and DPP are the members of LPP family.

All the pathways except the one involving PLC converge on dephosphorylation reaction of PA, indicating the importance of LPP in lipid metabolism and DAG-mediated signaling pathways. Due to its status as a gateway to DAG production, LPPs have been well studied and documented in diverse organisms ranging from yeast to plant and insects. In *Arabidopsis thaliana*, *AtLPP1* and *AtLPP2* are involved in stress response and regulation of stomatal movement through ABA signaling, respectively (Paradis et al., 2011; Pierrugues et al., 2001). LPP is shown to play important roles in germ

cell migration and tracheal development in insect (Ile et al., 2012; Zhang et al., 1996). In yeast, deletion of individual or both lipid phosphate phosphatase (*ScLPP1*) and diacylglycerol pyrophosphate phosphatase (*ScDPP1*) did not show any visible phenotypes compared to wild type strain, but both genes together controlled cellular reservoir of the phosphatidic acid (PA), lysophosphatidic acid (LPA) and diacylglycerol pyrophosphate (DGPP) (Toke et al., 1998a; Toke et al., 1998b). Despite the importance of LPP-mediated regulation of cellular DAGs in different organisms, its implication in fungal pathogenesis remains unexplored. Here we set out to investigate the role of LPP encoding enzymes in development and pathogenesis of a model plant pathogenic fungus, *Magnaporthe oryzae*.

M. oryzae is a filamentous fungus that causes the rice blast disease. The rice blast disease is one of the most devastating fungal diseases of rice throughout the world (Talbot, 2003). This disease causes 11 – 30% yield losses of the world rice production and is responsible for recurring epidemics throughout South East Asia and South America (Baker et al., 1997). Infection by this fungus begins when an asexual spore called conidium germinates following tight adherence to the surface of host plants. Upon recognition of environmental cues such as surface hydrophobicity, the tip of germ tube develops into a dome-shaped, specialized infection structure called an

appressoria (Howard et al., 1991). Using turgor pressure generated in appressorium, the fungus mechanically rupture the cuticular layer of the plant and gain access to host tissues (Bourett and Howard, 1990). Once inside the host cells, the fungus develops ramifying bulbous invasive hyphae that actively grow to result in visible disease lesion, from which massive number of conidia are produced as secondary inoculum (Balhadere et al., 1999; Kankanala et al., 2007; Urban et al., 1999).

Due to genetic tractability of rice blast pathogen and rice (Dean et al., 2005; Goff, 2005; Yu et al., 2002), signaling transduction pathways involving cAMP, calcium, and MAP kinase have been well documented for infection-related development in this fungus (D'Souza and Heitman, 2001; Jeon et al., 2008; Lee and Dean, 1993; Park et al., 2002; Rho et al., 2009; Xu and Hamer, 1996). Moreover, other studies suggested that DAG plays significant role in pathogenicity leading to appressorium formation in *M. oryzae* (DeZwaan et al., 1999; Thines et al., 1997). Although a number of studies suggested that lipids and their intermediates are implicated in pathogenesis of fungi including *M. oryzae* (Jenkins et al., 1997; Klose et al., 2004; Patton et al., 1992; Shea and Del Poeta, 2006; Spiegel et al., 1996; Wang et al., 2007; Wang et al., 2003), genetic analysis on regulation of cellular DAG, a key element of lipid metabolism and signaling has not been carried out.

As the first step to elucidate the biological functions of *LPP* genes in *M. oryzae*, we have identified genes encoding LPP family and functionally characterized those genes through gene deletion approach. Analyses of the deletion mutants showed that individual deletion of *MoLPP3* and *MoLPP5* caused defect in appressorium formation and pathogenicity. Exogenous addition of saturated DAG restored both appressorium formation and virulence defect in both mutants, indicating that maintaining DAG homeostasis is required for fungal pathogenesis. Our work shed light on the critical roles of lipid metabolism during fungal pathogenesis.

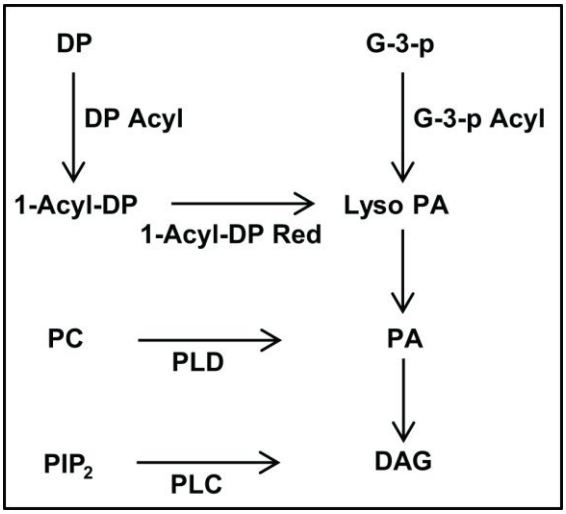

Fig. 1. The enzymatic reactions leading to DAG generation through different pathway.

DP, dihydroxyaceton phosphate; DP Acyl, dihydroxyaceton phosphate acyltransferase; 1-Acyl-DP, 1-Acyl-dihydroxyaceton phosphate; 1-Acyl-DP Red, 1-Acyl-dihydroxyaceton phosphate reductase; G-3-p, glycerol-3-phosphate; G-3-p Acyl, glycerol-3-phosphate acyltransferase; Lyso PA; lysophosphatidic acid; PC, phosphatidylcholine; PLD, phospholipase D; PA, phosphatidic acid; PIP_2, phosphatidyl inositol-4-5-bisphosphate; PLC, phospholipase C; DAG, diacylglycerol.

MATERIALS AND METHODS

I. Fungal isolates and culture conditions

M. oryzae wild type strain KJ201 was obtained from the Centre for Fungal Genetic Resources (CFGR: http://cfgr.snu.ac.kr) for this study. All strains used in this study were grown on V8 agar [V8; 8% V8 juice (v/v), 1.5% agar (w/v), adjusted to pH 6.0 using NaOH] or oatmeal agar [OMA; 5% oatmeal (w/v), 2% agar (w/v)] at 25ºC in constant light to promote conidial production (Park et al., 2010). All strains cultured on V8 juice agar media for 7 days or on OMA for 10 to 15 days at 25°C under the continuous light condition to observe the developmental and morphologic phenotypes and pathogenicity ability.

II. Nucleic acids manipulation and expression analysis

Fungal genomic DNA was isolated for two purposes using two different methods. For Southern DNA hybridization, genomic DNA was isolated from mycelia according to a standard protocol (Choi et al., 2007). For PCR-based screening of transformants, genomic DNA was extracted by quick and easy method (Chi et al., 2009). Southern DNA hybridization was performed on selected transformants to ensure correct gene replacement events and absence of ectopic integration. Genomic DNAs were digested with *Pst*I, *Bam*HI and

blots were probed with 1-kb 5'-flanking/ 3' flanking sequences. Southern DNA hybridization was done through a standard method (Sambrook and Russell, 2001). To perform the expression analysis by quantitative real-time PCR (qRT-PCR), cDNA synthesis was performed with 5µg of total RNA using the oligo dT primer with the ImProm-IITM Reverse Transcription System kit (Promega, Madison, WI, USA) following the manufacturer's instruction. Primer pairs used are listed in Table 1. To compare the relative abundance of *MoLPPs* (1-5) transcripts, average threshold cycle (Ct) was normalized to that of *β-tubulin* gene and germinating conidia for each sample.

III. Targeted deletion of genes and complementation experiments

Targeted deletions of genes were carried out by transforming wild-type protoplast with knockout constructs. Knockout construct of individual genes were obtained by double-joint PCR where ~1kb flanking sequences of target gene were amplified and fused to hygromycin cassette. The resulting transformants were subjected to PCR-based screening and gene replacement event in the genome of transformants were confirmed by Southern blot analysis using one of flanking sequences as a probe. Complemented strains for two mutants (Δ*Molpp3 and* Δ*Molpp5*) were generated by reintroducing 1.2 kb and 0.9 kb fragments carrying the *MoLPP3* and *MoLPP5* ORF, respectively.

Complemented strains were selected through PCR screening with specific primers (*MoLPP3*_NF, *MoLPP3*_NR, *MoLPP5*_NF and *MoLPP5*_NR) (Table 1) and also used “c” to indicate complementation strain through the whole manuscript.

IV. Developmental phenotype assay

Radial mycelial growth rate with three replications was measured on complete agar media and on minimal agar media 12 days after inoculation. For conidial germination and appressorium formation, conidia were harvested from 7-days-old V8 juice agar plates and passed through the two layer of miracloth with sterilized distilled water. Concentration of conidial suspension was adjusted to 2×10^4 conidia per mililiter and 40μl was dropped onto coverslips with three replicates and incubated in moistened box at room temperature. At two and four hours after incubation, germination rate was determined by observing at least a hundred of conidia per replicate under a microscope. The rate of appressorium formation was measured as the percentage of germinated conidia that developed appressoria at 8, 16 and 24 hours each after incubation. These assays were performed with three replicates in three independent experiments. For conidiation, conidia was collected from 7-days-old V8 juice agar plates with 5ml sterilized distilled water and measured by counting the

number of asexual spores within 10μl conidia suspension onto haemacytometer under a microscope.

V. Pathogenicity assay and infectious growth visualization

For spray inoculation, conidia were collected from 7-days-old V8 juice agar media with 10ml of filtered conidia suspension and adjusted to 1×10^5 conidia per mililiter containing Tween 20 (250 ppm final concentration) and sprayed onto the rice seedlings (*Oryza sativa* cv. Nakdongbyeo) in three to four leaf stage. Inoculated rice seedlings were placed in a dew chamber for 24 hours under the dark condition at 25°C. After then, they were transferred to the rice growth incubator that is maintained at 25°C, 80% humidity and with photoperiod of 16 hours using fluorescent lights (Valent and Chumley, 1991). For microscopic observation of invasive growth on rice tissue, excised rice sheath of Nakdongbyeo were prepared by standard process (Koga et al., 2004). Conidia suspension was injected in excised rice sheaths and incubated in moistened box for 24 and 48 hours at room temperature. After incubation, the infected rice sheaths were trimmed to remove chlorophyll enriched plant parts. Remaining epidermal layers of mid vein (three to four cell layers thick) were used for microscopic experiment.

VI. Computational analysis

All sequence information used in this study was taken and analyzed from the online database Comparative Fungal Genomics Platform (CFGP) (Choi et al., 2013), http://cfgp.snu.ac.kr/) and BLAST program provided at the National Center for Biotechnology Information, Bethesda, USA (http://www.ncbi.nlm.nih.gov/blast/) (McGinnis and Madden, 2004). Sequences were aligned by ClustalW algorithm (Thompson et al., 1994). Hydropathy plot was generated using TopPred 2 (http://www.sbc.su.se/~erikw/toppred2/) (von Heijne, 1992).

VII. Tests for sensitivity to reactive oxygen species and a cell wall perturbing agent

Sensitivity to reactive oxygen species was tested by growing fungi on complete media supplemented with H_2O_2 and methylviologen. Test for cell wall integrity was carried out by adding a cell wall perturbing agent, Congo red (200 ppm) to complete media that are inoculated with wild-type and mutant strains.

