Identification and Characterization of In planta–Expressed Secreted Effector Proteins from *Magnaporthe oryzae* That Induce Cell Death in Rice

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Interactions between rice and *Magnaporthe oryzae* involve the recognition of cellular components and the exchange of complex molecular signals from both partners. How these interactions occur in rice cells is still elusive. We employed robust-long serial analysis of gene expression, massively parallel signature sequencing, and sequencing by synthesis to examine transcriptome profiles of infected rice leaves. A total of 6,413 in planta–expressed fungal genes, including 851 genes encoding predicted effector proteins, were identified. We used a protoplast transient expression system to assess 42 of the predicted effector proteins for the ability to induce plant cell death. Ectopic expression assays identified five novel effectors that induced host cell death only when they contained the signal peptide for secretion to the extracellular space. Four of them induced cell death in *Nicotiana benthamiana*. Although the five effectors are highly diverse in their sequences, the physiological basis of cell death induced by each was similar. This study demonstrates that our integrative genomic approach is effective for the identification of in planta–expressed cell death–inducing effectors from *M. oryzae* that may play an important role facilitating colonization and fungal growth during infection.

Coevolution of plants and their pathogens in nature has led both sides to develop a battery of strategies to attack and defend. Plant pathogens may first use cell wall–degrading enzymes to digest the surface layers of cell walls to facilitate penetration. Successful pathogens can secrete a variety of extracellular molecules to modulate host defense circuitry. These extracellular molecules include apoplastic effectors such as virulence factors, toxins, and degradative enzymes that function as key virulence determinants to suppress host defense (Hogenhout et

al. 2009). On the other hand, plants have evolved unique mechanisms to defend themselves from most microbes using physical barriers, antimicrobial compounds, and the innate immune system. The plant immune system consists of two layers (Chisholm et al. 2006; Jones and Dangl 2006). The first layer is initiated by the perception of pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) by host membrane-associated pattern recognition receptors (PRR), which cause basal defense responses in plants, called PAMP-triggered immunity (Zhang and Zhou 2010). The second layer is initiated by rapid activation of a hypersensitive reaction (HR) upon recognition of the avirulence effectors by the cognate resistance (R) proteins, which causes strong race-specific resistance called effector-triggered immunity.

Over the past decade, extensive studies have led to the identification of many PAMP and avirulence effectors in plant pathogens and PRR and R proteins in plants (Dodds et al. 2009). Among them the majority of the well-characterized effectors are from prokaryotic bacteria that employ a type III secretion system to deliver effectors directly into host cells. Unlike bacteria, fungal and oomycete pathogens often secrete effector proteins to the extracellular milieu through the eukaryotic secretory pathway (Panstruga and Dodds 2009). Many secreted proteins function in the apoplast of plants, and some have been shown to be translocated into plant cells (Dou et al. 2008a; Whisson et al. 2007), in which they function in the host cytoplasm (Armstrong et al. 2005; Bos et al. 2006; Catanzariti et al. 2006; Dodds et al. 2004; Rehmany et al. 2005; Yoshida et al. 2009). Recent advances in genome sequencing technologies have led to a rapid discovery of numerous effectors in oomycete pathogens and have provided a wealth of information on their structure and function. For example, analysis of the *Phytophthora sojae* and *P. ramorum* genomes led to the discovery of conserved motifs RXLR and dEER (Tyler et al. 2006), required for the translocation of oomycete effectors into plant cells (Dou et al. 2008a; Whisson et al. 2007). The large family of predicted RXLR proteins thus provided a wealth of candidates for functional studies of effector activities in oomycetes. However, secreted proteins exported by fungi appear to lack such conserved motifs, and little is known about their functions.

The hemibiotrophic fungus *Magnaporthe oryzae* is the causal agent of rice blast, the most devastating disease that affects rice production worldwide (Dean et al. 2005; Ebbole 2007; Talbot 2003). In *M. oryzae*, a larger number of genes (up to

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1,306) coding for putative secreted proteins have been predicted from the genome of a laboratory strain, 70-15 (Dean et al. 2005; Yoshida et al. 2009). Seven secreted proteins, i.e., PwL1, PwL2 (Kang et al. 1995; Sweigard et al. 1995), AvrPita (Orbach et al. 2000), AvrPia, AvrPii, AvrPik/km/kp (Yoshida et al. 2009), and AvrPiz-t (Li et al. 2009), have been confirmed as avirulence (Avr) proteins presumably recognized by the corresponding resistance gene products. In addition, a few secreted proteins that are required for pathogenicity, i.e., MPG1 (Talbot et al. 1993), EMP1 (Ahn et al. 2004), MHP1 (Kim et al. 2005), MSP1 (Jeong et al. 2007), MC69 (Saitoh et al. 2012), and Slp1 (Mentlak et al. 2012), and four biotrophy-associated secreted proteins, BAS1 to BAS4 (Mosquera et al. 2009), have also been characterized. However, the majority of *M. oryzae* secreted proteins have not been experimentally tested for their functions in pathogenicity.

To identify *M. oryzae* genes encoding predicted secreted proteins that were expressed in blast-infected leaf tissue, we developed an integrative genome expression profiling approach that includes robust-long serial analysis of gene expression (RL-SAGE) (Gowda et al. 2004), massively parallel signature sequencing (MPSS) (Brenner et al. 2000; Meyers et al. 2004; Nobuta et al. 2007), and sequencing by synthesis (SBS) (German et al. 2008; Venu et al. 2011a). Infected tissues were collected from six timepoints so that fungal transcripts expressed in planta at a late infection stage can be included in the libraries. We used a protoplast transient expression assay to identify in planta-expressed secreted proteins from *M. oryzae* that induce cell death in rice. Among 42 tested proteins, five cell death-inducing proteins were functionally characterized. The integrative approach described here provides an efficient strategy for functional identification of fungal cell death-inducing proteins that are involved in plant and fungal interactions.

