Cellulases Belonging to Glycoside Hydrolase Families 6 and 7 Contribute to the Virulence of *Magnaporthe oryzae*

Ba Van Vu,¹ Kenji Itoh,¹ Quoc Bao Nguyen,^{1,2} Yukio Tosa,¹ and Hitoshi Nakayashiki¹

¹Laboratory of Plant Pathology, Graduate School of Agricultural Science, Kobe University, 1-1 Rokkodai-cho, Nada, 657-8501 Kobe, Japan; ²Research Institute of Biotechnology and Environment, Nong Lam University, Linh Trung Ward, Thu Duc District, Ho Chi Minh City, Vietnam

Submitted 19 February 2012. Accepted 28 April 2012.

Upon infection, phytopathogenic fungi secrete an array of hydrolytic enzymes that can degrade components of the host epidermis, including waxes, the cuticle, and cell walls. Cellulases, which can hydrolyze crystalline cellulose in the plant cell wall, are among these hydrolytic enzymes. Here, we provide RNAi-based evidence to show that cellulases belonging to glycosyl hydrolase (GH) families 6 and 7 contribute to the penetration of the host epidermis and further invasion by the phytopathogenic fungus *Magnaporthe oryzae*. The GH6 and GH7 cellulases likely include all members of the cellobiohydrolase family and some endoglucanases in *M. oryzae*. Quantitative reverse-transcriptase polymerase chain reaction analysis indicated that more than half of the cellulases were highly induced during infection. We constructed knock-down (KD) mutants of these cellulases using the building blocks method we reported previously. The transcript levels of the target genes and cellulase activity were considerably reduced in the KD mutants. The KD mutants resulted in fewer lesions, less penetration, and infection of fewer cells compared with the parent strain. Cytological analyses showed that a high rate of papilla formation blocked invasion of the KD mutants into host cells. These results suggest that the GH6 and GH7 cellulases play roles in the virulence of *M. oryzae*.

The most characteristic component of plant cell walls is cellulose, which is a linear polymer consisting of β -1,4-linked glucose residues. The plant cell wall serves as a physical support for cellular structures, and also as a mechanical barrier against environmental stresses, including pathogen infection. In fact, bacterial and viral pathogens can enter the host plants only through a natural opening or a wound, indicating that the cell wall barrier effectively prevents infection. However, many fungal pathogens penetrate plant cell walls by forming a series of infection structures represented by an appressorium (Mendgen et al. 1996), which generates turgor pressure to provide the mechanical force required to penetrate cell walls (Howard and Valent 1996).

Phytopathogenic fungi also produce cell-wall-degrading enzymes (CWDE) that are thought to aid their invasion into host cells (Walton 1994). A major group of CWDE consists of cellulases, which catalyze hydrolysis of the β -1,4-glycosidic

bonds in cellulose. Cellulases can be classified into three major types according to their mode of hydrolysis and substrate specificity: cellobiohydrolases (EC 3.2.1.91), endoglucanases (EC 3.2.1.4), and β -glucosidases (EC 3.2.1.21). The synergistic action of the three different types of cellulases is important for efficient degradation of cellulose (Henrissat and Bairoch 1996). Each type is involved in different but partly overlapping steps in cellulose degradation. Endoglucanases hydrolyze the internal bonds of the cellulose chains, producing new chain ends (Teeri 1997). Cellobiohydrolases are exoglucanases that liberate cellobiose from reducing or nonreducing ends of the polymeric cellulose chains in a processive manner (Teeri et al. 1998; Wood and Bhat 1988). Cellobiohydrolases can attack both crystalline and amorphous parts of cellulose. β -Glucosidases complete the hydrolysis of cellulose by cleaving the resulting cellobiose into two molecules of glucose.

The ascomycete fungus *Magnaporthe oryzae* is the causal agent of rice blast, the most devastating fungal disease of rice. Certain strains of *M. oryzae* can also infect other agriculturally important cereals, including wheat, barley, oat, and pearl millet. Wheat blast is a relatively new disease of wheat. It was first reported to occur in the northern region of Parana, Brazil (Urashima et al. 1993). Now, wheat blast has spread to other wheat-growing regions in Brazil and to other countries in South America.

This fungus invades host cells through appressorium-mediated cuticle penetration. Although the role of mechanical force in cuticle penetration by this fungus has been clearly demonstrated (Howard et al. 1991), it has been a matter of debate whether chemical degradation of plant cell wall components is required for its successful infection. A technical difficulty in addressing this question is the genetic redundancy of cellulases in the genome. In several previous attempts, the extensive genetic redundancy of CWDE appeared to mask the effects of single or even multiple knock-out mutations (Apel et al. 1993; Apel-Birkhold and Walton 1996; Keen et al. 1996; Wu et al. 1997). To overcome this problem, we have recently developed an RNAi strategy designated as the building blocks method. In this method, an artificial sequence composed of short nucleotide units, each of which is a sequence of 40 to 60 bp derived from a single target gene, is used as a silencing trigger to achieve the maximum average efficiency of gene silencing. In our previous study, this method allowed us to demonstrate that endoxylanases play significant roles in the penetration of cell walls and further invasion during infection with *M. oryzae* (Nguyen et al. 2011).