Table 1. Oligo sequences used in this study

Primer name	Sequence (5' → 3')
For generation and confirmation of *MoLPP* (*1-5*) mutants:	
*MoLPP1*_UF	ACC TTG CTT ACG AGT ACG
*MoLPP1*_UR	TGT GTG ACG CAG CTT CTT
*MoLPP1*_DF	GAT AGG TAA AGA CAG TGG GG
*MoLPP1*_DR	ATC AGG AGA GAT AGC TCG
*MoLPP1*_NF	GAC TTG TCA GGA CTC TTG
*MoLPP1*_NR	ACT ACT TGT AGA ACC CCG
*MoLPP2*_UF	CAG CGC CAT CAC GAT TAT
*MoLPP2*_UR	CAG AAG ACC AAT GTC TGG T
*MoLPP2*_DF	ACA GTC GAG GTC TTG GTA
*MoLPP2*_DR	ACA ACC TGC CCA ACA T
*MoLPP2*_NF	CCG TCA AAG TGT TTG TCG
*MoLPP2*_NR	ATG CCA CCA AGA AGT ACC
*MoLPP3*_UF	TTC GAA GGT GCC AAG T

*MoLPP3*_UR	GCA GCT TGA TCC ACA A
*MoLPP3*_DF	AGC AAC GAC TGT ACG A
*MoLPP3*_DR	TTT GGG CGA ATC ACA G
*MoLPP3*_NF	CCAACCTGCTTTCCAT
*MoLPP3*_NR	AAG CAC GCT TAC CCT TA
*MoLPP4*_UF	TTCGTCAGCAACTAGAGG
*MoLPP4*_UR	GTATTACATGTAGGAGCC
*MoLPP4*_DF	TTGCTAGAGCTCTCCTGT
*MoLPP4*_DR	GGATTTCAAAGGGGGGTT
*MoLPP4*_NF	TGATGGGGGAAAATCAGG
*MoLPP4*_NR	TCTAAACGAACGCTCGGA
*MoLPP5*_UF	ATC GAG GAT CTC TTG ACG
*MoLPP5*_UR	TCC GCG TAT TGC TTT C
*MoLPP5*_DF	TTG CGA GAC GTC TTG A
*MoLPP5*_DR	CTC CTT CAC TGT AGG CAA

*MoLPP5*_NF	TGCGGATATCCAGTGATG
*MoLPP5*_NR	AAT GCC AAC CCA CCA A
HPH_F	GGCTTGGCTGGAGCTAGTGGAGG
HPH_R	GTTGGTGTCGATGTCAGCTCCGGAG

For qRT-PCR:

*MoLPP1*_qRT-F	GGAGAAGCGAAGAGGTGTTT
*MoLPP1*_qRT-R	GGCGTTCAGATACAGATAGAGG
*MoLPP2*_qRT-F	TCATCGGAATTCTGTACTCGC
*MoLPP2*_qRT-R	ACAAACGTCGAGGAAGTGAG
*MoLPP3*_qRT-F	TCCTCAGCACCTTTTGTCAG
*MoLPP3*_qRT-R	GTAGCCAATGCCCTCTGTG
*MoLPP4*_qRT-F	GACATCTCCCGTCCCAATG
*MoLPP4*_qRT-R	AGATGAATATGGCACCGAAGG
*MoLPP5*_qRT-F	CAGCTTAGGATATTCGCCTACG

*MoLPP5*_QRT-F	CACCCTCTCCATCTTTCCAG
*MoPLC1*_qRT-F	GCTCCAACCGACATCTACATC
*MoPLC1*_qRT-R	TTATTGACTACCGCCATCGAC
*MoPLC2*_qRT-F	GATGCGAACTTATCCCTCTGG
*MoPLC2*_qRT-R	CCTGCAAACATCCCTTCATTG
*MoPLC3*_qRT-F	CTCATCCTTCATCCCCTACG
*MoPLC3*_qRT-R	CCCGTGCTCTTTATAGTGGTAC
B-tub_qRT-F	TCGACAGCAATGGAGTTTAC
B-tub_qRT-R	AGCACCAGACTGACCGAAGA

RESULTS

I. Identification of *LPP* genes in *M. oryzae*

To identify the members of PAP2 domain containing LPP encoding genes involved in DAG metabolism in *M. oryzae*, we searched the genome of *M. oryzae* for genes encoding phosphatidic acid phosphatase type 2/haloperoxidase (PAP2) domain (IPR000326), which is a signature of LPP enzymes (http://cfgp.riceblast.snu.ac.kr) (Choi et al., 2013). Our search found a total of eight genes encoding PAP2 domain. BLAST search using amino acid sequences encoded by these eight genes as queries showed that five of them were homologous to yeast *LPP1* (YDR503C) and *DPP1* (YDR284C) (*MoLPP1*; MGG_09330.8, *MoLPP2*; MGG_05988.8, *MoLPP3*; MGG_09994.8, *MoLPP4*; MGG_05650.8, *MoLPP5*; MGG_12462.8), and the rest of them to vanadium chloroperoxidase (*MoVAN*; MGG_02210.8), long chain base protein 3 (*MoLCBP3*; MGG_17385.8) and dolicylpyrophosphate phosphatase (*MoDoPP*; MGG_09184.8), respectively (Figure 2). Multiple sequence alignment and hydropathy plot revealed that the *MoLPP1* to *MoLPP5*, unlike the rest of PAP2 domain-containing proteins, have conserved three sequence motifs (KXXXXXXRP, PSGH and SRXXXXXHXXXD) (Figure 3) (Stukey and Carman, 1997) and six transmembrane domains (Figure 4), both of which

	ScDPP1	*ScLPP1*	*MoDoPP*	*MoLCBP3*	*MoVAN*	*MoLPP5*	*MoLPP4*	*MoLPP3*	*MoLPP2*
MoLPP1	28.12	26.09	0	0	0	25.56	28.57	59.92	53.85
MoLPP2	28.16	32.62	0	0	0	26.14	24.68	51.06	
MoLPP3	29.27	28.05	0	0	0	25.11	31.25		
MoLPP4	30.67	0	0	0	0	32.11			
MoLPP5	35.00	28.45	0	0	0				
MoVAN	0	0	0	0					
MoLCBP3	0	0	0						
MoDoPP	0	0							
ScLPP1	33.37								

Fig. 2. Sequence similarities of PAP2 domain of eight genes with each other and also with the yeast *ScLPP1* and *ScDPP1*.

Sequence similarities were measured by using BLAST2 with the amino acid sequences provided at Comparative Fungal Genomics Platform (CFGP), (http://cfgp.snu.ac.kr/).

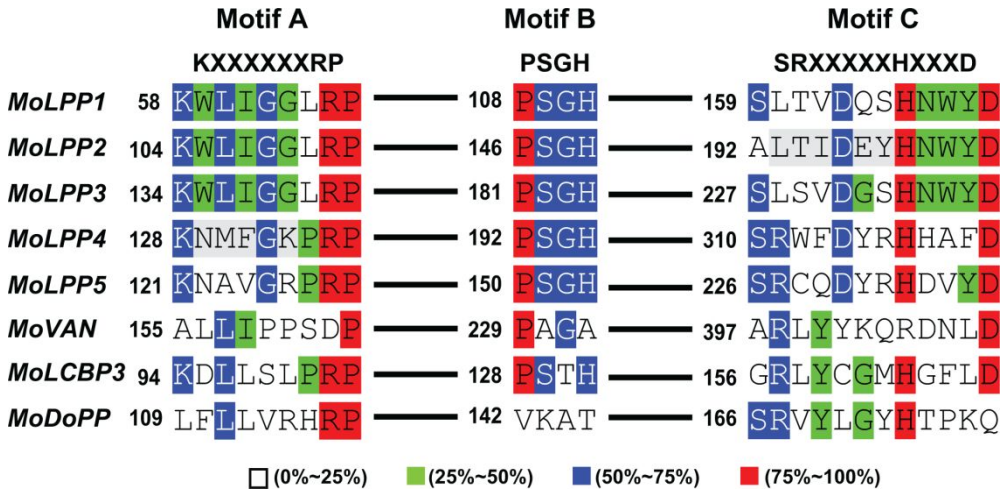

Fig. 3. Multiple sequence alignments of PAP2 domain containing proteins in *M. oryzae*.

Alignments of three novel sequence motifs among eight PAP2 domain containing proteins using ClustalW algorithm. Different colors indicate the conserved sequence among eight proteins and numbers (left of motifs) show the starting position of three motifs.

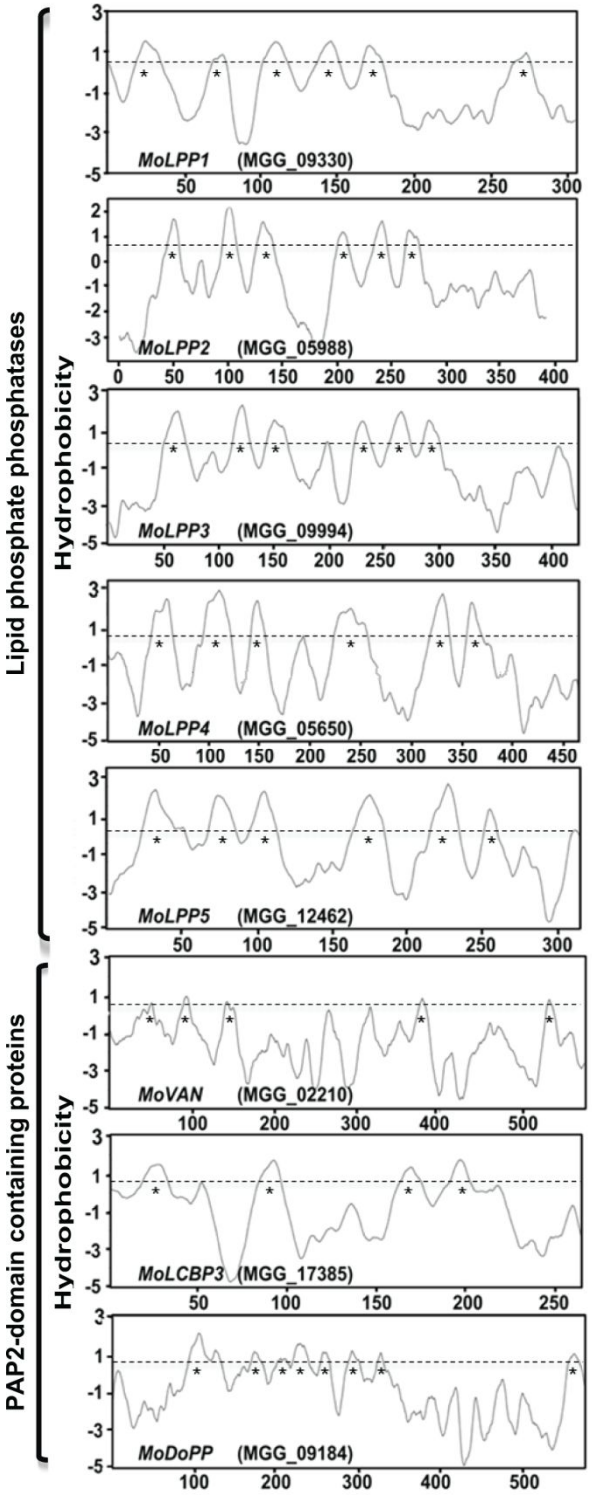
Lipid phosphate phosphatases
PAP2-domain containing proteins
Hydrophobicity
MoLPP1 (MGG_09330)
MoLPP2 (MGG_05988)
MoLPP3 (MGG_09994)
MoLPP4 (MGG_05650)
MoLPP5 (MGG_12462)
MoVAN (MGG_02210)
MoLCBP3 (MGG_17385)
MoDoPP (MGG_09184)

Fig. 4. Kyte-Doolittle hydropathy plots of LPP and PAP2 domain containing proteins in *M. oryzae*.

Hydropathy plots of *MoLPP* genes and PAP2-domain containing proteins showing potential transmembrane domains. Asterisks indicate the potential membrane-spanning domains. Hydropathy plot was generated using TopPred 2 (http://www.sbc.su.se/~erikw/toppred2/). Cutoff value (0.6) was indicated by dotted horizontal line.

are common features of LPP and DPP enzymes (Jia et al., 2003). Based on this data, we only focused on *MoLPP1* to *MoLPP5* in the following analyses. *De novo* synthesis of DAG is known to occur mainly in the endoplasmic reticulum (ER). In accordance with this, PSORT predicted that all *MoLPP*s are localized to ER (Horton et al., 2007).

II. Expression analysis of *MoLPP* genes

As an attempt to infer the implication of *MoLPP* genes in *M. oryzae*, qRT-PCR was done with cDNAs sampled from different developmental stages. In comparison with germinating conidia, most genes were down-regulated in conidia and appressoria stages. But interestingly, *MoLPP4* was the only up-regulated gene found in appressoria. However, they tended to be up-regulated during host infection. Especially, the expression of *MoLPP2* and *MoLPP3* remained relatively high, compared to the others (Figure 5). Expression profiling revealed that the transcript abundance of genes correlates with each other except with *MoLPP4*. Correlation coefficient of *MoLPP4* with expression of other *MoLPP* genes ranged from 0.29 to 0.73, while correlation coefficient for all pairs of other genes was higher than 0.75. This suggests that *MoLPP* genes except *MoLPP4* are likely to share overlapping regulatory mechanisms for transcription. In consistent with our observation,

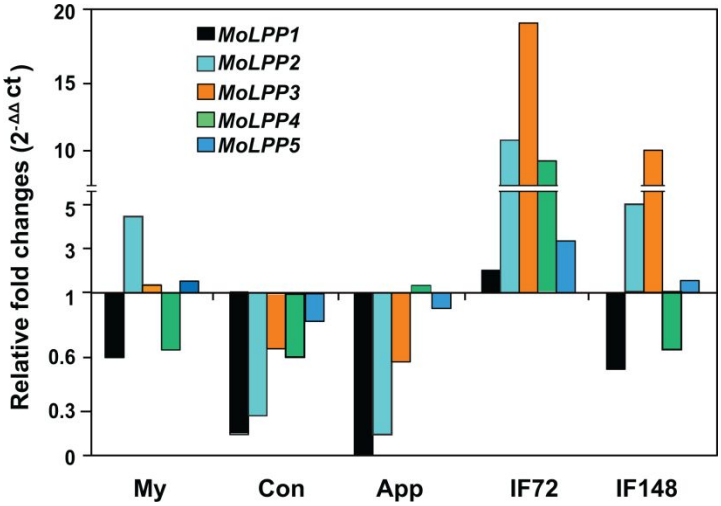

Fig. 5. Transcript abundance of *MoLPP* genes in development stages in *M. oryzae*.