RESULTS

Gene expression profiling in blast-infected rice leaves.

To obtain more comprehensive gene expression profiles of *M. oryzae* during compatible and incompatible interactions with rice, we constructed one RL-SAGE, eleven MPSS, and seven SBS libraries for deep sequencing. The RL-SAGE library was generated from rice (cv. Nipponbare) leaves inoculated with the compatible isolate Che86061 at 96 h postinoculation (hpi) (Fig. 1A). A total of 18,154 significant signatures were obtained from the library. Among them, 3,105 (17.1%) and 12,263 (67.5%) significant signatures matched to the *M. oryzae* and rice genomes, respectively. The unmatched signatures may be due to sequencing errors or are located in the sequencing gaps in both the genomes. Only 14 signatures matched to both the *M. oryzae* and rice genomes, signifying that the majority of signatures were genome specific. We identified 3,091 signatures specifically matching to the *M. oryzae* genome, which correspond to 3,000 previously annotated *M. oryzae* genes.

Eleven MPSS libraries were generated from the leaves of wild-type Nipponbare plants or transgenic Nipponbare plants carrying the blast resistance gene *Pi9* (Qu et al. 2006), which were inoculated with *M. oryzae* isolate KJ201 during the compatible (3, 6, 12, 24, 48, and 96 hpi) or incompatible interactions (3, 6, 12, 24, and 48 hpi) (Fig. 1A). As expected, more signatures matched to the *M. oryzae* genome in the compatible interactions compared with that in the incompatible interactions. A total of 57,671 significant signatures were obtained from the five incompatible interaction MPSS libraries. Among these, 724 (1.2%) and 38,024 (65.9%) significant signatures uniquely matched to the *M. oryzae* and rice genomes, respectively. From the six compatible interaction libraries, a total of 63,132 significant signatures were obtained. Among them,

2,545 (4%) and 41,784 (66.1%) significant signatures uniquely matched to the *M. oryzae* and rice genomes, respectively. Altogether, 3,216 annotated *M. oryzae* genes were identified from both the compatible and incompatible MPSS libraries.

The same leaf tissue used for the generation of the MPSS libraries (the compatible interaction at 6, 12, 24, and 96 hpi and the incompatible interaction at 6, 12, and 24 hpi) were used for the construction of the seven SBS libraries (Fig. 1A). A total of 65,299 significant signatures were obtained from the three incompatible-interaction SBS libraries. Among them, 3,492 (5.3%) and 49,706 (76.1%) significant signatures specifically matched to the *M. oryzae* genome and rice genome, respectively. A total of 68,825 significant signatures were obtained from the four compatible-interaction SBS libraries. Signatures matching to the *M. oryzae* genome numbered 5,283 (7.7%) and those matching to the rice genome numbered 50,756 (73.7%). The SBS signatures from both compatible and incompatible interactions together identified 4,781 annotated *M. oryzae* genes. Altogether, a total of 6,413 annotated *M. oryzae* genes expressed during infection process were identified using the three expression profiling technologies (Fig. 1A).

Identification of genes encoding putative secreted proteins expressed during infection.

Secreted proteins are known to play essential roles during fungal-plant interactions (Rep 2005; Zhang and Zhou 2010). Thus, we focused on the analysis of the secreted protein genes that were identified in the transcriptome libraries described above. To obtain most putative secreted protein genes from the in planta-expressed gene collections, we used two *M. oryzae* secreted protein datasets, one by Dean and associates (2005) (referred to as dataset I) and the other by Choi and associates (2010) (referred to as dataset II) as references for manual annotation. A total of 851 distinct secreted protein genes were identified from both datasets (Fig. 1A, Supplementary Table S1). About 85.7% (264/308) of the putative secreted protein genes identified in dataset I were present in dataset II (Fig. 1E). Among the three RL-SAGE, MPSS, and SBS libraries made from the rice leaves inoculated with compatible isolates at the same timepoint (96 hpi) (RL-SAGE-96 h, MPSS-96 h, and SBS-96 h), over two times more secreted protein genes were identified from the SBS-96 h library than from the other two libraries in both datasets I and II (Fig. 1C and D).

To gain more insight on the function of in planta-expressed secreted proteins involved in the rice-*M. oryzae* interaction, we conducted a gene ontology (GO)-based classification. This analysis revealed that most in planta-expressed secreted proteins are associated with metabolic process, followed by developmental process, cellular process, and multicellular organismal process (Fig. 2A). For molecular functions, most in planta-expressed secreted proteins are associated with catalytic activity and binding (Fig. 2B).

Transient expression assays identified five *M. oryzae* apoplastic effectors that induce cell death in rice cells.

Host cell death is a ubiquitous feature in plant-pathogen interactions. To identify *M. oryzae* secreted proteins involved in host cell death, we performed transient expression of *M. oryzae* secreted proteins in rice protoplasts, using our established method (Chen et al. 2006). In the assay, cell death is monitored by the reduced expression level of a cotransfected β -glucuronidase (GUS) reporter gene in rice protoplasts (Fig. 3A) (Dou et al. 2008b; Jia et al. 2000; Mindrinos et al. 1994; Yoshida et al. 2009). A total of 42 secreted protein genes were cloned for the transient expression assay (Supplementary Table S2). For each of the selected genes, two versions of transfection plasmids were constructed, one containing the full-length

open reading frame (ORF) (referred to as FL) and the other containing the truncated coding region without the signal peptide sequence but with an engineered ATG start codon (referred to as NS). The transient assay revealed that five out of 42 secreted protein genes of the FL version caused a significant reduction in GUS activity when expressed in rice protoplasts (Fig. 3B), suggesting that these five proteins can induce cell death in rice cells. On the other hand, transient expression of all 42 examined genes of the NS version did not result in any reduction in cell viability. We thus referred to the five secreted proteins, MGG_03356, MGG_05531, MGG_07986, MGG_08409, and, MGG_10234, as MoCDIP1 to MoCDIP5 (*M. oryzae* cell death-inducing proteins), respectively.