Here, we used the same strategy to examine the roles of cellulases belonging to glycosyl hydrolase (GH) families 6 and 7 (GH6 and GH7, respectively) in the pathogenicity of *M. ory-*

Corresponding author: H. Nakayashiki; Telephone and Fax: +81-78-803-5867; E-mail: hnakaya@kobe-u.ac.jp

*The e-Xtra logo stands for “electronic extra” and indicates that two supplementary figures and two supplementary tables are published online.

zae. Based on the *M. oryzae* genome sequence, there are three putative genes encoding GH6 cellulases and six encoding GH7 cellulases. Three GH6 and GH7 cellulase genes (designated as *MoCel6A*, *MoCel7A*, and *MgEGL1*) were previously cloned, and their expression patterns and biochemical characteristics were analyzed (Takahashi et al. 2010; Zhou et al. 2008). *MoCel6A* and *MoCel7B* were reported to be cellobiohydrolases and *MgEGL1* a putative endoglucanase. However, their roles in fungal pathogenicity remained unclear. Here, we conducted an RNAi-based study that indicated that the GH6 and GH7 cellulases contribute to the virulence of *M. oryzae* during infection.

RESULTS

Transcript levels of the GH6 and GH7 cellulase genes in *M. oryzae* during infection.

To identify putative GH6 and GH7 cellulase genes in the *M. oryzae* genome, we conducted BLAST searches with known fungal cellobiohydrolase sequences. Consequently, we identified three GH6 sequences (MGG_04499.6, MGG_05520.6, and MGG_07908.6) and six GH7 sequences (MGG_02532.6, MGG_06663.6, MGG_06834.6, MGG_07809.6, MGG_10712.6, and MGG_14954.6). *MoCel6A*, *MoCel7A*, and *MgEGL1* correspond to MGG_05520.6 (GH6), MGG_10712.6 (GH7), and MGG_02532.6 (GH7), respectively. Based on their amino acid sequences, cellobiohydrolases can be classified into three GH families (GH6, GH7, and GH48) (Henrissat 1991). We did not detect any genes belonging to the GH48 family in the *M. oryzae* genome by BLAST searches; therefore, we concluded that all of the *M. oryzae* cellobiohydrolases were in the GH6 and GH7 families. However, a previous phylogenetic analysis revealed that three GH7 cellulases (MGG_02532.6, MGG_06663.6, and MGG_14954.6) were more closely related to endoglucanases than to cellobiohydrolases (Zhou et al. 2008).

To examine the expression patterns of the GH6 and GH7 cellulases during *M. oryzae* infection, we monitored their transcript levels by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) using total RNA extracted from wheat leaves infected with *M. oryzae* (Br48) at 24, 48, and 72 h postinoculation (hpi) (Fig. 1). As the controls, their transcript levels were also determined in vegetative mycelia (Fig.

Fig. 1. Expression profiles of nine *Magnaporthe oryzae* cellulase genes belonging to glycosyl hydrolase families 6 (GH6) and 7 (GH7) in infected wheat leaves relative to those in vegetative mycelia. By quantitative RT-PCR with the internal control gene actin (MGG_03982), relative quantities of the cellulase genes were measured in vegetative mycelia and infected wheat leaves collected at 24, 48, and 72 h postinoculation (hpi). This experiment was performed with two biological replicates with duplicate technical replicates. Error bars represent standard deviations. N.D. = not detected.

1). In the vegetative mycelia, we detected transcripts of all the GH6 and GH7 cellulases except for MGG_14954 and MGG_07809. Among all of the cellulase genes, the most abundant transcript was MGG_06663 in the vegetative mycelia. In general, the other cellulase genes were expressed at low levels in the vegetative mycelia (mostly less than 0.1% relative to the transcript level of actin). Notably, however, some of the GH6 and GH7 cellulase genes were highly induced upon infection. qRT-PCR analysis showed that the expression levels of MGG_05520, MGG_10712, and MGG_14954 were upregulated more than 1,000-fold in infected leaves at 72 hpi compared with their respective expressions in the vegetative mycelia. The expression levels of MGG_07908 and MGG_06834 were also highly upregulated (more than 100-fold at 72 hpi). Interestingly, those highly inducible genes (MGG_05520, MGG_10712, MGG_14954, MGG_07908, and MGG_06834) showed similar expression patterns during infection; highly induced at 24 hpi, a little decreased at 48 hpi, and expressed at maximum levels at 72 hpi. This may indicate that there are two phases of cellulase induction during infection with *M. oryzae*. In contrast, the moderately inducible genes (MGG_04499 and MGG_02532) showed relatively high levels of expression in the vegetative mycelia, and their induction patterns during infection differed from those of the highly inducible genes, suggesting that the two cellulase groups might have different biological roles. The inducible nature of GH6 and GH7 cellulases was previously reported for *MoCel6A* and *MgEGL1*. Overall, the relatively high induction of cellulase gene expression during infection suggested their potential roles in the pathogenicity of the fungus.

Genetic knock-down of GH6 and GH7 cellulases in *M. oryzae* by the building-blocks method.