My, mycelia; Con, germinating conidia; App, appressoria; IF 72, early infection stage at 72 hour post inoculation (hpi); IF 148, late infection stage at 148 hour post inoculation (hpi).

the expression of *MoLPP2* and *MoLPP3* genes were detected in *in planta* EST library (Kim et al., 2010). We also checked the expression levels of *MoLPP* genes at 40-45 hpi (hours post inoculation) through RNA-Seq data (unpublished data). We found up-regulated expression of *MoLPP3* and *MoLPP5* compare to mycelial stage, whereas the rest three genes were down-regulated at the same stage. Our data suggest the possible involvement of *MoLPP* genes in post-penetration phase of host infection by the rice blast fungus.

III. Targeted gene disruption of *MoLPP* genes

To investigate the roles of *MoLPP* genes in *M. oryzae*, we generated deletion mutants of individual genes. Knockout constructs were prepared via double joint PCR. (Yu et al., 2004) and directly used for transformation of wild-type protoplasts. Correct gene replacement event in the resulting transformants was confirmed by PCR-based screening and subsequent Southern hybridization analysis (Figure 6). The deletion mutants were obtained for all but one gene. Despite our repeated efforts, we were not able to generate a deletion mutant for *MoLPP4*. Consequently, we analyzed four genes, of which deletion mutants are available.

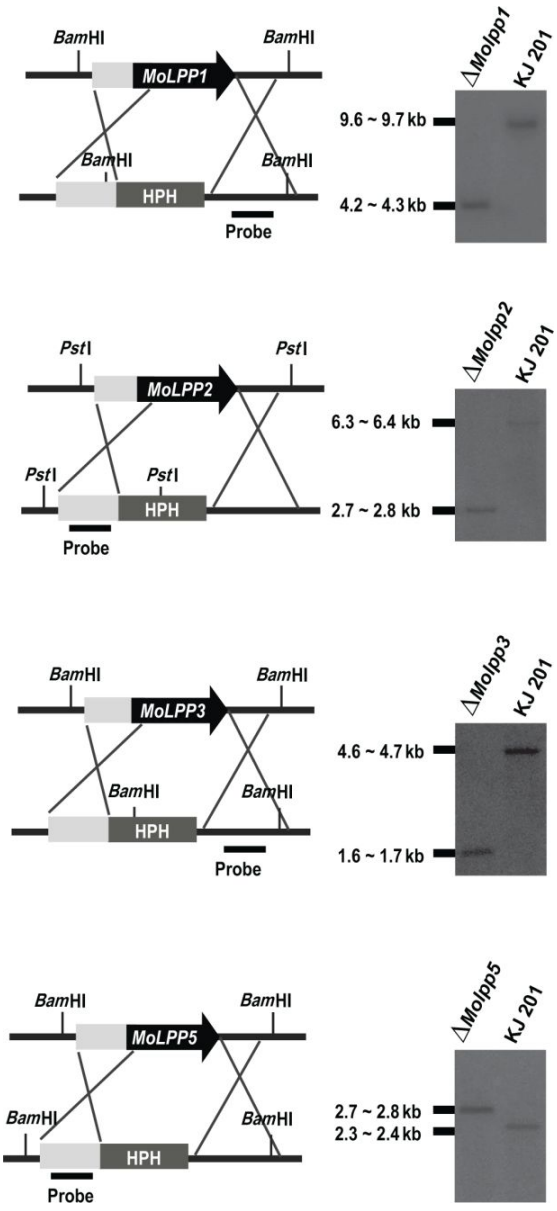

Fig. 6. Targeted disruption of *MoLPP1*, *MoLPP2*, *MoLPP3* and *MoLPP5*.

Schematic diagram of knockout of target gene (left) and Southern blot analysis of resulting mutant (right) are shown in pair for each gene.

IV. Vegetative growth, germination, and appressorium formation of the mutants

During phenotype analysis, we found that none of the *MoLPP*s were required for vegetative growth and asexual reproduction (Table 2). When the suspensions of mutant spores were incubated on inductive surface, spores of all mutants were capable of germinating and developing appressoria. However, we noticed that both germination and appressorium formation were delayed in Δ*Molpp3* and only - appressorium formation was delayed in Δ*Molpp5* (Figure 7). Despite - delayed appressorium formation by the two mutants, morphology of appressoria was indistinguishable from that of wild-type. Such delay in germination and appressorium formation in the mutants was complemented by introducing the sequences of *MoLPP3* and *MoLPP5* genes back into the genome of each deletion mutant (Figure 7).

V. Requirement of *MoLPP3* and *MoLPP5* for full virulence of the fungus

Next, we asked if *MoLPP* genes are involved in fungal pathogenesis on host plants as suggested by the expression analysis. To test this, conidial suspensions of the mutants were spray-inoculated onto rice plants of a susceptible cultivar, Nakdongbyeo. Pathogenicity test showed that Δ*Molpp1* and Δ*Molpp2* were comparable to the wild type in their ability to cause disease

Table 2. Vegetative growth and asexual reproduction of Δ*Molpp1*, Δ*Molpp2*, Δ*Molpp3* and Δ*Molpp5* in *M. oryzae* with complementation

	Mycelial growth[a]		Conidiation[b]
Strain	**CM (mm)**	**MM (mm)**	($\times 10^4$/ml)
KJ201	75.8 ± 1.3	70.3 ± 0.6	33.4 ± 5.3
Δ***Molpp1***	76.3 ± 0.6	70.7 ± 0.8	32.2 ± 5.6
Δ***Molpp2***	76.8 ± 0.3	70.3 ± 1.5	33.3± 6.3
Δ***Molpp3***	76.3 ± 1.5	70.2 ± 0.8	35.2 ± 5.2
Molpp3c	76.8 ± 0.8	71.1 ± 1.0	35.3 ± 0.3
Δ***Molpp5***	76.2 ± 0.3	69.5 ± 0.9	31.2 ± 2.9
Molpp5c	75.7 ± 0.6	71.5 ± 0.5	32.3 ± 3.5

[a] Vegetative growth was measured at 12 dpi on complete agar medium and minimal agar medium. Data were presented as mean ± sd from three independent experiments.

[b] Conidia was measured as the number of conidia from the culture flooded with 5ml of sterilized distilled water.

Data were presented as mean ± sd from three independent experiments.

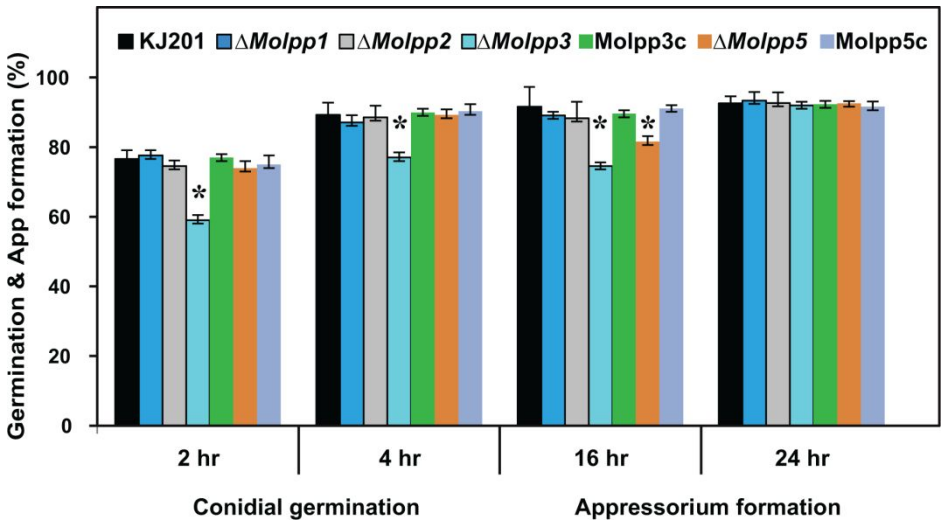

Fig. 7. Percentage of conidial germination and appressorium formation on hydrophobic surfaces with complemented strains.

Asterisks indicate significant difference observed in the mutants, compared to the wild-type (Tukey test, $p < 0.05$).

on rice plants, whereas Δ*Molpp3* and Δ*Molpp5* were not able to produce as large and many number of disease lesions on rice leaves as the wild-type (Figure 8A). Measurable and significant reduction in number and size of disease lesions were observed for the two mutants, resulting in 62 to 65% reduction in diseased leaf area (DLA), compared to the DLA calculated for leaves inoculated with wild-type strain (Figure 8B). The complementation strains of Δ*Molpp3* and Δ*Molpp5* were as virulent as the wild-type, indicating that *MoLPP3* and *MoLPP5* are required for full virulence of the fungus.

Defect in virulence observed for Δ*Molpp3* and Δ*Molpp5* could not be accounted for by minor delay in appressorium formation. Therefore, we monitored early infection process using sheath inoculation method (Koga et al., 2004). The mutant appressoria were able to penetrate plant cells as efficiently as the wild-type appressoria. However, invasive hyphae of both Δ*Molpp3* and Δ*Molpp5* were largely restricted to the primary infected cells at 48 hour post inoculation (hpi) in contrast to invasive hyphae of wild type actively growing to fill in the first host cell and moving to neighboring cells. (Figure 8C and 8D). This result indicates that *MoLPP3* and *MoLPP5* are necessary for the fungus to grow inside host plants in early infection stage.

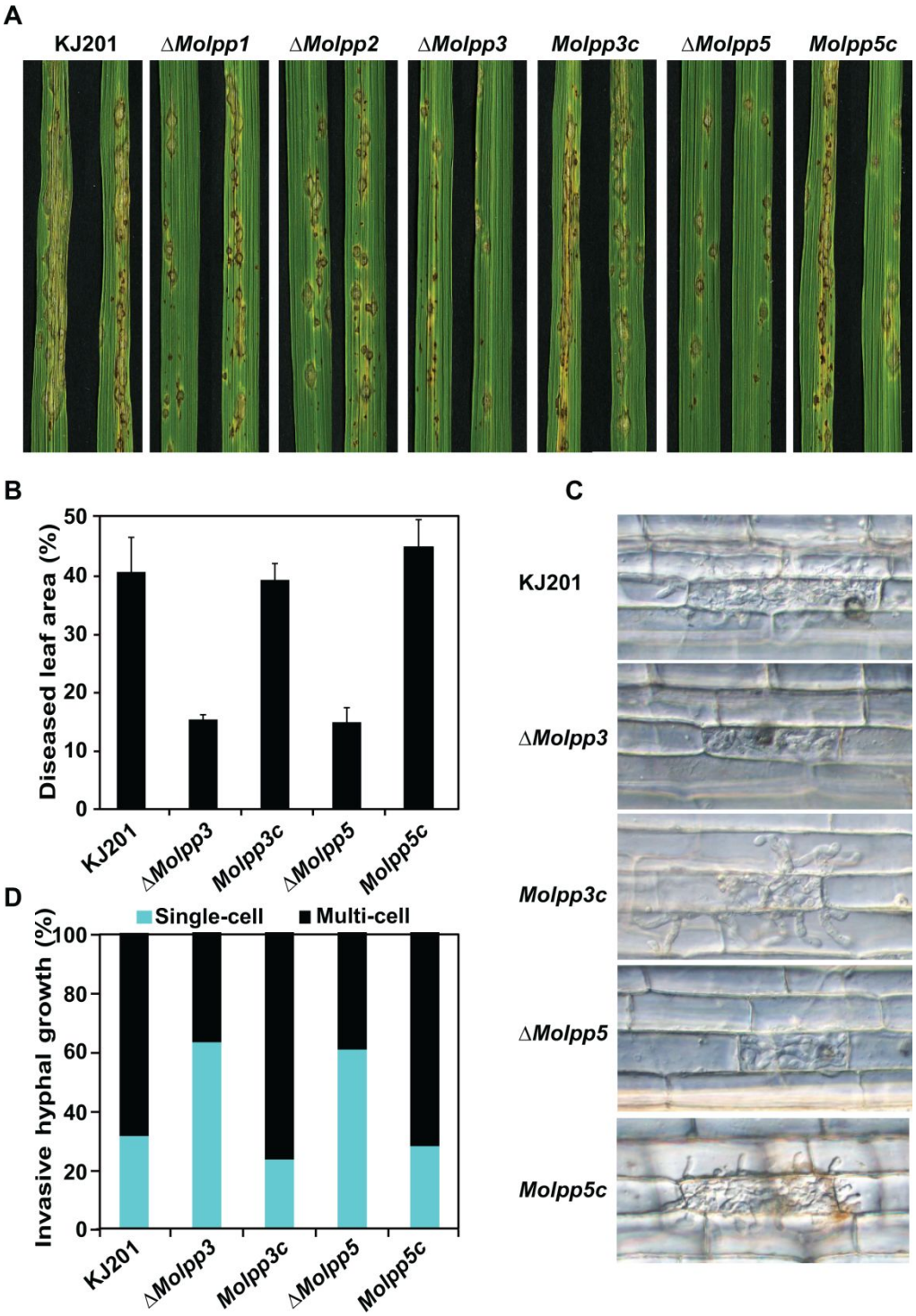
A
KJ201
ΔMolpp1
ΔMolpp2
ΔMolpp3
Molpp3c
ΔMolpp5
Molpp5c
B
Diseased leaf area (%)
0
10
20
30
40
50
KJ201
ΔMolpp3
Molpp3c
ΔMolpp5
Molpp5c
C
KJ201
ΔMolpp3
Molpp3c
ΔMolpp5
Molpp5c
D
Single-cell
Multi-cell
Invasive hyphal growth (%)
0
20
40
60
80
100
KJ201
ΔMolpp3
Molpp3c
ΔMolpp5
Molpp5c

Fig. 8. Pathogenicity assay of knockout mutants.