We further performed *Agrobacterium*-mediated transformation of rice calli with binary vectors containing the five *MoCDIP* genes to see whether it was possible to obtain stable transgenic

lines with the expression of these genes for functional analysis. About 600 calli were used for the transformation of each construct. As expected, no resistant transgenic calli were obtained after transforming with *FL-MoCDIP1*, *FL-MoCDIP2*, *FL-MoCDIP3*, or *FL-MoCDIP4*, individually (data not shown). By contrast, around 50 to 80 transgenic calli were obtained from the transformations with *NS-MoCDIP1*, *NS-MoCDIP2*, *NS-MoCDIP3*, or *NS-MoCDIP4*, with efficiencies similar to normal transformation efficiencies (around 8 to 15% for other constructs) in our lab. Interestingly, transformation with *FL-MoCDIP5* at first yielded a normal efficiency of about 40 transgenic calli with no cell-death phenotypes. However, when they were cultured on the selection medium for 2 weeks or longer, the transgenic calli expressing *FL-MoCDIP5* started to show cell-death phenotypes (Fig. 3C). Similar to other *NS-MoCDIP*, no cell death was observed in the transgenic calli

Fig. 1. Gene expression profiling of *Magnaporthe oryzae* during its interaction with rice. **A**, Summary statistics for gene expression profiling by robust-long serial analysis of gene expression (RL-SAGE), massively parallel signature sequencing (MPSS), and sequencing by synthesis (SBS), and identification of *in planta*-expressed *M. oryzae* genes encoding putative secreted proteins. **B**, Clustering analysis of all *M. oryzae* genes identified in the libraries of RL-SAGE-96 h, MPSS-96 h, and SBS-96 h, respectively. **C**, Clustering analysis of putative *M. oryzae* secreted protein genes retrieved from dataset I (Dean et al. 2005) in RL-SAGE-96 h, MPSS-96 h, and SBS-96 h, respectively. **D**, Clustering analysis of putative *M. oryzae* secreted protein genes retrieved from dataset II (Choi et al. 2010) in RL-SAGE-96 h, MPSS-96 h, and SBS-96 h, respectively. **E**, Clustering analysis of putative *M. oryzae* secreted protein genes in all timepoints libraries retrieved from datasets I and II, respectively.

expressing *NS-MoCDIP5* (Fig. 3C). These results, consistent with the transient expression assay in rice protoplasts, confirmed that the five MoCDIP induced cell death when the full-length proteins are expressed in rice cells.

To functionally investigate the predicted secretion feature of the five identified MoCDIP, a yeast secretion assay was performed, following the method previously published (Lee et al. 2006). The sequences of both *FL-MoCDIP* and *NS-MoCDIP*

Fig. 2. Gene ontology (GO) annotation of in planta-expressed *Magnaporthe oryzae* putative secreted proteins. **A**, Classification based on biological process. **B**, Classification based on molecular function.

Fig. 3. Identification of five in planta-expressed putative secreted proteins that induce cell death in rice cells. **A**, Schematic representation of the rice protoplast transient expression assay approach to the identification of *Magnaporthe oryzae* secreted proteins that can induce rice cell death. Rice protoplasts were cotransfected with a reporter β -glucuronidase (GUS) construct (*Promoter-GUS-Tnos*) and the other construct (*Promoter-M.o gene-Tnos*) carrying a *M. oryzae* secreted protein gene. Rice cell viability was detected based on monitoring the reduced GUS expression level. MoCDIP = *M. oryzae* cell death inducing protein. **B**, Ectopic expression of the full length of five MoCDIP in rice protoplasts resulted in reduction in cell viability. CK = protoplast sample cotransfected with a GUS reporter and an empty vector control; 1 to 5 = protoplast samples cotransfected with a GUS reporter and the other construct carrying MoCDIP1 to MoCDIP5, respectively. Data bars show averages from three triplicate samples in one experiment. Each experiment was repeated at least three times with similar results. **C**, Ectopic expression of a full-length but not truncated non-signal peptide version of MoCDIP5 resulted in cell death in transgenic rice calli. CK = rice calli transformed with empty vector; NS-MoCDIP5 = rice calli transformed with construct carrying a truncated non-signal peptide version of MoCDIP5; FL-MoCDIP5 = rice calli transformed with construct carrying a truncated full-length MoCDIP5. Pictures were taken at about 20 days when the newly obtained transgenic calli were maintained on selection media. Transformation experiments were repeated two times, and similar results were observed. **D**, Reverse transcription-polymerase chain reaction (RT-PCR) analysis of expression of *NS-MoCDIP5* or *FL-MoCDIP5* in transgenic rice calli. CK = RT-PCR result from the rice calli transformed with the empty vector control; 1 to 3 = RT-PCR result from three independent transgenic calli lines.

were fused in frame to the N-terminal end of a yeast invertase gene (*suc2*) lacking its own signal peptide sequence. The fusion constructs were transformed into the yeast strain DBY α 2445, an invertase-deficient mutant (Lee et al. 2006), and the transformed yeasts were grown directly on sucrose medium and were assayed for secretion. As expected, the yeast strain transformed with the constructs containing the *NS-MoCDIP-suc2* fusions did not grow on sucrose medium (Supplementary Fig. S1), due to the lack of secreted invertase to catalyze the decomposition of sucrose into fructose and glucose as the carbon source. In contrast, all five constructs containing the fusions of the *FL-MoCDIP-suc2* enabled the yeast mutant strain to grow on sucrose medium, confirming that the predicted signal peptides of the five MoCDIP are functional to direct the invertase fusions to the secretory pathway. These results, together with the results showing that the signal peptide is required for MoCDIP expressed in rice cells to induce rice cell death, indicate that these proteins most likely function in the plant apoplastic space. However, the exact target site of these proteins in plant cells remains to be elucidated.