To construct knock-down (KD) mutants of the GH6 and GH7 cellulase genes in *M. oryzae*, we used the building blocks method using the gene-silencing vector pSilent-Dual1 (pSD1), as described previously (Nguyen et al. 2008, 2011). As a silencing trigger, we constructed a 450-bp artificial sequence composed of nine units of a 50-bp sequence from each one of

Fig. 2. Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) analysis of nine glycoside hydrolase families 6 (GH6) and 7 (GH7) cellulase genes in vegetative mycelia of knock-down (KD) *Magnaporthe oryzae* mutants (KD_CEL1, and KD_CEL2). Values in the graph indicate relative expression levels of nine cellulase genes compared with those in the parent strain (onefold). cDNA quantities were normalized to the actin gene (MGG_03982). This experiment was performed with at least three biological replicates with duplicate technical replicates. Error bars represent standard deviations. N.D., not detected; * and ** indicate $P < 0.05$ and 0.01, respectively; significant difference from the parent strain (two-tailed t test).

the nine GH6 and GH7 cellulase genes (discussed below). The artificial silencing trigger was introduced into pSD1G, which is a pSD1 derivative with an enhanced green fluorescent protein (eGFP) sequence for GFP-based co-silencing screening, yielding pSD1G-CEL. We initially screened 10 to 15 transformants on the basis of their reduced GFP fluorescence, then conducted further screening by evaluating extracellular cellulose activity on media containing 0.5% carboxymethylcellulose and 0.5% Congo Red. Two transformants (KD_CEL1 and KD_CEL2) with pSD1G-CEL were finally selected as KD mutants for further studies.

The KD mutants KD_CEL1 and KD_CEL2 were first subjected to qRT-PCR to examine the expression levels of the nine GH6 and GH7 cellulase genes in the vegetative mycelia. Seven of the nine cellulases were expressed at detectable levels in the parent strain (Br48-GFP) (Fig. 1). In the KD mutants, the seven expressed cellulase genes were all silenced, mostly at levels below 50% relative to those in the parent strain (Fig. 2). The levels of gene silencing differed significantly among the KD mutants and also among the target genes. Next, we examined cellulase activity in the KD mutants. Total cellulase activity was estimated by the Nelson-Somogyi assay for reducing sugars released from carboxymethylcellulose. Cellulase activity was decreased by 44% in KD_CEL1 and by 46% in KD_CEL2 (Fig. 3). The difference between KD_CEL1 and KD_CEL2 was not statistically significant. Those results clearly showed that the GH6 and GH7 cellulases were considerably silenced in the KD mutants.

We also monitored the levels of cellulase transcripts in the KD mutants during infection by qRT-PCR using total RNA extracted from infected leaves at 48 hpi (Fig. 4). Consistent with the data obtained from vegetative mycelia (Fig. 2), the expressions of the cellulases were generally downregulated during infection, suggesting that RNAi could function even against genes that were highly induced upon infection (Fig. 4). However, in some cases (MGG_10712, MGG_07980, and MGG_04499), the degrees of gene silencing during infection were lower than those in the vegetative mycelia. The levels of cellulase transcripts in the KD mutants at 24 and 72 hpi were very similar to those at 48 hpi (data not shown).

To assess the specificity of gene silencing, we then performed qRT-PCR analysis of 15 endoglucanase genes in the GH61 family and 11 endoxylanase genes in the GH10 and GH11 families. The expression levels of the endoglucanase and endoxylanase genes were not significantly different between

Fig. 3. Cellulase activity in cytosolic protein extracted from knock-down (KD) *Magnaporthe oryzae* mutants (KD_CEL1 and KD_CEL2) and their parent strain (Br48-GFP). Values in the graph indicate relative cellulase activity of the KD mutants compared with the parent strain (100%). The experiment was performed with three biological replicates. Error bars represent standard deviations. Letters in the graph indicate significant differences (Tukey's multiple comparison; $P < 0.01$).

the parent strain and KD mutants in most cases (Supplementary Fig. S1). However, one endoglucanase gene (MGG_06069) was highly upregulated and another endoglucanase gene (MGG_08066) was significantly downregulated in the KD_CEL1 mutant. Consistently, the KD mutants showed enzymatic activity toward xylan at levels comparable with that in the parent strain (Supplementary Fig. S2). In contrast, the enzymatic activity toward barley 1,3-1,4- β -glucan was significantly reduced in the KD mutants. This may be due to the fact that barley 1,3-1,4- β -glucan could partly contain cellulose. In fact, MoCel6A was shown to hydrolyze barley 1,3-1,4- β -glucan (Takahashi et al. 2010). We also examined the rates of vegetative growth and appressorium formation in the KD mutants, and found no significant difference between the KD mutants and the parental strain (Supplementary Table S1).

GH6 and GH7 cellulase KD mutants

of *M. oryzae* showed reduced pathogenicity on host plants.

To examine the effect of genetic KD of the GH6 and GH7 cellulases on pathogenicity, we conducted inoculation assays using two cultivars of wheat, 'Norin 4' and 'Chinese Spring', as described previously (Nguyen et al. 2008). The KD mutants KD_CEL1 and KD_CEL2 caused a few blast lesions on the host plant (Norin 4), indicating that the pathogenicity of the KD mutants was not completely lost (Fig. 5). However, compared with the parent strain, there were markedly fewer and smaller lesions on leaves infected with the KD mutants, suggesting that the KD mutants were impaired in virulence because of the knocked-down GH6 and GH7 cellulase genes. Similar infection types were also observed on Chinese Spring infected with the KD mutants (data not shown). The degrees of reduction in virulence appeared to be comparable between the two KD mutants. This was consistent with the levels of accumulation of cellulase mRNAs and enzymatic activity in the KD mutants (Figs. 2, 3, and 4).