(A) Disease development after spraying conidial suspension onto rice leaves. Conidia (1×10^5/ml) was sprayed onto the leaves and incubated for 7 days. (B) Comparison of diseased leaf area (DLA). The DLA was calculated relative to the total leaf area using the Axiovision image analyzer. (C) Invasive hyphal growth through sheath assay. Rice sheath was injected with 2×10^4 conidia/ml and observed under microscope at 48 hour post inoculation (hpi). (D) Quantification of invasive growth. Number of cells that are invaded by the fungus was counted using sheath inoculation at 48 hour post inoculation (hpi).

To date, one of the major causes of defective invasive growth is sensitivity to or inability of the fungus to scavenge/suppresses reactive oxygen species (ROS) that are produced by host plants as a defense response during host-pathogen interaction (Torres et al., 2006; Wojtaszek, 1997). In order to test this possibility, we examined sensitivity of the fungus to oxidative stress in the form of H_2O_2 or methyl viologen (MV). On complete media containing varying concentration of H_2O_2 and MV, radial growth of both mutants was comparable to that of wild-type, suggesting that defect in invasive growth is not ROS-dependent (Figure 9A). In addition, we also checked the sensitivity of the fungus to a cell wall perturbing agent, congo red (CR) but no significant differences were detected between the wild-type and the mutants (Figure 9B).

VI. Complementation of Δ*Molpp3* and Δ*Molpp5* by DAG

Since LPPs are involved in production of DAG using lipids as substrates, we reasoned that imbalance in cellular reservoir of DAG underlies in both pre-penetration and post-penetration defects observed in the mutants. It is known that LPPs are implicated in production of monounsaturated/saturated DAG, whereas PI-PLC-mediated pathway is responsible for polyunsaturated DAG (Nishizuka, 1992). To test such imbalance in the mutants, we

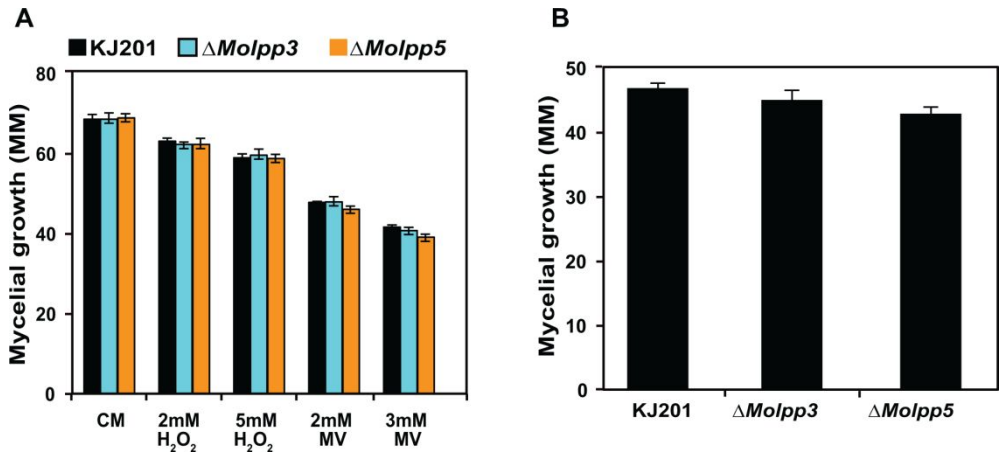

Fig. 9. Mycelial growth in different stress conditions.

(A) Oxidative stress (B) Congo red. Mycelial block was inoculated in CM agar plate containing different concentration of hydrogen peroxide, methyl viologen and congo red. Data was taken at 10 dpi with three independent experiments with three replications.

exogenously added saturated DAG (1,2-dicotnly-sn-glycerol: DOG, Sigma-Aldrich) in the conidial suspension and checked appressorium formation at different time points. We also checked appressorium formation after adding $CaCl_2.2H_2O$ alone or combination of DAG and $CaCl_2.2H_2O$ to examine whether calcium signaling pathway is implicated. We found that addition of saturated DAG was able to restore appressorium formation without delay in the mutants, while $CaCl_2.2H_2O$ had little effect in isolation or combination with DAG (Figure 10A). More importantly, when conidial suspensions of the mutants supplemented with DAG were used for spray-inoculation onto rice plants, virulence of both Δ*Molpp3* and Δ*Molpp5* were recovered to the wild-type level (Figure 10B and 10C). These data suggest that *MoLPP3* and *MoLPP5* are involved in regulation of cellular DAG and such regulation is important for fungal pathogenesis.

VII. Transcriptional expression pattern of genes involved in cellular DAG production

Given the different phenotypic consequences of deletion of *MoLPP* genes, we investigated the relationship among the genes by checking the transcript abundance of genes in each deletion mutant background. Our transcript

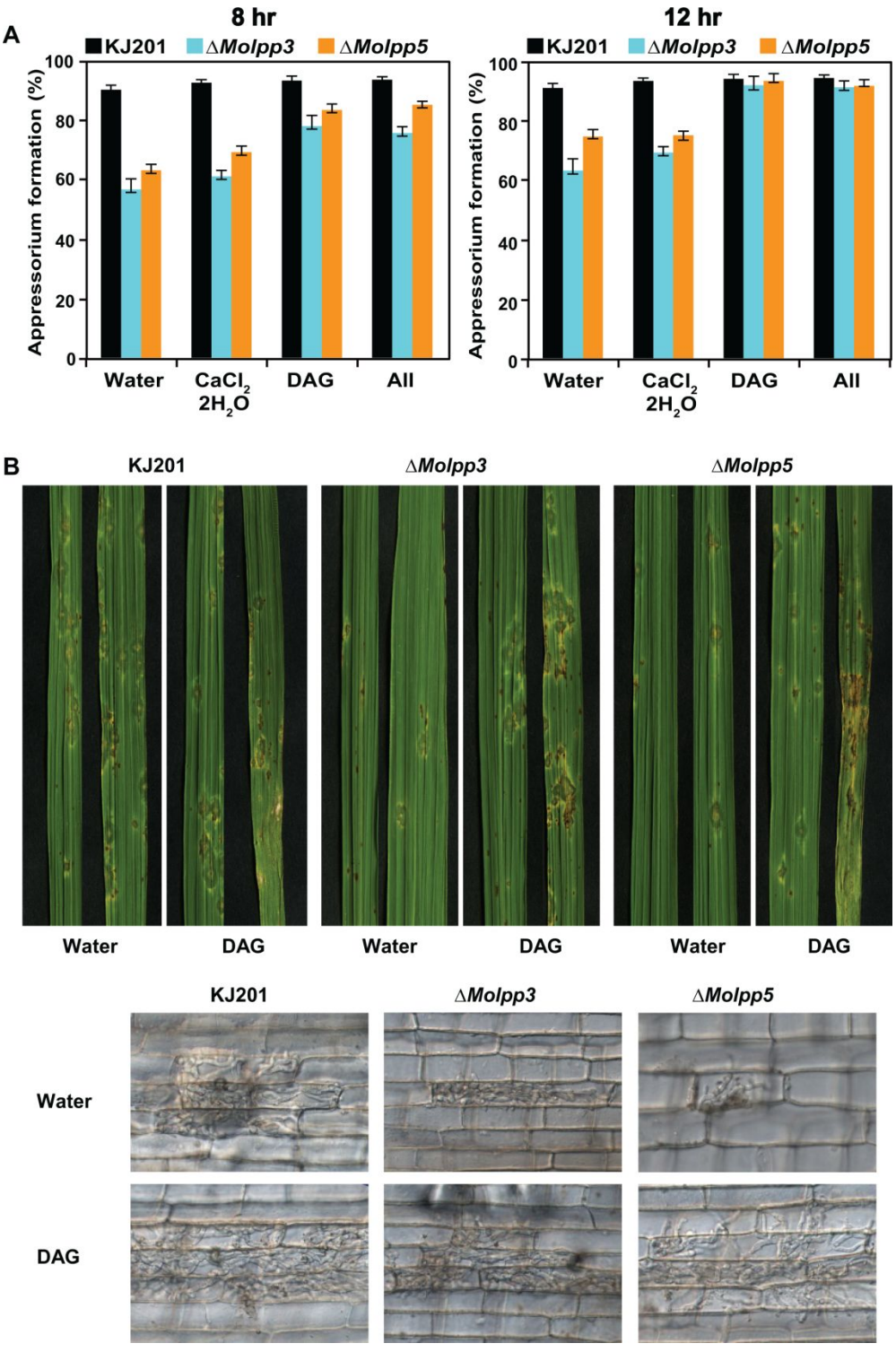
A
8 hr
12 hr
KJ201
ΔMolpp3
ΔMolpp5
Appressorium formation (%)
100
80
60
40
20
0
Water
CaCl2 2H2O
DAG
All
B
KJ201
ΔMolpp3
ΔMolpp5
Water
DAG

Fig. 10. Restoration of appressorium formation and pathogenicity by addition of DAG.

(A) Appressorium formation of wild-type and mutants in the presence of DAG and/or $CaCl_2$. Conidial suspension (2×10^4 conidia/ml) was placed the hydrophobic side of cover slips and mixed with DAG and/or $CaCl_2$ to final concentrations of 20 μg/ml and 10 mM, respectively. Appressorium formation was observed under a microscope 8 and 12 h after incubation. (B) Spray inoculation (upper panels) and sheath assay (lower panels) with conidial suspensions supplemented with 20 μg/ml of DAG.

analysis showed that deletion of *MoLPP1* or *MoLPP2* increased the expression of rest of the genes, whereas deletion of *MoLPP3* or *MoLPP5* decreased the expression of other genes (Figure 11). It appeared that depletion of *MoLPP1* or *MoLPP2* can be complemented by elevating the expression of *MoLPP3* and/or *MoLPP5* but the reverse could not be achieved. These data suggest that transcriptional regulations of LPP genes are intertwined in a network where expressions of other genes are dependent on expression of *MoLPP3* or *MoLPP5*.

Although PI-PLC-mediated pathway is known to produce different type of DAGs in mammals, we could not exclude the possibility that this pathway contributes to maintaining homeostasis by responding to perturbation in cellular reservoir of DAG. However, when we checked the expression of three PLC encoded genes (*MoPLC1*, *MoPLC2* and *MoPLC3*) in Δ*Molpp3* and Δ*Molpp5* background, none of them showed significant difference relative to the wild type strain, suggesting that *MoLPP*-dependent and PI-PLC-mediated pathways are under independent regulation (Figure 12). Therefore, we rule out the possibility that depletion of cellular DAG level resulted from deletion of LPP genes can be complemented by up-regulation of the alternative pathway.

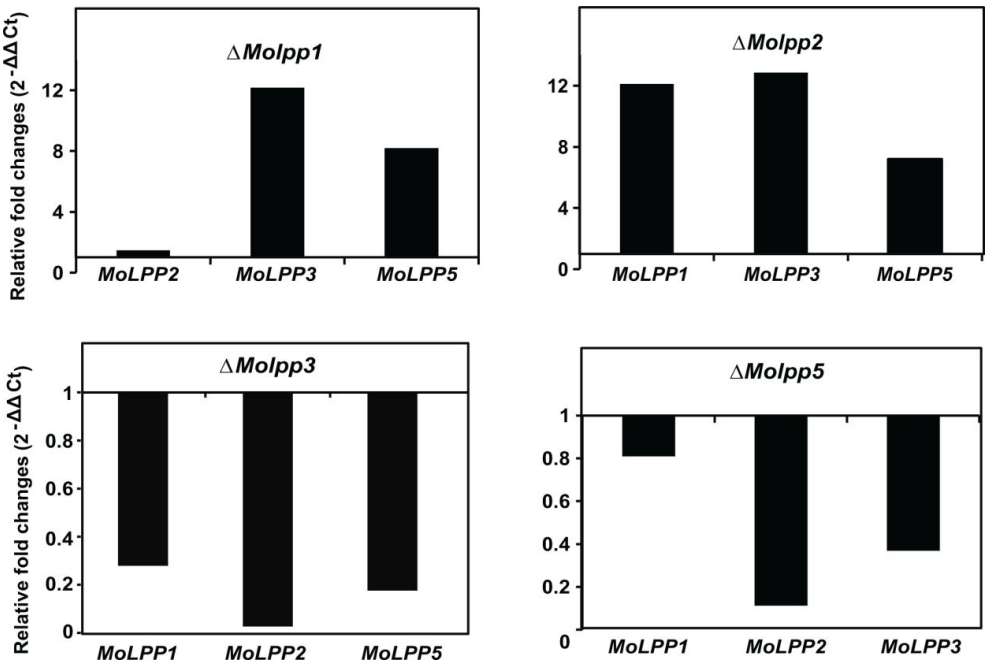

Fig. 11. Transcriptional profiling of *MoLPPs* in knockout mutant background.