MoCDIP genes are expressed in infected leaves and appressoria.

To experimentally confirm that the MoCDIP genes are expressed in infected leaves and to determine their expression pattern in appressoria and mycelia, reverse transcription-polymerase chain reaction (RT-PCR) was carried out using the RNA extracted from Nipponbare rice leaves inoculated with the compatible blast isolate KJ201 and from *M. oryzae* appressoria and mycelia. Because of the low proportion of the fungal mass in the infected leaves at early infection stages, the Mo28S transcript was not detected before 72 hpi. Similar to the Mo28S transcript, the MoCDIP2 and MoCDIP4 transcripts were detected from the infected rice leaves only at 72 hpi, and the transcripts of MoCDIP1, MoCDIP3, and MoCDIP5 were detected from the infected rice leaves at 96 hpi (Fig. 4). This result confirmed that all five MoCDIP were expressed during infection stages. The transcripts of MoCDIP1 and MoCDIP2 were detected in both appressoria and mycelia with relatively higher expression in the former, and the transcripts of MoCDIP3, MoCDIP4, and MoCDIP5 were detected only in appressoria (Fig. 4).

MoCDIP induce cell death in nonhost plant cells.

Many pathogen effectors induce cell death in nonhost plant cells (Rep 2005). To determine whether the five MoCDIP have cell death effects in nonhost plant cells, we performed transient expression assays of the *MoCDIP* genes in the protoplasts of three model plants, maize (*Zea mays*), *Arabidopsis thaliana*, and *Nicotiana benthamiana*. Consistent with the results observed in rice protoplasts, transient expression of the five *FL-MoCDIP* but not the *NS-MoCDIP* induced a significant reduction in cell viability in maize protoplasts (Fig. 5A). As for transient assays in protoplasts of dicot plants *Arabidopsis* and *N. benthamiana*, expression of the *FL-MoCDIP*, except for *FL-MoCDIP2*, caused significant cell-viability reduction. Similar to the result in rice cells, expression of all five *NS-MoCDIP* did not reduce cell viability in either *Arabidopsis* or *N. benthamiana* protoplasts (Fig. 5B and C).

We further tested the five MoCDIP in *N. benthamiana* leaves via an *Agrobacterium*-mediated transient-expression approach. *A. tumefaciens* containing the empty vector pGD and a pGD recombinant expressing *WtsE* were used as negative and positive controls, respectively. *WtsE* is a bacterial type III effector that induces cell death in both host and nonhost plants (Ham et al. 2008). Consistent with results from the protoplast assays, infiltration of *N. benthamiana* leaves with the

Agrobacterium strains expressing *FL-MoCDIP1*, *FL-MoCDIP3*, *FL-MoCDIP4*, or *FL-MoCDIP5* resulted in cell-death responses (Fig. 5D). On the contrary, infiltration of the *FL-MoCDIP2* strain, as well as infiltration of the strains carrying the constructs expressing the *NS-MoCDIP*, did not result in cell death in the infiltrated area. RT-PCR analysis was performed to examine the transient expression of the *MoCDIP* genes, and the results showed that both the *FL-MoCDIP* and *NS-MoCDIP* genes were expressed at similar levels in the infiltrated *N. benthamiana* leaves (Fig. 5E). These results confirmed that MoCDIP1, MoCDIP3, MoCDIP4, and MoCDIP5, but not MoCDIP2 induce cell death in both monocot and dicot species.

The timing and appearance of the cell death in *N. benthamiana* leaves induced by the *FL-MoCDIP* strains were not as strong as that induced by the *WtsE* strain. The cell death symptom induced by the *WtsE* strain usually started at 36 to 48 h after agroinfiltration, and the symptoms induced by the *FL-MoCDIP1*, *FL-MoCDIP3*, and *FL-MoCDIP4* strains generally appeared at 2 to 3 days after agroinfiltration, with a severe cell death around the infiltrated site. However, the symptoms induced by *FL-MoCDIP5* generally were visible at 4 to 6 days after agroinfiltration, with weak necrotic spots in the infiltrated area. This result together with the observation of cell death only in relative long-term cultured rice calli expressing *FL-MoCDIP5* suggests that *FL-MoCDIP5* induces weak cell death in a delayed pattern.

Physiological basis of the MoCDIP-induced cell death.

Previous studies have shown that plant cell death induced by some microbial toxins or effectors share some conserved mechanisms (Asai et al. 2000; Qutob et al. 2006). To further characterize the physiological properties of the cell death induced by the MoCDIP, we performed inhibition assays in rice protoplasts and *N. benthamiana* leaves. Two cell death-inducing proteins, i.e., *WtsE* and *Bax*, were also included in the assays as the controls.

Calcium signaling has been shown to play an important role in the cell death process (Boudsocq et al. 2010; Lecourieux et al. 2002). To determine whether calcium signaling is required for the MoCDIP-induced cell death, LaCl_3 , a calcium channel inhibitor, was applied in the inhibition assays. Application of LaCl_3 blocked cell death induced by the transient expression of all *MoCDIP*, suggesting that the cell-death process mediated by these proteins is dependent on a calcium signaling pathway.

Fig. 4. In planta expression pattern of the five *Magnaporthe oryzae* cell death-inducing protein genes (*MoCDIP*). Total RNA samples extracted from infected rice leaves 0, 24, 48, 72, 96, or 120 h after inoculation, from in vitro-grown *M. oryzae* appressorium (A) and mycelium (M) were subjected to reverse transcription-polymerase chain reaction using specific primers.