We then performed cytological examination of leaves infected with the cellulase KD mutants as well as those infected with a xylanase KD mutant (KD_sd1) that we had constructed previously (Nguyen et al. 2011). At least 100 spores with an appressorium were observed at 40 hpi, and the cellular re-

Fig. 4. Quantitative reverse-transcriptase polymerase chain reaction analysis of nine glycosyl hydrolase families 6 (GH6) and 7 (GH7) cellulase genes in infected leaves with knock-down (KD) *Magnaporthe oryzae* mutants (KD_CEL1 and KD_CEL2). Infected leaf samples were collected at 48 h postinoculation (hpi). Values in the graph indicate relative expression levels of the nine cellulase genes compared with those in the parent strain (onefold). cDNA quantities were normalized to the actin gene (MGG_03982). This experiment was performed with two biological replicates and with duplicate technical replicates. Error bars represent standard deviations. N.D., not detected; * and ** indicate $P < 0.05$ and 0.01 , respectively; significant difference from the parent strain (two-tailed t test).

sponses at the sites were classified into the following four types—no reaction (type A), papilla formation (B), hypersensitive reaction (HR) (type C), and hyphal growth (type D)—as described previously (Murakami et al. 2000). In leaves infected with the parent strain, the incompatible responses (types A, B, and C) were relatively infrequent events (13.7, 12.7, and 5.8%, respectively), and the compatible response (type D) was the most common (67.6%) (Table 1). In contrast, the compatible response occurred much less frequently in the leaves infected with cellulase and xylanase KD mutants (Table 1), suggesting that those CWDE significantly contributed to the virulence of the fungus. The lower rates of infection of the cellulase KD mutants were attributed to higher frequencies of type B (papilla formation) and type C (HR) responses (Table 1), suggesting that the cellulase KD mutants might have some defects in their penetration processes. However, the cellulase KD mutants showed significantly higher rates (38.3 and 35.3%) of successful infection (type D) compared with that of the xylanase KD mutant (10.3%). In addition, the frequency of the type A response, where an appressorium formed but no further fungal invasion was observed, was much higher for the xylanase KD mutant (50.3%) than for the cellulase KD mutants (16.7 and 15.7%). This trend was also observed with the other xylanase KD mutants we reported previously (Nguyen et al. 2011). These results indicated that the cellulase and xylanase KD mutants showed significantly reduced virulence but the reduction in virulence was more severe in the xylanase KD mutants than in the GH6 and GH7 cellulase KD mutants. It should be noted that the frequency of the type B response (papilla formation) was significantly higher after infection with the cellulase KD mutants (31.3 and 30.7%) than with the xylanase KD mutant (11.0%) (Table 1).

Next, we conducted a time-course experiment to analyze the rate and extent of fungal invasion in leaves infected with the

cellulase KD mutants. Epidermal cells of infected wheat leaves (Norin 4) were observed by epifluorescence microscopy at 24, 40, and 64 hpi, and the extent of infection was classified into the following five categories: no infection hypha, and infection hyphae in one, two to three, four to five, and more than five cells, as described previously (Nguyen et al. 2011) (Table 2).

In leaves infected with the parent strain, the extent of fungal invasion progressed over the experimental time course (Table 2). Fungal expansion was significantly slower in leaves infected with the KD mutants than in those infected with the parent strain. For example, 37.3% of germings of the parent strain infected one cell at 24 hpi and a similar number of germings infected more than five cells at 64 hpi. In contrast, 18.7 and 28% of germings of KD_CEL1 and KD_CEL2, respectively, infected one cell at 24 hpi but only 1.7 and 8.3% infected more than five cells at 64 hpi (Table 2). These data suggested that the cellulase KD mutants were also impaired in horizontal expansion even after vertical penetration into a host cell. These results indicated that the GH6 and GH7 cellulases play roles in both vertical penetration and horizontal expansion of *M. oryzae* in infected leaves.

DISCUSSION

Many phytopathogenic microorganisms produce enzymes capable of hydrolyzing the polymeric carbohydrate constituents of plant cell walls. Such enzymes, which are known as CWDE, are considered to be important pathogenicity factors. Several studies have shown a correlation between production of CWDE and aggressiveness of the plant pathogen (Campion et al. 1997; Novo et al. 2006). In addition, comparative genomics studies have revealed that genes encoding CWDE are generally expanded in phytopathogenic fungi compared with related saprobic fungi, supporting their roles in the pathogenicity from an evolutionary viewpoint. In contrast, recent genome sequencing of powdery mildew fungi has revealed that cellulases or hemicellulases are largely absent, suggesting that CWDE are not essential for fungal pathogenicity in some obligate biotrophs (Spanu et al. 2010).

Functional redundancy among gene family members can mask the effects of a mutation. Therefore, there is very little genetic evidence about the biological roles of CWDE in fungal pathogenicity. Using an RNAi approach, we have recently demonstrated that the GH10 and GH11 endoxylanases are important pathogenicity factors involved in both vertical penetration and horizontal expansion of *M. oryzae* in infected leaves (Nguyen et al. 2011). In this study, we showed that the GH6 and GH7 cellulases also contribute to the virulence of the fungus at the stages of vertical penetration and horizontal expansion.