In each knockout mutant, relative transcript abundance of genes was measured for *MoLPP* genes.

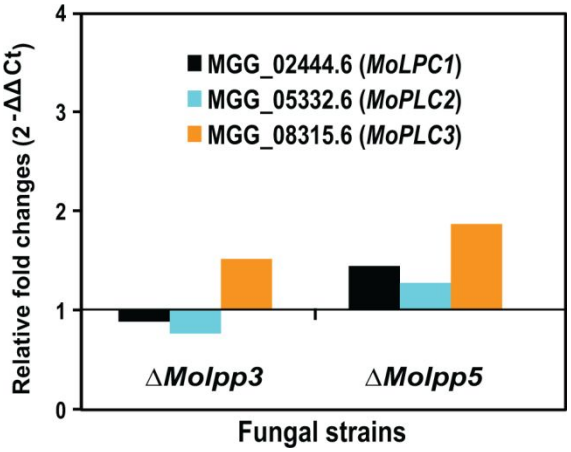

Fig. 12. Transcriptional profiling of phospholipase genes of *M. oryzae* in Δ*Molpp3* and Δ*Molpp5* knockout mutants.

Transcriptional expressions of *MoPLC* genes in knock-out mutants were compared with the expression of wild type strain following normalization using β-tubulin.

DISCUSSION

In many pathogenic fungi, it has been shown that DAG acts as a second messenger involved in regulation of developmental processes as a part of PLC-mediated signaling pathways (Choi et al., 2011; Ghannoum, 2000; Rho et al., 2009; Rhome and Del Poeta, 2009). However, roles of DAG are not limited to a second messenger but as diverse as a basic component of membranes, a precursor in lipid metabolism and a central element in lipid-mediated signaling pathway (Carrasco and Merida, 2007). Such a broad implication of DAG in cellular processes implies that cellular DAG level should be tightly regulated. A critical point at which DAG production and clearance are regulated is the dephosphorylation of PA by LPP.

Here we identified LPP encoding genes using three criteria: presence of PAP2 domain, conservation of three sequence motifs, and presence of six trans-membrane domains in gene-coded proteins. Among the eight genes encoding PAP2 domain, the five that meet the rest of criteria, were selected as candidate LPP genes in *M. oryzae* and named *MoLPP1* to *MoLPP5*. The fact that three distinct motifs and six trans-membrane domains are the hallmark properties of LPP enzymes, suggesting that *MoLPP1* to *MoLPP5* are likely to be bona fide LPPs. It is not clear whether or not *MoVAN*, *MoLCBP3*, and *MoDoPP* have

phosphatase activity, considering poor conservation of three motifs that are important for catalytic activity. Among the five putative LPPs, *MoLPP4* and *MoLPP5* were more closely related to *DPP1* than *LPP1* in *S. cerevisiae*, suggesting that their substrates may include DGPP (Figure 3). Of note, *MoLPP4* seemed to be particularly highly divergent from the yeast *LPP1*. In conjunction with that, the expression profile of *MoLPP4* deviating the most from the other genes suggests sub-functionalization of *MoLPP4*. It is currently difficult to further address the implication of such divergence due to our failure to generate a knockout mutant strain for the gene.

One possibility of the failure in generation of mutant lacking *MoLPP4* is lethality of gene deletion. However, based on the studies of yeast *LPP1* and *DPP1*, together with our own work, it is not likely that deletion of individual LPP genes including *MoLPP4* is lethal. In *M. oryzae,* as with the other filamentous fungi, it is known that efficiencies of gene deletion by homologous recombination are generally low and dependent on the locus (Talbot and Foster, 2001; Villalba et al., 2008). Therefore, we presume that the genomic environment of the locus harboring *MoLPP4* is not favorable for homologous recombination to occur.

Among the remaining four *LPP* genes, deletion of either *MoLPP3* or *MoLPP5* resulted in the fungus that was significantly impaired in pathogenesis, in

contrast to deletion of *MoLPP1* or *MoLPP2* having no effects on fungal developments and pathogenicity. This discrepancy in effect of deletion between two sets of genes could be explained by our expression analysis that revealed complex regulatory relationship among *LPP* genes. Since *LPPs* are not able to directly regulate expression of genes, the nature of such regulatory relationship should be dependent on lipid signaling. Removal of terminal phosphomonoester group from bioactive lipid molecules such as phosphatidic acid through *LPP* activities is known to result in functional inactivation of these lipids, which otherwise have multitude of effects on cells through interaction with their targets (Sciorra and Morris, 2002). We conjecture that each *LPP* might have different but overlapping substrate specificity and that regulation of bioactive lipids by different *LPP*s would underlie the observed transcriptional network among *LPP* genes.

Notably, effect of deletion of *MoLPP3* or *MoLPP5* was specific to plant infection. This may be possibly due to functional divergence among *MoLPP* genes after duplication events leading to paralogs in the genome. However, it is still possible that *MoLPP*s function in combinations, making it difficult for us to evaluate their functions independently of other genes via deletion of individual genes. For example, deletion of *MoLPP3* together with other *MoLPP*(s) may reveal roles of *MoLPP3* in vegetative growth. Unfortunately,

however, introducing more than three targeted mutations into the fungal genome is technically challenging and our efforts to make double deletion mutant for *MoLPP3* and *MoLPP5* genes were unsuccessful.

We showed that virulence defect of Δ*Molpp3* and Δ*Molpp5* are attributed to the reduced ability of the mutant to grow inside host cells. However, both mutants were comparable to the wild-type in sensitivity to reactive oxygen species and a cell wall perturbing agent, leaving an open question of why invasive growth is significantly impaired in the mutants. In a human pathogen, *Cryptococcus neoformans*, DAG produced by inositol phosphorylceramide (IPC) synthase is known to regulate production of melanin and antiphagocytic protein, two important virulence factors, in at least two ways (Rhome and Del Poeta, 2009). First, DAG can bind to C1 domain of Pkc1 leading to increase in Pkc1 activity, which in turn mediates melanin production. The second way is binding of DAG to the transcription factor *Atf2* that promote production of antiphagocytic protein 1. Although the genome of *M. oryzae* does not encode an orthologs of IPC synthase, it may be possible that *LPP*-mediated production of DAG may have an impact on regulation of some virulence factors in *M. oryzae* through its interaction with kinases or transcription factors as shown in *C. neoformans*.

Alternative possibility is related to the effects of *LPP* activities on the

membrane lipid bilayer through production of DAG. Under equilibrium conditions, DAG contributes no significant proportion of cell membranes. However, local and transient accumulation of DAG in the membranes may lead to changes in physical properties of membrane itself and membrane-associated proteins, influencing important cellular processes such as membrane trafficking and exocytosis (Gomez-Fernandez and Corbalan-Garcia, 2007; Goni and Alonso, 1999). In addition, production of DAG will alter lipid compositions in the membranes as well, since DAG is a byproduct of dephosphorylation reaction of lipids. In many intracellular pathogens of animals, lipid rafts, sterol and sphingolipid-rich membrane microdomains, have been shown to control their virulence (Goldston et al., 2012). Even in human pathogenic fungi, potential role of such membrane domain in pathogenesis was alluded. For example, *Candida albicans* was shown to produce many GPI-anchored virulence factors that are induced along with sterol-rich domain (SRD) (Alvarez et al., 2007). Similarly, it was suggested that membrane localization and release of the virulence factors such as superoxide dismutase and phospholipase B are regulated by special membrane domains in *C. neoformans* (Siafakas et al., 2006). Our expression data shows specific up-regulation of *MoLPP3* and *MoLPP5* during interaction with the host plants. Considering the impact of DAG production on the membranes, it

is tempting to speculate that *in planta* specific activities of *LPP*s may enable the fungus to modulate or remodel physical properties and lipid composition of membranes for successful colonization of rice plants.

In this study, we investigated how regulation of cellular DAG levels is implicated in development and pathogenesis of the plant pathogenic fungus through deletion of genes that encode proteins involved in a key step of DAG biosynthesis. Our results demonstrated that proper regulation of DAG is pivotal for fungal pathogenesis, independently of the PIP_2-PLC pathway. In particular, we showed that *LPP*-mediated DAG production has profound impact on invasive growth, during which the most intimate interaction with the host plant occurs. Currently other cellular targets of DAG than PKC1 are not known in *M. oryzae*. Furthermore, there are growing bodies of evidences that suggest the importance of membrane functions in fungal pathogenesis. In light of this, we believe that our work will not only illuminate the importance of DAG production in virulence of plant pathogenic fungi but also have many ramifications on studies regarding lipid metabolisms and membrane-associated processes in fungal pathogenesis.

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CHAPTER 2

Analysis of *in Planta* Expressed Orphan Genes in the Rice Blast Fungus, *Magnaporthe oryzae*

ABSTRACT

Genomes contain a large number of genes no orthologs of which have been found in other species. Although the origin of such "orphan" genes remains unclear, they are thought to be involved in species-specific adaptive processes. Here, we analyzed seven orphan genes (*MoSPC1* to *MoSPC7*) prioritized based on *in planta* expressed sequence tag (EST) data in the rice blast fungus, *Magnaporthe oryzae*. Expression analysis using qRT-PCR confirmed the expression of four genes (*MoSPC1*, *MoSPC2*, *MoSPC3* and *MoSPC7*) during plant infection. However, individual deletion mutants of these four genes did not differ from the wild-type strain for all phenotypes examined, including pathogenicity. The length, GC contents, codon adaptation index and expression during mycelial growth of the four genes suggest that these genes formed during the evolutionary history of *M. oryzae*. Synteny analyses using closely related fungal species corroborated the notion that these genes evolved *de novo* in the *M. oryzae* genome. In this report, we discuss our inability to detect phenotypic changes in the four deletion mutants. Based on these results, the four orphan genes may be products of *de novo* gene birth processes, and their adaptive potential is in the course of being tested for retention or extinction through natural selection.

INTRODUCTION

Orphan genes are protein-coding regions that lack any recognizable orthologs in other organisms (Ekman and Elofsson, 2010). Based on the large number of genome sequences available, orphans are a universal feature of all genomes (Khalturin et al., 2009). Two major models have been proposed for the origin of new genes; the duplication-divergence model and *de novo* evolution model (Tautz and Domazet-Loso, 2011). In the first model, new genes emerge through gene duplication followed by rapid divergence leading to loss of similarity to the gene from which it was duplicated (Domazet-Loso and Tautz, 2003). This model has been well-supported and is considered the major mechanism for creating evolutionary novelties. However, within the framework of this model, it is difficult to explain how natural selection can choose one gene for divergence while retaining the duplicate to maintain the ancestral function (Conant and Wolfe, 2008; Lynch and Katju, 2004). Furthermore, this model is based on the extensive accumulation of substitutions over the entire length of the protein during divergence to the point at which paralogous relationships cannot be detected. This assumption rarely holds due to the existence of functional protein domains in many genes. The second model postulates that a new gene can emerge directly from non-

coding sequences via a random combination of sequences, giving rise to functional sites such as transcription initiation regions and polyadenylation sites (Siepel, 2009).

Although such *de novo* gene birth is considered very rare, recent reports provided evidence for this type of gene birth in a variety of organisms (Begun et al., 2007; Cai et al., 2008; Donoghue et al., 2011; Heinen et al., 2009; Levine et al., 2006; Li et al., 2010a; Yang and Huang, 2011; Zhou et al., 2008). Furthermore, comparative analyses of genome sequences revealed that orphan genes emerge at high rates and account for 10-20% of total genes within individual eukaryotic genomes (Domazet-Loso and Tautz, 2003; Khalturin et al., 2009). Characteristics of orphan genes are well-documented in diverse eukaryotes. They are relatively short (both with respect to gene and ORF length), contain a low number of exons and the detectable domains and are less expressed and evolve more rapidly than non-orphan or old genes (Carvunis et al., 2012; Neme and Tautz, 2013).

Orphan genes are believed to be involved in species-specific processes of adaptation (Kaessmann, 2010). A number of studies provide examples supporting such roles of orphan genes. In human, *FLJ33706* that encodes newly organized genes is associated with brain function (Li et al., 2010a). In the yeast *Saccharomyces cerevisiae*, the newly born *BSC4* gene is suggested to

be involved in the DNA damage repair pathway during the stationary phase, whereas another new gene, *MDF1*, promotes vegetative growth and decreases mating efficiency in rich media (Cai et al., 2008; Li et al., 2010b). The *EED1* gene, which is found only in *Candida albicans*, is crucial for hyphal extension and maintenance of filamentous growth both on solid surfaces and during the interaction with host cells (Martin et al., 2011).