Light intensity has been demonstrated to be an important factor for cell-death induction triggered by some toxins or effectors (Asai et al. 2000; Qutob et al. 2006). To test whether MoCDIP-induced cell death is light-dependent, we transfected MoCDIP in rice protoplasts in the light or in the dark. There was no difference of cell viability between the protoplast samples incubated in either condition, indicating that the cell death process induced by the MoCDIP in rice protoplasts is light-independent (Table 1). In contrast, transient expression of the *MoCDIP* did not induce any cell-death lesions in the leaves of *N. benthamiana* kept in the dark (Table 1), suggesting that the MoCDIP-induced cell death in *N. benthamiana* leaves is light dependent.

Studies have shown that some antiapoptotic proteins such as BCL-2 family proteins and bax inhibitor-1 (BI-1) can suppress various types of cell death and are functionally conserved in yeast, plants, and mammals (Watanabe and Lam 2009). Over-expression of antiapoptotic proteins was shown to inhibit cell death induced by multiple stimuli, revealing that various types of cell death may have a common downstream mechanism (Dickman et al. 2001). We tested Bcl-xl, a member of the BCL-2 family, to determine whether MoCDIP-induced cell death can be inhibited by the antiapoptotic protein. Preinfiltration with *A. tumefaciens* cells harboring the *Bcl-xl* expression vector did not produce cell-death symptoms after infiltration

Table 1. Inhibition assays of *Magnaporthe oryzae* cell death-inducing proteins in rice protoplasts and *Nicotiana benthamiana* leaves^a

Effector	Applications or treatments									
	Catalase		DPI		LaCl ₃ ^b		Dark		Bcl-xl ^c	
	RP	NBL	RP	NBL	RP	NBL	RP	NBL	RP	NBL
MoCDIP1	+	+	+	+	–	–	+	–	ND	–
MoCDIP2	–	ND	+	ND	–	ND	+	ND	ND	ND
MoCDIP3	+	+	+	+	–	–	+	–	ND	–
MoCDIP4	–	–	+	+	–	–	+	–	ND	–
MoCDIP5	–	–	+	+	–	–	+	–	ND	–
WtsE	–	–	+	+	–	–	+	+	ND	–
Bax	–	–	+	+	–	–	+	+	ND	–

^a RP = rice protoplasts, NBL = *N. benthamiana* leaves, DPI = diphenyleneiodonium sulfate, + = cell death, – = no cell death, and ND = not determined.

^b LaCl₃ was dissolved in distilled water, and the same volume of water was applied to rice protoplasts or *N. benthamiana* leaves as control.

^c An empty vector pGD was applied as control. No effects of controls on cell-death assays was observed.

Fig. 5. Transient expression of the full-length but not truncated non-signal peptide version of *Magnaporthe oryzae* cell death inducing protein (MoCDIP) induced cell death in nonhost plant cells. **A**, **B**, and **C**, Transient expression assay of the five MoCDIP in protoplasts of maize, *Arabidopsis*, and *Nicotiana benthamiana*, respectively. CK = protoplast sample cotransfected with a β -glucuronidase (GUS) reporter and an empty vector control; 1 to 5 = protoplast samples cotransfected with a GUS reporter and the other construct carrying MoCDIP1 to MoCDIP5, respectively. **D**, Transient expression assay of the five MoCDIP in *N. benthamiana* leaves by using an agroinfiltration approach. Agroinfiltration was performed on the same leaf side by side with *Agrobacterium tumefaciens* carrying an empty vector control (pGD), a positive control (WtsE), constructs with full-length MoCDIP (FL-MoCDIP), or constructs with non-signal peptide sequence MoCDIP (NS-MoCDIP), respectively. **E**, Reverse transcription-polymerase chain reaction (RT-PCR) analysis of MoCDIP expression in agroinfiltrated *N. benthamiana* leaves. Total RNA was extracted from *N. benthamiana* leaves at 36 h postinoculation. CK = RT-PCR result from tissues infiltrated with the empty vector control; 1 = RT-PCR result from tissues infiltrated with FL-MoCDIP; 2 = RT-PCR result from tissues infiltrated with NS-MoCDIP.

Fig. 6. Structural analyses of *Magnaporthe oryzae* cell death inducing proteins (MoCDIP). Schematic views of MoCDIP and their deletion mutants are shown on the left. The predicted domains or motifs of MoCDIP are represented as color rectangles. The deleted regions are represented as dotted lines. MoCDIP and their deletion mutants were transiently expressed in *Nicotiana benthamiana* leaves or rice protoplasts. Assay results from agroinfiltrated *N. benthamiana* leaves or transfected rice protoplasts are shown on the right. The signs +, +-, and - indicate obvious cell death, weak cell death, and no cell death, respectively. Data bars of protoplast results show averages from three triplicate samples in one experiment. Each experiment was repeated at least three times with similar results.

with *A. tumefaciens* cells carrying *MoCDIP*, *WtsE*, or *Bax*. In contrast, *N. benthamiana* leaves preinfiltrated with the culture containing the empty vector showed obvious cell-death symptoms induced by the transient expression of *MoCDIP*, *WtsE*, or *Bax*. This result indicated that cell death in *N. benthamiana* leaves induced by *MoCDIP* is suppressed by the antiapoptotic protein (Table 1).

Sequence and structural analysis of MoCDIP.