The major targets in the current study were cellobiohydrolases, because GH6 and GH7 cellulases likely include all members of the cellobiohydrolase family in the *M. oryzae* genome, and probably some endoglucanases also. The KD mutants of the GH6 and GH7 cellulases induced incompatible responses

Fig. 5. Inoculation test of knock-down (KD) *Magnaporthe oryzae* mutants (KD_CEL1, KD_CEL2, and KD_sd1) and their parent strain Br48-GFP (C). Infection assay was performed using wheat ‘Norin 4’ at 22°C. Five days after inoculation, symptoms on the inoculated plants were evaluated. This experiment was repeated at least three times, and representative samples are presented.

Table 1. Cellular responses of wheat leaves (‘Norin 4’) to *Magnaporthe oryzae* knock-down (KD) mutants of cellulases (KD_CEL1 and KD_CEL2) and xylanases (KD_sd1)

Strain	Percentage of cellular responses (type) ^a			
	No reaction (A)	Papilla formation (B)	HR (C)	Hyphal growth (D)
Br48-GFP	13.7 ± 4.5	12.7 ± 2.9	5.8 ± 4.0	67.6 ± 4.5
KD_CEL1	16.7 ± 5.1	31.3 ± 1.5**	13.7 ± 4.0**	38.3 ± 8.7**
KD_CEL2	15.7 ± 3.5	30.7 ± 3.1**	18.3 ± 7.5**	35.3 ± 7.8**
KD_sd1	50.3 ± 4.8**	11.0 ± 2.7	28.0 ± 6.4**	10.3 ± 3.0**

^a Samples were collected 40 h postinoculation and observed under an epifluorescence microscopy; * and * indicate $P < 0.05$ and 0.01 , respectively; significant difference from the parent strain (two-tailed t test after angular transformation).

such as papilla formation (type B) and the HR (type C) at higher frequencies than did the parent strain. This may have been because the slower invasion of the KD mutants, which was likely due to their reduced virulence, allowed the host cells to mount an effective defense. In fact, symptom development in leaves infected with the KD mutant was considerably delayed compared with those infected with the parent strain (Table 2). However, compared with the KD mutants of the GH10 and GH11 endoxylanases, the degree of reduction in symptom development appeared to be lower for the KD mutants of the GH6 and GH7 cellulases (Table 2) (Nguyen et al. 2011).

Interestingly, the KD mutants of the GH6 and GH7 cellulases induced higher frequencies of papilla formation than did the xylanase KD mutants. Conversely, the frequency of type A response (no response) was much higher among plants infected with the xylanase KD mutants. Papilla formation is a typical resistance response to fungal penetration of host cell walls. Papillae consist of callose, cross-linked proteins, and phenolics (Brown et al. 1998; McLusky et al. 1999). Hydrogen peroxide is detectable during their formation, and is thought to be involved in signaling for further resistance responses and also in cross-linking reactions that could make cell walls resistant to CWDE (Bradley et al. 1992; Brown et al. 1998; Ikegawa et al. 1996; Soyly et al. 2005). Papilla formation is considered to be a wound response, because it can be induced by wounding only. This suggested that the majority of germ-lings of the cellulase KD mutants penetrated cell walls at least partially, triggering papilla formation. In addition, slower penetration of cell walls by the cellulose KD mutants might allow the host defense response (that is, papilla formation) to reach a stage where it effectively prevents further infection. In contrast, more than half of the xylanase KD mutant germ-lings did not induce any detectable response as determined by our cytological analysis, suggesting that their penetration was blocked completely or at very early stages. These results indicated that the xylanase KD mutant was more severely impaired in cell wall penetration than were the cellulase KD mutants. The xylanase KD mutant consistently showed a markedly lower frequency of successful infection (type D) than did the cellulase KD mutants.

There are several possible reasons why the reduction in virulence was milder for the KD mutants of the GH6 and GH7 cellulases than for those of the GH10 and GH11 endoxylanases. First, the GH10 and GH11 endoxylanases might have more important roles in cell wall penetration than do the GH6 and GH7 cellulases. In the *M. oryzae* genome, there are at least 15 endoglucanases belonging to the GH61 family and 1 belonging to the GH45 family. Even though it is likely that all of the cellobiohydrolases were targeted for gene silencing in this

study, some cellulases in other classes may have compensated for the silenced GH6 and GH7 cellulase genes during infection. In fact, the mRNA levels of most GH61 cellulases were not significantly reduced in KD_CEL1 and KD_CEL2. An alternative explanation is that xylan degradation may be an important part of the infection process. Xylan is a major constituent of graminaceous plants such as rice and wheat. Because the lower layer of the cuticle is composed of polysaccharides, including hemicelluloses (Domínguez et al. 2011), it is possible that degradation of xylan-related polysaccharides may be crucial at an early stage of cuticle penetration.

Another possibility is that the degree of gene silencing affected the results. The xylanase KD mutant (KD_sd1) constructed in our previous study showed better overall gene silencing than the cellulase KD mutants in this study (Nguyen et al. 2011). The enzymatic activity was reduced by 60 to 70% in KD_sd1 (Nguyen et al. 2011) whereas the reduction was approximately 45% in KD_CEL1 and KD_CEL2. However, another xylanase KD mutant, KD_fa10, also showed an infection phenotype (high frequency of the type A response) similar to that of KD_sd1 in our previous study (Nguyen et al. 2011). KD_fa10 was constructed using a conserved sequence in GH10 xylanases as an RNAi trigger. Therefore, the GH11 xylanase was not silenced or upregulated in the KD mutant (Nguyen et al. 2011), and the enzymatic activity was reduced by approximately 40%. Although the degree of the reduction in enzymatic activity in KD_fa10 was comparable with those in KD_CEL1 and KD_CEL2, the phenotypic characteristics of KD_fa10 were more similar to those of the other xylanase KD mutants than to those of the cellulase KD mutants. Therefore, it is unlikely that the degree of gene silencing explained the difference in virulence between the two types of mutants. Instead, it is more likely that the target gene family was the main factor causing phenotypic changes in the CWDE KD mutants.