In this report, we analyzed orphan genes and determined whether they are involved in fungal pathogenesis in a model plant pathogenic fungus, *Magnaporthe oryzae*. The fungus is a causal agent of the rice blast disease, which is one of the most devastating global fungal diseases of cultivated rice (Talbot, 2003; Valent and Chumley, 1991). Leaf infection by this fungus is initiated by landing of a conidium on the leaf surface. Melanized appressorium develops to mechanically penetrate the cuticular layer, following conidial germination (Wilson and Talbot, 2009). Rice blast is used as a model system to investigate host-pathogen interactions due to the genetic tractability and availability of genome sequences for both organisms (Dean et al., 2005; Goff, 2005; Yu et al., 2002). In this study, we report for the first time the identification and analysis of orphan genes in *M. oryzae*.

MATERIALS AND METHODS

I. Computational analysis

Orphan genes were identified using BLASTP searches against the NCBI non-redundant dataset using protein sequences of *M. oryzae* as queries. The E-value threshold was set at 10^{-3}. The BLAST matrix function embedded in CFGP 2.0 was used to visualize the presence of the gene in *M. oryzae* among species with genome sequences archived in CFGP 2.0 (Choi et al., 2013) (http://cfgp.snu.ac.kr/). Comparison between orphan and non-orphan genes based on gene length, GC content, transcript abundance, and codon adaptation index was performed using R (http://www.R-project.org/).

II. Fungal isolates and culture conditions

Magnaporthe oryzae strain KJ201 was provided by the Center for Fungal Genetic Resources (CFGR, http://genebank.snu.ac.kr) and was used as the wild-type strain for this study. All strains including mutants were grown on V8 agar [V8; 8% V8 juice (v/v) and 1.5% agar (w/v), adjusted to pH 6.0 using NaOH] or oatmeal agar [OMA; 5% oatmeal (w/v) and 2% agar (w/v)] at 25ºC in constant light to promote conidial production (Park et al., 2010). For conidial production, strains were cultured on V8 juice agar medium for 7 days

or on OMA for 10 to 15 days at 25°C under continuous light conditions. To compare mycelial growth, modified complete agar medium (MCA) or modified minimal agar medium (MMA) (Talbot et al., 1993) was used.

III. Nucleic acid manipulation and expression analysis

Two methods were used for fungal genomic DNA isolation based on two different purposes. A quick and easy genomic DNA extraction method was used for PCR-based screening of transformants (Chi et al., 2009). Genomic DNA was isolated from mycelia according to a standard protocol for southern hybridization (Sambrook and Russell, 2001). Southern DNA hybridization was performed with the selected transformants to ensure correct gene replacement events and absence of ectopic integration. Genomic DNA was digested with *Bam*HI, *Pst*I, *Xho*I and *Nhe*I, and blots were probed with 1-kb 5'-flanking or 3'-flanking sequences. Southern DNA hybridization was performed using a standard method (Sambrook and Russell, 2001). To perform expression analysis using quantitative real-time PCR (qRT-PCR), cDNA synthesis was performed with 5 μg of total RNA using the oligo dT primer with the ImProm-IITM Reverse Transcription System kit (Promega, Madison, WI, USA) following the manufacturer's instructions. Primer pairs used in this study were listed in Table 1.

IV. Targeted gene deletion

Knock-out constructs of individual genes were generated by double-joint PCR where ~1 kb flanking sequences of each gene was amplified and fused with the hygromycin resistance gene (HPH) cassette. The knock-out constructs were transferred to wild-type protoplasts and the resulting transformants were primarily selected by PCR-based screening using specific primer pairs *MoSPC1*_NF, *MoSPC1*_NR, *MoSPC2*_NF, *MoSPC2*_NR, *MoSPC3*_NF, *MoSPC3*_NR, *MoSPC7*_NF and *MoSPC7*_NR (Table 1). Knock-outs were confirmed by Southern blot analysis using one of the flanking sequences as a probe.

V. Developmental phenotype assays

Radial mycelial growth was measured on modified complete agar medium (MCA) or modified minimal agar medium (MMA) 12 days after inoculation (DAI) with three replications (Kim *et al*., 2014). Conidia were harvested from 7-day-old mycelia grown on V8 juice agar plates and the conidial suspension was adjusted to 2×10^4 conidia/ml. For conidial germination and appressorium formation, 40 μl of conidial suspension was dropped onto plastic coverslips with three replicates and incubated in a moistened box at room temperature. At 2 and 4 h after incubation, the frequency of conidial germination was

determined by counting at least 100 conidia per replicate under a microscope. The frequency of appressorium formation was measured from germinated conidia at 8 h after incubation. Conidiation was determined by counting the number of conidia using a hemacytometer under a microscope. These assay processes were performed with three replicates in three independent experiments.

VI. Pathogenicity assay

For the pathogenicity assay by spray inoculation, conidia were collected from 7-day-old V8 juice agar medium and 10 ml of conidial suspension were adjusted to 1×10^5 conidia/ml containing Tween 20 (250 ppm final concentration) and sprayed onto the rice seedlings (*Oryza sativa* cv. Nakdongbyeo) of three to four leaf stages. Sprayed rice seedlings were placed in a dew chamber for 24 h under dark conditions at 25°C, then transferred to a rice growth incubator at 25°C, 80% humidity and a 16-h photoperiod with fluorescent lights.

Table 1. Oligo sequences used in this study

Primer name	Sequence (5' → 3')
Primers used for generating gene knock-out constructs:	
*MoSPC1*_UF	CAGTGCACCTGCAAATGTTCC
*MoSPC1*_UR	TGGCAGACAGACGTGTGGTTT
*MoSPC1*_DF	CTACTTGGGAAGGGGACAGGAA
*MoSPC1*_DR	CTTCAGCATCTCCGAATGACTCTGC
*MoSPC1*_NF	ACACCGGGCAACCTCAGCA
*MoSPC1*_NR	TCG CCCTCCACCTCTGA
*MoSPC2*_UF	GTC CAG GTA GCT GTT TGC CAG
*MoSPC2*_UR	GTG TTG CGA TTG GGA GAG CTT
*MoSPC2*_DF	CGC TCT ACG ACG AAG AGT AGA
*MoSPC2*_DR	TCA CAC GGC ATC GAG AAT ACC
*MoSPC2*_NF	ATA TTC CGG CCG ATG GGC CC
*MoSPC*2_NR	GCC ATC GAG CGT CCG CTG TA
*MoSPC*3_UF	GGC TTG GTA CGG AGT GTA CTA GTT C
*MoSPC*3_UR	TGA TGC CTC TAA CGC TAC TCA GTC
*MoSPC*3_DF	CCA AGC CGA TCA ATG TGG ATG TG
*MoSPC*3_DR	TAG CGG GCA AGG TGC AGA T
*MoSPC*3_NF	AGC ACG GAA CTG GCT GCA AGA

*MoSPC*3_NR	AGC ACG GAA CTG GCT GCA AGA
*MoSPC*7_UF	TCC TGC GCA ACT CCT TGT TG
*MoSPC*7_UR	TAT ACG CGC CGA AGA GGC
M*oSPC*7_DF	ATG ATA ACC TGA GCG CCG ACT
*MoSPC*7_DR	ATG ATA ATG ACG CGG CAA ACG G
*MoSPC*7_NF	ATC ATC CCC TCA CCA GCT GGC
*MoSPC*7_NR	GTG CAA CAG CCG CCG CAA
HPH_F	GGCTTGGCTGGAGCTAGTGGAGG
HPH_R	GTTGGTGTCGATGTCAGCTCCGGAG

For qRT-PCR*:*

*MoSPC*1_qRT-F	ACGCACTATGAGCTAAACGAG
*MoSPC*1_qRT-R	CTGCTCTTCCCATCTGGC
*MoSPC*2_qRT-F	CGCGTACATGATTGGAATCG
*MoSPC*2_qRT-R	TTAGGTAAGCTCTCGTGTTGC
*MoSPC*3_qRT-F	GTGCCTAAACAAAGACCATCG
*MoSPC*3_qRT-R	CCTGGGTACTATCTTTCAGCG
*MoSPC*4_qRT-F	GGGCAGCTTCTCTAGGTTTAG
*MoSPC*4_qRT-R	GCTGTCCGTTGTTCTCTGTAT
*MoSPC*5_qRT-F	ACACCAAATTATCTCCACCCC

*MoSPC*5_qRT-R	GAGGTGGATAGACGTGCATAAG
*MoSPC*6_qRT-F	CCTTGATTACCTTGTGCTTGC
*MoSPC*6_qRT-R	TTTCTGGTCTATTCTTATTCCCGG
*MoSPC*7_qRT-F	CTGGCTGAAGTGGTCGTATT
*MoSPC*7_qRT-R	GGTAAACAAATGGCAAGCTCC
B-tub_qRT-F	TCGACAGCAATGGAGTTTAC
B-tub_qRT-R	AGCACCAGACTGACCGAAGAC

RESULTS

I. Identification of orphan genes with transcriptional activity in *M. oryzae*

To identify orphan genes in the *M. oryzae* genome, we performed BLASTP searches against the NCBI non-redundant database (NCBI nr) with an e-value threshold of 10^{-3}. This search demonstrated that 2,740 out of 12,991 genes (~21%) encode putative proteins with no match in the NCBI nr, indicating that they are likely orphan genes. We hypothesized that there should be evidence of transcription of orphan genes with roles in the host plant interaction. Thus, we utilized a previously reported *in planta* EST library (Kim *et al*., 2010). Although a total of 712 genes were initially reported to have *in planta* ESTs, we found only 542 genes in *M. oryzae* genome version 8. Comparison of orphan genes and *in planta* EST data showed that seven genes were shared between the two datasets. Since these genes were specific to *M. oryzae*, we named them *MoSPC*s (*Magnaporthe oryzae* specific). These genes were typically less than 1 kb in length (excluding *MoSPC6*), and *MoSPC3* was predicted to contain a signal peptide (Table 2).

II. Expression analysis of *in planta* expressed *M. oryzae* orphan genes

The presence of ESTs is a good indication of transcriptional activity. However,

Table 2. Structural characteristics of *MoSPC* genes

Gene name	Gene size (bp)	No. of exons	Longer exon (bp)	Secretion possibility
MoSPC1	809	3	330	No
MoSPC2	546	2	251	No
MoSPC3	704	3	198	Yes
MoSPC4	233	2	134	No
MoSPC5	984	1	298	No
MoSPC6	1979	7	218	No
MoSPC 7	777	2	222	No

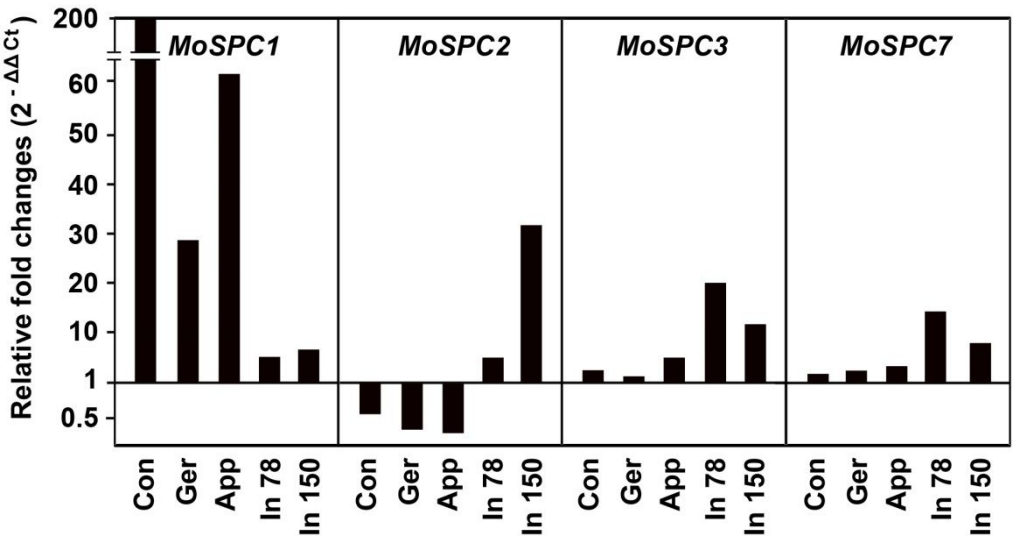

Fig. 1. Transcript abundance of *MoSPC* genes in different development stages of *M. oryzae*.

Con, conidia; Ger, germinating conidia; App, appressoria; In 78, infection stage at 78 h post-inoculation (hpi); In 150, infection stage at 150 hpi.

it does not provide information on differential expression of a gene, which is associated with the gene's role. Therefore, we examined the transcriptional activity of seven genes during different developmental stages, including plant infection, using qRT-PCR. Expression profiling suggested that four genes (*MoSPC1*, *MoSPC2*, *MoSPC3* and *MoSPC7*) among the seven are differentially expressed during plant infection (appressorium formation and invasive growth) compared to mycelial growth conditions, whereas expression of *MoSPC6* was downregulated (Fig. 1). However, *MoSPC4* was not differentially expressed under the same conditions (data not shown). Based on this expression analysis, we selected *MoSPC1*, *MoSPC2*, *MoSPC3* and *MoSPC7* for targeted deletion and further functional studies.