Sequence analysis revealed that the five identified *MoCDIP* are highly diverse in their sequences and have no sequence homology with known cell death-inducing effectors in different pathogens, such as Nep1-like proteins (Qutob et al. 2006), INF1 (Kamoun et al. 1997), ToxA (Ciuffetti et al. 1997), ToxB (Martinez et al. 2001), or Nip (Mattinen et al. 2004). BLAST searches against the *M. oryzae* database and the NCBI non-redundant database revealed that *MoCDIP2* and *MoCDIP4* have a relatively large number of homologs in the sequenced genomes of *M. oryzae* and of other organisms (Supplementary Fig. S2). In contrast, *MoCDIP1* and *MoCDIP5* have homology to proteins only from other microorganisms (Supplementary Fig. S2), and *MoCDIP3* has no homologs in the *M. oryzae* genome or in the sequenced genomes of other organisms. Homology searches also revealed that *MoCDIP2* belonged to a family of CFEM-containing proteins that may function as a cell-surface receptor, signal transducers, or as adhesion molecules in host-fungi interactions (Kulkarni et al. 2003); *MoCDIP4* was a highly conserved homolog to glycosyl hydrolase family 61 proteins (Davies and Henrissat 1995); and *MoCDIP1* and *MoCDIP5* shared similarity to ricin B lectin proteins, although there was no sequence similarity between the two proteins.

To further delineate the properties of the *MoCDIP* that are involved in cell-death induction, we searched conserved domains in the proteins and performed functional analysis of deletion mutants based on domain prediction. Deletion mutant constructs of *MoCDIP*, except for *MoCDIP3*, because it does not contain any predicted domain, were tested in both rice protoplasts and *N. benthamiana* leaves (Fig. 6). Transient expression assays revealed that a *MoCDIP1* mutant of the N-terminal region, amino acids (aa) 1 to 185, was sufficient for inducing cell death in plant cells. However, the N-terminal region, aa 1 to 162, which lacks the PbH1 motif in the aa 1 to 185 fragment, lost the ability to induce cell death, suggesting that the PbH1 motif may play an important role in inducing cell death in plants. The N-terminal, CFEM domain-containing region of *MoCDIP2* induced rice cell death efficiently, suggesting that the predicted GPI anchor was not required for cell-death induction. As for *MoCDIP4*, a deletion mutant lacking the cellulose binding domain (CBD) domain was found to induce only a weak cell death, and the mutant lacking both the linker fragment and the CBD domain failed to induce cell death, suggesting that the C-terminus of the linker fragment and the CBD domain are functionally important in inducing cell death in plants. The assays also showed that *MoCDIP5* mutants lacking the C-terminus or lacking the potential zinc-binding site motif failed to induce cell death in both rice protoplasts and *N. benthamiana* leaves, suggesting that the full length of *MoCDIP5* is required for cell-death induction in plants.

DISCUSSION

Transcriptome profiling in the rice-*M. oryzae* interaction.

Using different gene-prediction algorithms, about 12% of the annotated genes (1,546) are predicted to be putative effector proteins in the *M. oryzae* genome (Soanes et al. 2008). Whether these predicted genes are expressed in infected rice plants is largely unknown. By applying expressed sequence tag

(EST) analysis, RL-SAGE, and SuperSAGE analysis to infected rice leaves at the early infection stages, the defense transcriptome in response to *M. oryzae* infection was characterized (Ebbolle et al. 2004; Gowda et al. 2007; Kim et al. 2001; Matsumura et al. 2003; Rauiyaree et al. 2001). However, these studies only identified a small number of fungal genes, mainly because the proportion of the fungal RNA in the infected tissue was relatively low and the sequencing coverage was not deep enough. Recently, improved approaches for enrichment of fungal RNA from infected plant tissues have been employed to profile the interaction transcriptome of *M. oryzae* and rice. Kim and associates (2010) applied EST analysis coupled with subtractive hybridization of a cDNA library from infected leaves at very late stages of infection. A total of 712 uniEST were identified from the fungus, representing up to 31% of the total uniEST. Mosquera and associates (2009) developed a procedure to produce heavily infected rice sheaths that allowed isolation of total RNAs with a high proportion of RNA (up to 20%) originating from biotrophic invasive hyphae of *M. oryzae*. By applying microarray analysis, the authors identified 262 fungal genes and 210 rice genes that were induced up to 10 fold during biotrophic invasion.

In this study, we employed three high-throughput technologies, i.e., RL-SAGE, MPSS, and SBS, to profile the transcriptome of *M. oryzae*-infected rice tissue. While RL-SAGE was only used to study gene-expression profiles from rice leaf tissue infected with a compatible isolate at 96 hpi, both MPSS and SBS were applied to study gene expression profiles of rice leaf tissue inoculated with either a compatible or incompatible isolate at different postinoculation timepoints. In the rice leaves at 72 hpi, inoculated with both incompatible and compatible isolates, only a very limited number of fungal genes were identified, as there were no visible symptoms or only a very few brown lesions appeared on the rice leaves. When inoculated with a compatible isolate at 96 hpi, rice leaves developed typical susceptible blast lesions. Consistently, a relatively large number of fungal transcripts were detected from heavily infected leaves. We identified 3,091 (17%), 2,327 (12.3%), and 5,099 (11.4%) significant *M. oryzae* tags from the libraries of RL-SAGE-96 h, MPSS-96 h, and SBS-96 h, respectively. By combining results from three technologies, we identified a total of 6,413 *M. oryzae* genes, including 851 genes that are predicted to encode putative secreted proteins and might be expressed in planta. Our results together with those previously reported will provide valuable information for future studies of the molecular mechanisms underlying the rice-*M. oryzae* interaction.