As we discussed here, imperfect gene silencing by RNAi can make it difficult to interpret experimental results. However, the effectiveness of multiple gene knock-outs is limited because of the number of available marker genes, as well as time and effort. There are often more than 10 or even 20 genes in a group of CWDE in the genome of a phytopathogenic fungus. Thus, despite its weaknesses, we assume that RNAi is one of the most useful approaches to address the biological roles of CWDE.

MATERIALS AND METHODS

Fungal strains, culture condition, and transformation.

The *M. oryzae* strains used in this study were Br48 (wheat infecting isolate) (Urashima et al. 1999) and Br48-GFP, a transformant of Br48 with the eGFP gene driven by the *Aspergillus nidulans* *TrpC* promoter (Kadotani et al. 2003; Nguyen et al.

Table 2. Extent of fungal invasion in wheat leaves infected with *Magnaporthe oryzae* knock-down (KD) mutants of glycosyl hydrolase families 6 and 7 cellulases^a

Strain	Time (hpi)	Infection hyphae found in				
		0 cell	1 cell	2 or 3 cells	4 or 5 cells	>5 cells
Br48-GFP	24	62.3 ± 9.5	37.3 ± 9.0	0.3 ± 0.5	0	0
KD_CEL1	...	81.3 ± 5.0*	18.7 ± 5.0*	0	0	0
KD_CEL2	...	72.0 ± 8.7	28.0 ± 8.7	0	0	0
Br48-GFP	40	41.3 ± 4.6	58.3 ± 11.9	4.3 ± 1.5	0	0
KD_CEL1	...	70.0 ± 4.6**	29.7 ± 4.0*	0.3 ± 0.6*	0	0
KD_CEL2	...	65.7 ± 3.1**	34.3 ± 3.1*	0	0	0
Br48-GFP	64	5.7 ± 1.2	14.3 ± 2.5	16.7 ± 4.0	23.3 ± 3.2	40.3 ± 7.0
KD_CEL1	...	23.3 ± 2.9**	25.0 ± 4.4*	36.3 ± 3.8**	13.7 ± 2.9*	1.7 ± 0.8**
KD_CEL2	...	11.7 ± 2.1*	17.0 ± 2.0	37.0 ± 1.7**	25.0 ± 4.6	8.3 ± 1.5**

^a Inoculated leaves were collected at the indicated times and observed under a microscope; dpi = days postinoculation; * and * indicate $P < 0.05$ and 0.01, respectively; significant difference from the parent strain (two-tailed *t* test after angular transformation).

2008). They were maintained on potato dextrose agar or sterile barley seed media as described previously (Nakayashiki et al. 1999). For RNA extraction and spheroplast preparation, *M. oryzae* was grown in 100 ml of complete medium liquid broth (0.3% casamino acids, 0.3% yeast extract, and 0.5% sucrose) at 26°C. Fungal transformation and screening of KD mutants were performed as described previously (Nguyen et al. 2011).

Construction of silencing vectors.

To generate an artificial DNA fragment composed of 50-bp sequences from the nine cellulase genes, 200- and 250-bp fragments were first amplified by PCR using sets of three (Cello-1F, Cello-2F, and Cello-3R) or four (Cello-4F, Cello-5F, Cello-6R, and Cello-7R) primers, respectively. The resulting two DNA fragments were inserted into the *Sma*I and *Eco*RV sites, respectively, in pBluescript SK+II (Statagene, La Jolla, CA, U.S.A.). Then, the insert of approximately 450 bp in the resulting plasmid was PCR amplified using the SK and KS primers and cloned into the *Eco*RV site in pSD1G to establish pSD1G-CEL.

Infection assay and microscopic observation.

The infection assay was carried out as described previously (Murakami et al. 2000) using 14-day-old seedlings of *Triticum aestivum* (L) Thell. Norin 4 and Chinese Spring. For cytological observation, samples were made by the lactophenol fixation method and observed under bright and fluorescent fields of a fluorescence microscope with an excitation filter B, as described previously (Nguyen et al. 2011). Conidial germination and appressorium formation were examined by microscopic observation of at least 100 spores. The incidence of the cellular responses was statistically analyzed by a *t* test after angular transformation. The experiments were repeated at least three times independently.

RNA isolation and qRT-PCR.

RNA isolation and cDNA synthesis were performed as described previously (Nguyen et al. 2011). The qRT-PCR assay was carried out using FastStart SYBR Green Master (Roche, Mannheim, Germany) according to the manufacturer's instructions, with specific primers for targets and an internal control gene (actin) (Supplementary Table S2). Fluorescence from DNA-SYBER Green complex was monitored by the Thermal Cycler Dice Realtime System (Takara Bio USA, Otsu, Japan) throughout the PCR reaction. The level of target mRNA relative to the mean of the reference housekeeping gene was calculated by the comparative cycle threshold method.

Enzymatic assay.