III. Targeted deletion of selected genes and phenotypes analysis

To investigate the possible roles of the four selected genes in *M. oryzae*, we generated deletion mutants of individual genes. Gene deletion constructs were prepared through double joint PCR (Yu et al., 2004), in which the HPH cassette was combined with ~1-kb 5'/3' flanking regions of the target gene (Figure 2). The gene deletion constructs were used directly for transformation with wild-type protoplasts. The resulting hygromycin-resistant transformants were screened by PCR and correct gene replacement, and the resulting

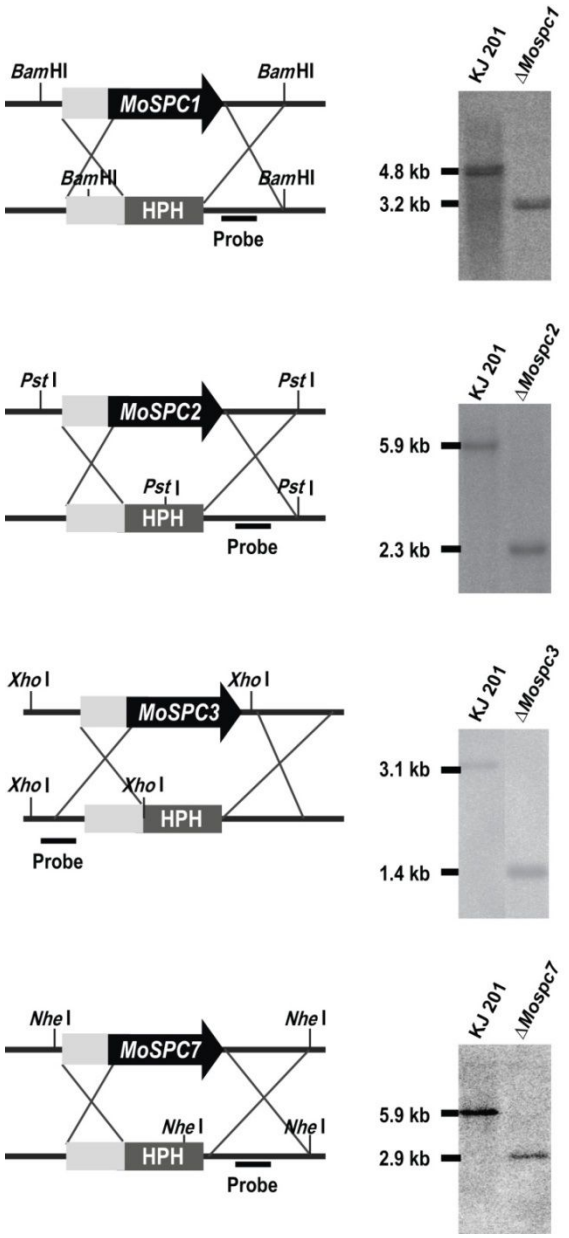

Fig. 2. Targeted disruption of *MoSPC1*, *MoSPC2*, *MoSPC3* and *MoSPC7*. Schematic diagram of knockout strategy for target genes (left) and Southern blot analysis of the resulting mutants (right) are shown in pairs for each gene.

transformants were confirmed using Southern hybridization analysis (Figure 3).

When examining the phenotypes of the deletion mutant in each of the four genes, we found that deletion mutants of *MoSPC*s were comparable to the wild-type with respect to mycelial growth and conidiation (Table 3). Conidia of all mutants were capable of germinating and developing appressorium on a germ tube tip. The morphology of appressoria formed by the four mutants was indistinguishable from the wild-type. Furthermore, when conidial suspensions of the mutants were spray-inoculated onto rice plants of a susceptible cultivar, Nakdongbyeo, all mutants showed virulence similar to the wild-type strain (Fig. 3A and 3B). These results suggested that *MoSPC*s are not required for the traits examined, including mycelial growth, conidiation, conidial germination, appressorium formation, and pathogenicity.

IV. Genome-wide analysis of orphan genes in *M. oryzae*

In parallel to the targeted deletion of the four selected genes, we analyzed orphan genes at the genome scale by comparing the features of orphan and non-orphan genes, including gene length, GC contents, transcription and codon adaptation index. Our analysis showed that orphan genes including *MoSPC*s (excluding *MoSPC6*) were relatively short (Figure 4A), showed a

Table3. Phenotypes of wild-type, Δ*Mospc1*, Δ*Mospc2*, Δ*Mospc3* and Δ*Mospc7*

	Mycelial growth (mm)[a]		**Conidiation**	**Conidial**	**Appressorium**
Strain	**MCA**	**MMA**	**(× 10^4/ml) [b]**	**germination (%)[c]**	**formation (%)[d]**
KJ201	83.6± 0.6	78.6 ± 0.6	27.8 ± 4.6	90.2 ± 1.1	84.3 ± 5.4
Δ***Mospc1***	83.2 ± 0.7	78.0 ± 0.6	24.3 ± 9.6	90.2 ± 1.1	79.4 ± 3.9
Δ***Mospc2***	82.2 ± 0 .3	77.3 ± 0.3	28.3 ± 3.3	88.9 ± 0.9	79.4 ± 3.9
Δ***Mospc3***	81.6 ± 1.6	77.0 ± 1.3	29.2 ± 4.2	90.3 ± 1.3	82.3 ± 3.3
Δ***Mospc7***	82.3 ± 0.6	76.0 ± 1.5	26.6 ± 0.6	87.1 ± 3.3	81.2 ± 3.5

[a] Mycelial growth was measured at 12 days after inoculation (DAI) on modified complete agar medium (MCA) and minimal agar medium (MMA).

[b] Conidiation was measured as the number of conidia from a culture flooded with 5 ml of sterilized distilled water.

[c] Percentage of conidial germination was measured on plastic coverslips under a light microscope using conidia harvested from 7-day-old V8 juice agar plates.

[d] Percentage of appressorium formation was measured on plastic coverslips using conidia harvested from 7-day-old V8 juice agar plates.

Data are presented as means ± standard deviation from three independent experiments.

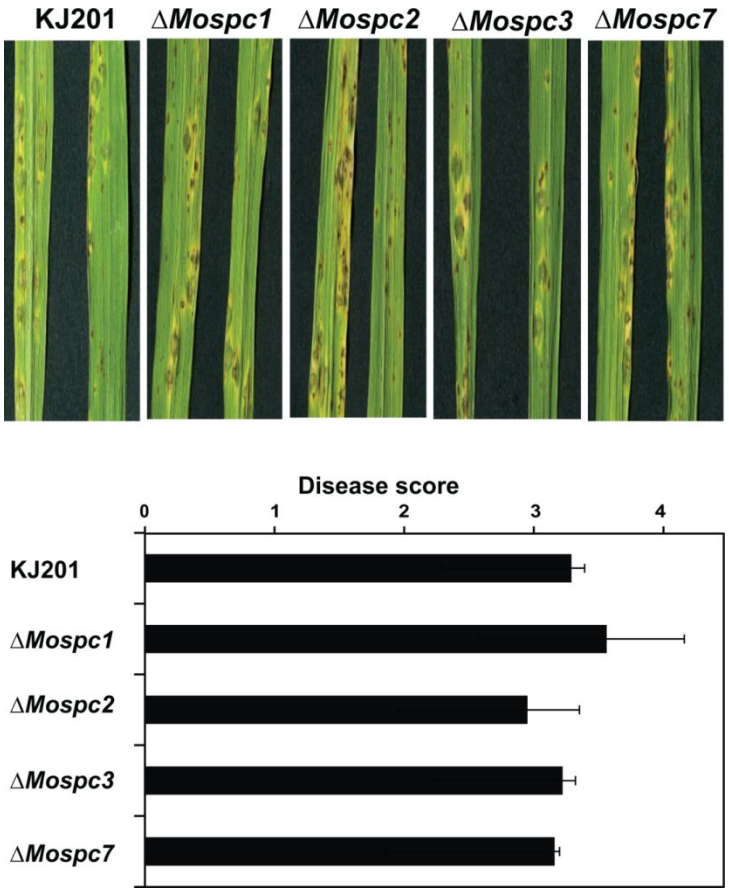

Fig. 3. Pathogenicity assay of wild-type, Δ*Mospc1*, Δ*Mospc2*, Δ*Mospc3* and Δ*Mospc7*.

(A) Disease development after spraying conidial suspension onto rice leaves. Conidial suspension (1×10^5/ml) was sprayed onto the leaves and diseased leaves were harvested 7 days after inoculation. (B) Disease score measurement was performed on 7-day diseased leaves of all strains, as described by Valent et al., 1991. The data are the mean ± standard deviation of three independent experiments and all data are statistically analyzed using Tukey test ($p < 0.05$).

low GC content (Figure 4B), low transcription (Figure 4C) and less-biased codon usage (Figure 4D) than their non-orphan counterparts. Gene length is positively associated with both GC content and gene expression (Jansen and Gerstein, 2000; Pozzoli et al., 2008). We also observed such a positive correlation among gene features. The differences between orphan and non-orphans in our analysis are in concordance with the results of previous studies on *de novo* emergence of orphan genes from model organisms (Cai et al., 2008; Carvunis et al., 2012; Li et al., 2010b; Neme and Tautz, 2013).

V. Synteny analysis of orphan genes

The most stringent criterion for involvement of *de novo* processes in explaining orphan genes requires that syntenic blocks spanning an orphan gene are present in outgroup organisms as non-coding sequences that are not transcribed (Cai et al., 2008; Knowles and McLysaght, 2009). To further support *de novo* gene birth of the four selected genes, we performed synteny analysis with phylogenetically closely related fungal species, including *Gaeumannomyces graminis* (a member of the Magnaporthaceae family) (Table 4). Our synteny analysis demonstrated that the selected genes (excluding *MoSPC2*) were present in the well-conserved synteny block (Figure 5). We did not detect a synteny block containing *MoSPC2,* even by expanding the number

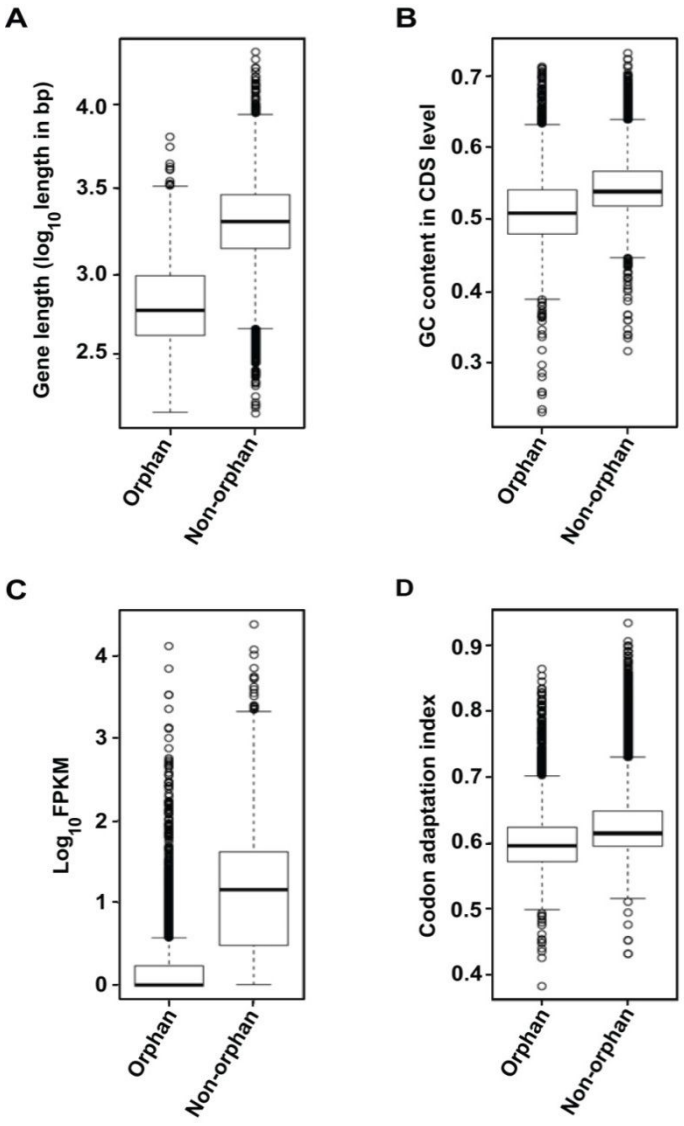

Fig. 4. Genomic features of new born genes in *M. oryzae*.