Clustering analysis of the three 96-h libraries showed that the transcripts recovered from the RL-SAGE, MPSS, and SBS were partially overlapping, similar to previous results of transcriptional profiles of *M. oryzae* using MPSS, RL-SAGE, and oligoarray methods (Gowda et al. 2006). This result also suggests that using multiple different approaches can provide more comprehensive gene expression profiles. When considering individual technologies, SBS appeared to be the most efficient method for transcript profiling. While similar numbers of *M. oryzae* genes were identified from RL-SAGE-96 h and MPSS-96 h (3,008 and 2,401 unique *M. oryzae* genes from the two libraries, respectively), a total of 4,730 *M. oryzae* genes were identified from SBS-96 h (Fig. 1B), almost twice the gene numbers identified by the other two methods. Moreover, the *M. oryzae* transcripts recovered from SBS-96 h included most of the *M. oryzae* transcripts from RL-SAGE-96 h or MPSS-96 h. Our results indicated that many weakly expressed transcripts of both rice and *M. oryzae* were recovered in the libraries, due to the deep coverage of the transcriptome. As the sequencing cost is rapidly declining, the SBS-based ultra-fast sequencing method or other next-generation sequencing plat-

forms will allow for more in-depth characterization of plant-pathogen interaction transcriptomes.

Functional identification of *M. oryzae* effectors using the rice protoplast transient expression system.

Transcriptional analysis of the *M. oryzae* effector genes in infected rice plants has provided a starting point for functional analysis of the in planta-expressed genes in the rice-*M. oryzae* interaction. Over the past two decades, several *M. oryzae* avirulence or pathogenicity effector genes have been isolated by map-based cloning (Kang et al. 1995; Orbach et al. 2000; Sweigard et al. 1995), genetic association analysis (Yoshida et al. 2009), or loss-of-function (Ahn et al. 2004; Jeong et al. 2007; Kim et al. 2005; Talbot et al. 1993) approaches. The first two procedures are time-consuming, tedious, and expensive. As for loss-of-function approach, it is often hampered by the fact that many genes may have overlapping functions. For example, many knockout mutants of secreted protein genes have no identifiable phenotype (Mosquera et al. 2009). Thus, a cost-efficient and high-efficiency gain-of-function method would be a valuable alternative approach to the identification of *M. oryzae* effectors.

As for gain-of-function identification, the agroinfiltration transient assay is a widely used approach for characterizing function of phytopathogen effectors in many solanaceous plants, especially in *N. benthamiana* and *N. tabacum* (Munkvold and Martin 2009). However, this agroinfiltration method is not applicable in monocot plants. We previously reported a protoplast transient expression system to perform assays directly in rice cells (Chen et al. 2009a). Recently, Yoshida and associates (2009) and Okuyama and associates (2011) detected HR in rice protoplasts coexpressing *R* gene and its cognate *Avr* gene from *M. oryzae*. Using the system, they successfully screened the candidates of *M. oryzae* *Avr-Pia*, *Avr-Pii*, *Avr-Pik/km/kp*, and rice blast resistance gene *Pia*. In this study, we identified five *M. oryzae* effectors that induce cell death in rice cells. The results demonstrate that the rice protoplast expression assay is an efficient method for large-scale screening of putative effectors that induce cell death or HR reaction.

Role of MoCDIP in the interaction between *M. oryzae* and rice.

Unlike many bacterial pathogens that deliver effector proteins inside host cells via a type III secretion system, eukaryotic plant pathogens, like oomycetes and fungi, seem to secrete a large number of extracellular proteins via the eukaryotic (type II) secretory pathway (Panstruga and Dodds 2009). Some secreted proteins are translocated into host cells and function in the host cytoplasm to suppress host defenses. Many others function in the host apoplastic space to facilitate the parasitic lifestyle of pathogens. The latter include degradative enzymes, toxins, or inhibitors of plant enzymes. More recently, a broader definition of the term effector was suggested to include these secreted proteins, as they exert some effect on plant cells (Hogenhout et al. 2009). Over the past few decades, several apoplastic effectors with toxin or elicitor activity that can induce cell death in plants have been identified from eukaryotic plant pathogens (Rep 2005). Many of these apoplastic effectors play a positive role in the virulence of the hemibiotrophic or necrotrophic plant pathogens (Ottmann et al. 2009; Qutob et al. 2006). From 42 in planta-expressed *M. oryzae* putative secreted proteins, we successfully identified five novel effectors MoCDIP1 to MoCDIP5 that induce plant cell death. Given the fact that these genes are expressed during infection stages, especially 96 hpi (Fig. 4), we speculate that some of these cell death-inducing effectors may facilitate the colonization of *M. oryzae* during the late necrotrophic phase of the

blast infection, which is a common mechanism among different pathosystems (Gijzen and Nurnberger 2006). It will be interesting to identify the receptor of these effectors in rice cells and define the interactions that trigger rice cell necrosis.

The five MoCDIP are highly diverse in their sequences. While MoCDIP3 does not share any significant similarity with any known proteins, MoCDIP1, MoCDIP2, MoCDIP4, and MoCDIP5 have closely related homologs from *M. oryzae* or other microorganisms. Our analysis also revealed that cell death induced by the five different MoCDIP share similar physiological phenotypes, such as the response to light, to inhibitors of calcium channel, and to Bcl-x1-mediated cell death suppression. These results together suggest that the cell death-inducing mechanism of the five MoCDIP might be similar, though their sequences are quite different. Among the five MoCDIP, MoCDIP4 belongs to a large family of cellulolytic enzymes from a wide variety of microorganisms. Related homologous proteins include endoglucanase, xylanase, and acetylxyloesterase. Several previous studies have observed that the expression of genes encoding members of this family of enzymes was modulated by mitogen-activated protein kinase signaling pathways, which play critical roles in regulating pathogenesis as well as other features (Lev and Horwitz 2003; Madhani et al. 1999; Roberts et al. 2000). However, because of redundancy among cellulolytic enzymes, knock-out of one or even several genes encoding cellulolytic enzymes had little or no consequences for virulence (Lev and Horwitz 2003). In this report, we demonstrate that MoCDIP4, a putative endoglucanase, induces cell death when it is expressed in plant cells. Hence, our finding provides a new clue to the mechanism by which the cellulolytic enzymes can aid invasion of microorganisms. MoCDIP4 contains two conserved domains, a glycosyl hydrolase family 61 domain and a fungal CBD. Recently, the fungal CBD have been shown to be important for the function of a *Phytophthora* elicitor lectin CBEL in plant cell-death induction (Gaulin et al. 2006) and for the function of a *Trichoderma* swollenin in plant root colonization (Brotman et al. 2008). More interestingly, the CBD domain was identified as a novel type of PAMP in microorganisms (Brotman et al. 2008; Gaulin et al. 2006). Consistent with previous reports, the CBD domain of MoCDIP4 was found to be important in inducing plant cell death (Fig. 6), strengthening the notion that the CBD plays a functionally conserved role, such as serving as a PAMP elicitor among different plant-pathosystems.