After washing with distilled water, fungal mycelia were homogenized in buffer composed of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1% Nonidet P-40. After centrifugation at $10,000 \times g$ for 10 min, protein concentration in the supernatant was determined by Bio-Rad Protein Assay Kit (Bio-Rad, Munich). Cellulase activity was measured by the Somogyi-Nelson method (Nelson 1944; Somogyi 1952) using carboxymethyl cellulose (CMC) (Nacalai tesque, Kyoto, Japan), barley 1,3-1,4- β -glucan (Megazyme, Wicklow, Ireland), and xylan (Nacalai tesque) as substrates. The protein sample was incubated in 0.2 ml of 50 mM sodium acetate buffer (pH 5.0) containing 0.5% CMC at 40°C. After incubating for 60 min, the reaction was terminated by boiling for 5 min. After cooling, 0.2 ml of Somogyi reagent was added and the mixture was boiled for 10 min. After cooling, 0.2 ml of Nelson reagent was added and mixed, and then the total volume was adjusted to 2.0 ml with distilled water. After incubation at room temperature for 30 min, optical density at 490 nm was measured using Avro-SX.

ACKNOWLEDGMENTS

We are grateful to T. Takeda (Iwate Biotechnology Research Center, Japan) for barley 1,3-1,4- β -glucan and technical advice on cellulase assays. This work was supported in part by a Grant-in-Aid for Scientific Research and Special Coordination Funds for Promoting Science and Technology Japan, and a postdoctoral fellowship to Q. B. Nguyen from the Japan Society for the Promotion of Science.

LITERATURE CITED

- Apel, P. C., Panaccione, D. G., Holden, F. R., and Walton, J. D. 1993. Cloning and targeted gene disruption of *XYL1*, a β 1,4-xylanase gene from the maize pathogen *Cochliobolus carbonum*. *Mol. Plant-Microbe Interact.* 6:467-473.
- Apel-Birkhold, P. C., and Walton, J. 1996. Cloning, disruption and expression of two endo β 1,4-xylanase genes, *XYL2* and *XYL3*, from *Cochliobolus carbonum*. *Appl. Environ. Microbiol.* 62:4129-4135.
- Bradley, D., Kjellbom, P., and Lamb, C. 1992. Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: A novel, rapid defense response. *Cell* 70:21-30.
- Brown, I., Trethowan, J., Kerry, M., Mansfield, J., and Bolwell, G. P. 1998. Localization of components of the oxidative cross-linking of glycoproteins and of callose synthesis in papillae formed during the interaction between non-pathogenic strains of *Xanthomonas campestris* and French bean mesophyll cells. *Plant J.* 15:333-343.
- Campion, C., Massiot, P., and Rouxel, F. 1997. Aggressiveness and production of cell-wall degrading enzymes by *Pythium violae*, *Pythium sulcatum* and *Pythium ultimum*, responsible for cavity spot on carrots. *Eur. J. Plant. Pathol.* 103:725-735.
- Domínguez, E., Heredia-Guerrero, J. A., and Heredia, A. 2011. The biophysical design of plant cuticles: An overview. *New Phytol.* 189:938-949.
- Henrissat, B. 1991. A classification of glycosyl hydrolases based on amino acid sequence similarity. *Biochem. J.* 280:309-316.
- Henrissat, B., and Bairoch, A. 1996. Updating the sequence-based classification of glycosyl hydrolases. *Biochem. J.* 316:695-696.
- Howard, R. J., and Valent, B. 1996. Breaking and entering: Host penetration by the fungal rice blast pathogen *Magnaporthe grisea*. *Annu. Rev. Microbiol.* 50:491-512.
- Howard, R. J., Ferrari, M. A., Roach, D. H., and Money, N. P. 1991. Penetration of hard substrates by a fungus employing enormous turgor pressure. *Proc. Natl. Acad. Sci. U.S.A.* 88:11281-11284.
- Ikegawa, T., Mayama, S., Nakayashiki, H., and Kato, H. 1996. Accumulation of diferulic acid during the hypersensitive response in oat leaves and its role in enhanced resistance to cell-wall degrading enzymes. *Physiol. Mol. Plant Pathol.* 48:245-255.
- Kadotani, N., Nakayashiki, H., Tosa, Y., and Mayama, S. 2003. RNA silencing in the phytopathogenic fungus *Magnaporthe oryzae*. *Mol. Plant-Microbe Interact.* 16:769-776.
- Keen, N. T., Boyd, C., and Henrissat, B. 1996. Cloning and characterization of a xylanase gene from corn strains of *Erwinia chrysanthemi*. *Mol. Plant-Microbe Interact.* 9:651-657.
- McLusky, S. R., Bennett, M. H., Beale, M. H., Lewis, M. J., Gaskin, P., and Mansfield, J. W. 1999. Cell wall alterations and localized accumulation of feruloyl-3-methoxytyramine in onion epidermis at sites of attempted penetration by *Botrytis allii* are associated with actin polarization, peroxidase activity and suppression of flavonoid biosynthesis. *Plant J.* 17:523-534.
- Mendgen, K., Hahn, M., and Deising, H. 1996. Morphogenesis and mechanisms of penetration by plant pathogenic fungi. *Annu. Rev. Phytopathol.* 34:367-386.
- Murakami, J., Tosa, Y., Kataoka, T., Tomita, R., Kawasaki, J., Chuma, I., Sesumi, Y., Kusaba, M., Nakayashiki, H., and Mayama, S. 2000. Analysis of host species specificity of *Magnaporthe grisea* toward wheat using a genetic cross between isolates from wheat and foxtail millet. *Phytopathology* 90:1060-1067.
- Nakayashiki, H., Kiyotomi, K., Tosa, Y., and Mayama, S. 1999. Transposition of the retrotransposon MAGGY in heterologous species of filamentous fungi. *Genetics* 153:693-703.
- Nelson, N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. *J. Biol. Chem.* 153:375-380.
- Nguyen, Q. B., Kadotani, N., Kasahara, S., Tosa, Y., Mayama, S., and Nakayashiki, H. 2008. Systemic functional analysis of calcium signaling proteins in the genome of the rice blast fungus, *Magnaporthe oryzae*, using a high-throughput RNA silencing system. *Mol. Microbiol.* 68:1348-1365.
- Nguyen, Q. B., Itoh, K., Vu, V. B., Tosa, Y., and Nakayashiki, H. 2011. Simultaneous silencing of endo- β 1,4 xylanase genes reveals their roles in the virulence of *Magnaporthe oryzae*. *Mol. Microbiol.* 81:1008-1019.