(A) Gene length, (B) GC content in coding DNA sequence (CDS) level, (C) expression pattern and (D) codon adaptation between new and old genes using the Mann-Whitney Wilcoxon test ($p<2.2e-16$).

of fungal species included in the analysis. Based on our analysis, *MoSPC3* was the orphan gene that best fit our stringent criterion. In synteny blocks harboring *MoSPC1* and *MoSPC7*, predicted ORFs (GGTG_00636 and GGTG_00730) of *G. graminis* were found in a region corresponding to the location of *MoSPC1* and *MoSPC7*. However, these two ORFs did not have any similarity to *MoSPC1* or *MoSPC7* (data not shown). BLASTP searches against the NCBI nr database showed that GGTG_00636 and GGTG_00730 were also likely orphan genes in the *G. graminis* genome. Based on our observations, at least one of the selected orphan genes (*MoSPC3*) originated *de novo* during the evolutionary history of *M. oryzae*.

Table 4. List of organisms used in synteny relationship with *MoSPC* genes

Species	**Kingdom**	**Phylum**	**Type**
Fusarium graminearum	Fungi	Ascomycota	Plant Pathogen
Fusarium oxysporum	Fungi	Ascomycota	Plant Pathogen
Blumeria graminis	Fungi	Ascomycota	Plant Pathogen
Colletotrichum graminicola	Fungi	Ascomycota	Plant Pathogen
Botrytis cinerea	Fungi	Ascomycota	Plant Pathogen
Mycosphaerella graminicola	Fungi	Ascomycota	Plant Pathogen
Gaeumannomyces graminis	Fungi	Ascomycota	Plant Pathogen
Magnaporthe poae	Fungi	Ascomycota	Plant Pathogen
Histoplasma capsulatum	Fungi	Ascomycota	Animal Pathogen
Coccidioides immitis	Fungi	Ascomycota	Animal Pathogen
Aspergillus fumigatus	Fungi	Ascomycota	Animal Pathogen
Candida albicans	Fungi	Ascomycota	Animal Pathogen
Aspergillus nidulans	Fungi	Ascomycota	Model Fungi
Neurospora crassa	Fungi	Ascomycota	Model Fungi
Podospora anserina	Fungi	Ascomycota	Model Fungi
Saccharomyces cerevisiae	Fungi	Ascomycota	Model Fungi
Schizosaccharomyces pombe	Fungi	Ascomycota	Model Fungi

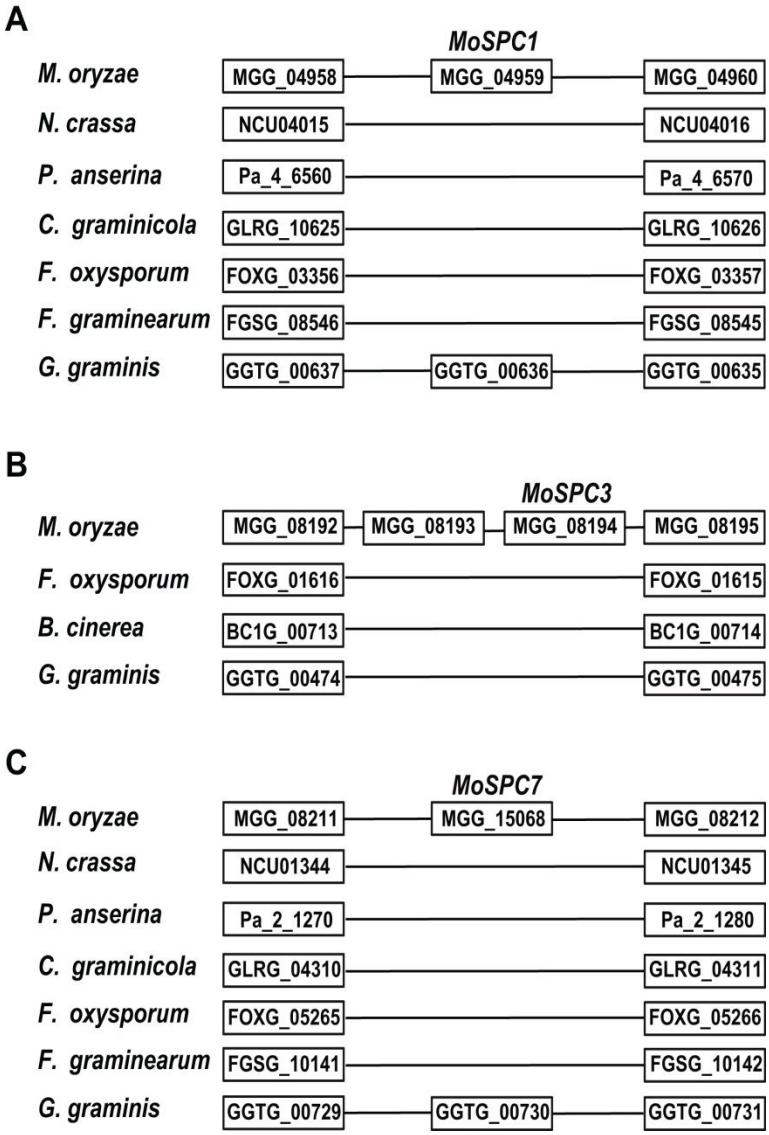

Fig. 5. Synteny relationships of *MoSPC* genes with other fungal species. Protein sequences from the upstream and downstream flanking regions of *MoSPC* genes were used for BLASTP searches with a cutoff e-value of 10^{-5} to identify homologous genes in other organisms. Gene birth of *M. oryzae* is shown in the upper region.

DISCUSSION

De novo gene birth is an important mechanism in species-specific adaptation processes (Cai et al., 2008; Kaessmann, 2010). Examples of newborn genes have shown that they quickly become essential and play pivotal roles during development, reproduction and survival (Ding et al., 2010; Li et al., 2010a). However, there have been no fully documented cases in plant pathogenic fungi regarding *de novo*-originated genes, although it is possible that such novel genes contribute to fungal pathogenesis, host specificity and host jumping.

To identify orphan genes in *M. oryzae*, we performed BLASTP searches against the NCBI nr database. To characterize orphan genes, it is important to understand whether a gene is absent in other species or whether the observed absence is the result of technical limitations of the methods used. This concern was addressed by a recent study showing that BLAST is sufficiently sensitive to detect the majority of remote homologues (Alba and Castresana, 2007). Furthermore, the NCBI nr database includes a variety of sequences, including those from metagenomic studies (Pruitt et al., 2007). A gene is considered to be absent in the genomes of other species if there is no match to the corresponding gene product in a BLASTP search against the NCBI nr database. Using this approach, we identified ~ 2,700 orphan genes that account for ~21%

of genes annotated in the genome. Considering that orphan genes account for approximately 10-20% of total genes in other organisms (Khalturin et al., 2009), the observed proportion of orphans in the genome of *M. oryzae* was reasonable. Our analysis of orphan versus non-orphan genes showed that orphan genes of *M. oryzae* possess typical characteristics of genes formed via *de novo* evolution; namely, short length, low GC contents, low transcriptional activity, and less bias in codon usage.

In this report, we targeted four orphan genes of *M. oryzae* among ~ 2,700 orphan genes prioritized by *in planta* EST data and qRT-PCR to explore the involvement of orphan genes in fungal pathogenesis. Synteny analysis of these four genes indicated that at least one of these genes (*MoSPC3*) has a *de novo* origin. Our data suggest that the other three genes may have originated *de novo*. It is highly likely that homologous genes of *MoSPC2* flanking genes are spread in other organisms during evolution, in contrast to *M. oryzae* in which these genes remain linked. Predicted ORFs of *G. graminis* (GGTG_00636 and GGTG_00730) are present in the corresponding location of *MoSPC1* and *MoSPC7* without sequence similarities, which is suggestive of two possibilities. First, the two genes are orthologous between *M. oryzae* and *G. graminis* but underwent rapid divergence after the speciation event. Second, each gene emerged *de novo* from non-coding sequences independently in both

species. We argue that the second possibility is more parsimonious than the first considering the small amount of time since speciation events and that the divergence is significant enough to be undetectable using a BLAST search.

Deletion of individual genes (*MoSPC1*, *MoSPC2*, *MoSPC3* and *MoSPC7*) did not result in phenotypic changes for traits we examined in this study. This suggests that these four genes are not required for fungal development and pathogenesis. It is also possible that assays we performed to examine phenotypes were not sufficiently sensitive to detect subtle differences that exist between the mutants and wild-type. The absence of phenotypes in deletion mutants of an orphan gene was reported previously in *M. grisea*. The authors investigated a gene called *MIR1*, which is unique to *M. grisea* (Li et al., 2007). Deletion of *MIR1* did not cause changes in phenotypes examined. Based on its upregulation during plant infection, *MIR1* may be important for the fitness and virulence of *M. grisea* under field conditions, although it is dispensable for plant infection under laboratory conditions. This may also be true for the four orphan genes investigated in this study.

Alternatively, the absence of phenotypes could be related to evolutionary processes through which orphan genes are gained and retained in the genome. The *de novo* evolution model of orphan genes suggests a balance between gene emergence and gene loss over time (Palmieri et al., 2014). These genes

can be retained if they confer selective advantages, otherwise they are quickly lost. Thus, we may have observed orphan genes that are being selected for retention or extinction through natural selection. In addition, orphan genes present in the majority of field isolates are more likely to have an impact on the fungal life cycle.

In summary, we investigated the role of orphan genes during pathogenesis of *M. oryzae* through gene deletions. Our data demonstrated that none of these genes are important for fungal development and pathogenicity, despite their upregulation during plant infection processes. We confirmed that at least one of the genes has a *de novo* origin based on our analyses, including synteny comparison. These results suggest that a significant proportion of the fungal genome is comprised of orphan genes. These genes should be examined to determine their origin and increase our understanding of fungal pathogenesis and evolution.

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벼 도열병균 인지질 탈인산화 효소 유전자와 식물체내 발현 orphan 유전자의 특성 구명

사닷 엠디 아부

초 록

물질대사와 신호전달 체계에 diacylglycerol (DAG)이 미치는 영향을 고려한다면 세포 내 적정 수준의 DAG 농도를 유지하는 것은 곰팡이의 항상성 유지와 발달과정에 중요하다. PIP_2-PLC 경로를 제외하고, DAG 생합성 경로는 인지질 탈인산화 효소 (LPP)에 의한 phosphatidic acid의 탈인산화로 수렴한다. 곰팡이의 발달과 병원성에서 LPP의 역할을 이해하기 위해, 모델 식물 병원균인 *Magnaporthe oryzae*의 5개 LPP 추정 유전자 (*MoLPP1*~*MoLPP*5) 를 동정하고 기능 분석하였다. qRT-PCR을 활용한 유전자 발현 분석 결과 5개 유전자는 식물 침입과정에서

발현되었다. *MoLPP4*를 제외하고 유전자 삭제 변이체를 만들어 관찰한 결과 *MoLPP3*와 *MoLPP5*는 특이적 침입 구조 발달과 기주 식물 내 증식에 필수적이었던 반면에 *MoLPP1*과 *MoLPP2*는 곰팡이 병원성에 필요하지 않았다. 게다가 외부에서의 포화 DAG 첨가는 부착기 형성의 결함을 복구할 뿐만 아니라 *⊿Molpp3* and *⊿Molpp5* 두 변이체의 병원성 결함을 보완하였다. 이러한 결과는 *MoLPP*에 의해 생성되는 DAG가 곰팡이 발달과 병원성에 중요함을 나타낸다. 곰팡이 병원성에 대해 식물체내 발현 유전자들의 기능을 이해하기 위해 식물체내에서 발현되면서 벼 도열병균에 특이적으로 존재하는 7개의 유전자 (*MoSPC1~MoSPC7*)를 동정하였다. 비록 이러한 orphan 유전자의 기원은 아직 밝혀지지 않았지만 이 유전자들은 종 특이적 적응 과정에 관련되어 있다고 여겨진다. qRT-PCR을 활용한 유전자 발현 분석 결과 7개 유전자 중 4개는 식물 침입과정에서 발현되었다. 하지만 이 4개의 유전자 (*MoSPC1*, *MoSPC2*, *MoSPC3*, *MoSPC7*)에 대한 유전자 삭제 변이체는 침입 발달과 병원성에서 야생형과 유의한 차이를 보이진 않았다. 유전자 길이, GC 비율, 코돈 적응 지수와 균사 생장 시 발현량 정보는 4개 유전자들이 *M. oryzae*의 진화 역사에서 새롭게 생겨난 유전자 일

수 있다는 가능성을 제시한다. 유연관계가 있는 곰팡이 종과 synteny 분석을 수행한 결과 해당 유전자들은 *de novo* 진화과정을 거쳤다는 것을 입증했다. 이 자료에 의거하여 4개의 orphan 유전자들이 *de novo* 유전자 생성 과정의 산물이라는 것을 추측할 수 있으며 이 유전자들의 적응 잠재력은 자연선택에 의해 유지되거나 도태되는 과정에 있다고 할 수 있다.

주요어: 벼 도열병균 (*Magnaporthe oryzae*), diacylglycerol, 인지질 탈인산화 효소, orphan 유전자, 곰팡이 발달과 병원성

학 번: 2009-31285