MATERIALS AND METHODS

Plant materials and fungal strains.

Rice (*Oryza sativa*) materials used in this study were wild-type Nipponbare plants and transgenic Nipponbare plants carrying a blast resistance gene *Pi9* (Qu et al. 2006). *M. oryzae* isolates used in this study include Che86061 and KJ201.

Construction and data analyses of RL-SAGE, MPSS, and SBS libraries.

RNA samples isolated from rice leaves inoculated with *M. oryzae* isolates were used for library construction. The RL-SAGE library was constructed following previously described procedures (Gowda et al. 2004). MPSS library construction was carried out at Illumina (San Diego, CA, U.S.A.), as described (Brenner et al. 2000; German et al. 2008; Meyers et al. 2004). The same total RNAs used for the MPSS library construction (except samples from leaves collected at 3 and 48 hpi) were used for the SBS library construction using our published protocols (Venu et al. 2011a).

RL-SAGE clones were sequenced at the Arizona Genome Institute (Tucson, AZ, U.S.A.). RL-SAGE signatures were

identified using the SAGEspy program. MPSS tag processing was carried out at Illumina (Brenner et al. 2000; Meyers et al. 2004; Lu et al. 2005). The distinct RL-SAGE, MPSS, and SBS tag sequences were matched to the *M. oryzae* reference sequences, including the whole genomic sequences, annotated genes, and 500 bp upstream (putative 5' untranslated region [UTR]) and downstream (putative 3' UTR) regions that are available from the Broad Institute (version 6.0) (Gowda et al. 2006; Venu et al. 2007; Venu et al. 2010, 2011a and b). Rice and *M. oryzae* transcript signatures from RL-SAGE, MPSS, and SBS experiments were separated after matching signatures to the whole genomic sequence of both rice (Michigan State University's Plant Biology directory) and *M. oryzae*. To identify in planta-expressed *M. oryzae* genes encoding for putative secreted proteins, the experimental RL-SAGE, MPSS, and SBS signatures were matched to two computational prediction datasets of *M. oryzae* secreted proteins (Choi et al. 2010; Dean et al. 2005). Clustering analysis using Microsoft access was performed to identify the putative secreted protein genes expressed specifically or commonly among the three platforms. The RL-SAGE data were deposited at the *Magnaporthe oryzae* community annotation database and the MPSS and SBS data were deposited at Arabidopsis MPSS Plus database.

Cloning of *M. oryzae* genes encoding putative secreted protein and construction of *MoCDIP*-related vectors.

We first selected about 100 putative secreted protein genes with high expression levels from the libraries. We had difficulty cloning some of the genes, mainly because the proportion of fungal RNA in the total RNA from infected rice leaves was relatively low. Some of the genes were cloned from *M. oryzae* EST clones or from *M. oryzae* genomic DNA, for those with no intron. A total of 42 in planta-expressed *M. oryzae* putative secreted protein genes were selected for functional characterization based on the profiling data. The genes were amplified by PCR, using specific primers, and were cloned into plant expression vector pXUN (Chen et al. 2009b). *MoCDIP*-related vectors were generated based on fragments cloned into pXUN.

Protoplast transient expression assays.

Transient expression assays in the protoplasts of rice, maize, *Arabidopsis*, and *N. benthamiana* were carried out following previously described procedures (Chen et al. 2006; Sheen 2001). For transient assays in rice and maize cells, protoplasts were cotransfected with a *maize ubiquitin1 promoter-gus* construct (Chen et al. 2006) and the pXUN-based construct of *M. oryzae* secreted protein genes, and for transient assays in *Arabidopsis* and *N. benthamiana* cells, protoplasts were cotransfected with a *Cauliflower mosaic virus 35S promoter-gus* construct (Odell et al. 1985) and the pGD-based construct (Goodin et al. 2002) of *M. oryzae* secreted protein genes. After transfection, protoplasts were incubated at room temperature for 16 to 24 h. GUS activity was detected essentially as described (Jefferson et al. 1987), using 4-methylumbelliferyl- β -D-glucuronide (Sigma-Aldrich, St Louis) as the substrate.

More details of materials and methods are available in the Supplementary Materials and Methods published online.

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manuscript. M. Gowda, M. Bellizzi, J. Hu, and W. Liu were involved in performing part of experiments; D. Ebbola, B. C. Meyers, and T. Mitchell were involved in interpretation of results and writing the manuscript. G.-L. Wang was responsible for conception and planning of the study, interpretation of results, and writing the manuscript. All authors read and approved the final manuscript. The authors declare that they have no competing interests.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- Arabidopsis MPSS Plus database: mpss.udel.edu/#rice
- Broad Institute (version 6.0) download sequence webpage: www.broadinstitute.org/annotation/genome/magnaporthe_grisea/MultiDownloads.html
- Illumina website: www.illumina.com
- Magnaporthe oryzae* community annotation database: www.mgosdb.org
- Michigan State University's Plant Biology directory: ftp.plantbiology.msu.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/pseudomolecules/version_6.0
- SAGEspy program: www.osc.edu/research/bioinformatics/projects/sagespy/index.shtml