- Novo, M., Pomar, F., Gayoso, C., and Merino, F. 2006. Cellulase activity in isolates of *Verticillium dahliae* differing in aggressiveness. *Plant Dis.* 90:155-160.
- Somogyi, M. 1952. Notes on sugar determination. *J. Biol. Chem.* 195:19-23.
- Soylu, S., Brown, I., and Mansfield, J. W. 2005. Cellular reactions in *Arabidopsis* following challenge by strains of *Pseudomonas syringae*: From basal resistance to compatibility. *Physiol. Mol. Plant Pathol.* 66:232-243.
- Spanu, P. D., Abbott, J. C., Amselem, J., Burgis, T. A., Soanes, D. M., Stüber, K., Ver Looren van Themaat, E., Brown, J. K. M., Butcher, S. A., Gurr, S. J., Lebrun, M.-H., Ridout, C. J., Schulze-Lefert, P., Talbot, N. J., Ahmadinejad, N., Ametz, C., Barton, G. R., Benjdia, M., Bidzinski, P., Bindschedler, L. V., Both, M., Brewer, M. T., Cadle-Davidson, L., Cadle-Davidson, M. M., Collemare, J., Cramer, R., Frenkel, O., Godfrey, D., Harriman, J., Hoede, C., King, B. C., Klages, S., Kleemann, J., Knoll, D., Koti, P. S., Kreplak, J., López-Ruiz, F. J., Lu, X., Maekawa, T., Mahanil, S., Micali, C., Milgroom, M. G., Montanna, G., Noir, S., O'Connell, R. J., Oberhaensli, S., Parlange, F., Pedersen, C., Quesneville, H., Reinhardt, R., Rott, M., Sacistán, S., Schmidt, S. M., Schön, M., Skamnioti, P., Sommer, H., Stephens, A., Takahara, H., Thordal-Christensen, H., Vigouroux, M., Weßling, R., Wicker, T., and Panstruga, R. 2010. Genome expansion and gene loss in powdery mildew fungi reveal tradeoffs in extreme parasitism. *Science* 330:1543-1546.
- Takahashi, M., Takahashi, H., Nakano, Y., Konishi, T., Terauchi, R., and Takeda, T. 2010. Characterization of a cellobiohydrolase (MoCel6A) produced by *Magnaporthe oryzae*. *Appl. Environ. Microbiol.* 76:6583-6590.
- Teeri, T. T. 1997. Crystalline cellulose degradation: New insight into the function of cellobiohydrolases. *Trends Biotechnol.* 15:160-167.
- Teeri, T. T., Koivula, A., Linder, M., Wohlfahrt, G., Divne, C., and Jones, T. A. 1998. *Trichoderma reesei* cellobiohydrolases: why so efficient on crystalline cellulose? *Biochem. Soc. Trans.* 26:173-178.
- Urashima, A. S., Igarashi, S., and Kato, H. 1993. Host range, mating type and fertility of *Pyricularia grisea* from wheat in Brazil. *Plant Dis.* 77:1211-1216.
- Urashima, A. S., Hashimoto, Y., Don, L. D., Kusaba, M., Tosa, Y., Nakayashiki, H., and Mayama, S. 1999. Molecular analysis of the wheat blast population in Brazil with a homolog of retrotransposon MGR583. *Ann. Phytopathol. Soc. Jpn.* 65:429-436.
- Walton, J. D. 1994. Deconstructing the cell wall. *Plant Physiol.* 104:1113-1118.
- Wood, T. M., and Bhat, K. M. 1988. Methods for measuring cellulase activities. *Methods Enzymol.* 160:87-112.
- Wu, S. C., Ham, K. S., Darvill, A. G., and Albersheim, P. 1997. Deletion of two endo- β -1,4-xylanase genes reveals additional isozymes secreted by the rice blast fungus. *Mol. Plant-Microbe Interact.* 10:700-708.
- Zhou, J., Zheng, X. Z., Lan, L., Lin, C. Z., Wu, Y. B., Lin, X. J., Ebbole, D., Lu, G. D., and Wang, Z. H. 2008. Biochemical and molecular characterization of a putative endoglucanase in *Magnaporthe grisea*. *Curr. Genet.* 53:217-224.