MST12* Regulates Infectious Growth But Not Appressorium Formation in the Rice Blast Fungus *Magnaporthe grisea

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In the rice blast fungus *Magnaporthe grisea*, a mitogen-activated protein kinase gene, *PMK1*, is known to regulate appressorium formation and infectious hyphae growth. Since *PMK1* is homologous to the *FUS3* and *KSS1* genes that regulate the transcription factor *STE12* in yeast, we functionally characterized the *STE12* homologue in *M. grisea* (*MST12*). A polymerase chain reaction–based approach was used to isolate the *MST12* gene that is homologous to yeast *STE12*. Four *mst12* deletion mutants were isolated by gene replacement. No obvious defect in vegetative growth, conidiation, or conidia germination was observed in *mst12* mutants. However, *mst12* mutants were nonpathogenic on rice and barley leaves. In contrast to *pmk1* mutants that did not form appressoria, *mst12* mutants produced typical dome-shaped and melanized appressoria. However, the appressoria formed by *mst12* mutants failed to penetrate onion epidermal cells. When inoculated through wound sites, *mst12* mutants failed to cause spreading lesions and appeared to be defective in infectious growth. These data indicate that *MST12* may function downstream of *PMK1* to regulate genes involved in infectious hyphae growth. A transcription factor or factors other than *MST12* must exist in *M. grisea* and function downstream from *PMK1* for appressorium formation.

Additional keyword: fungal pathogenicity.

Rice blast, caused by the heterothallic ascomycete *Magnaporthe grisea*, is one of the most severe diseases of rice throughout the world (Ou 1985). *M. grisea* infects all above-ground parts of rice plants in a manner typical of many foliar pathogens. Conidia are dispersed in the air and attach tightly after landing on the leaf surface (Hamer et al. 1988). Germ tubes produced from conidia differentiate into specialized infection structures called appressoria. The fungus uses the enormous turgor pressure generated in appressoria to penetrate the underlying plant surface (Howard et al. 1991). Once inside plants, bulbous infectious hyphae grow inter- and intracellu-

larly. Blast lesions develop in less than a week after the initial infection and release new conidia to reinitiate the infection cycle. The blast fungus attacks rice plants throughout the season and causes severe yield losses (Ou 1985).

In the past few years, several signal transduction pathways involved in regulating appressorium formation have been reported in *M. grisea* (Dean 1997; Xu 2000). The *PMK1* mitogen-activated protein (MAP) kinase appears to be essential for appressorium formation and infectious growth after penetration (Xu and Hamer 1996). *PMK1* is homologous to *Saccharomyces cerevisiae* *FUS3/KSS1*, and it can functionally complement a yeast *fus3 kss1* double mutant for mating (Xu and Hamer 1996). While there is no defect in vegetative growth and asexual reproduction, *pmk1* mutants of *M. grisea* fail to form appressoria and fail to grow infectiously in rice plants. In addition to the *PMK1* MAP kinase, the cyclic AMP (cAMP) signaling pathway has also been implicated in regulating appressorium formation and penetration. In *M. grisea*, cAMP stimulates appressorium formation on hydrophilic surfaces (Lee and Dean 1993), and the catalytic subunit of protein kinase A (PKA) is critical for the formation of functional appressoria (Mitchell and Dean 1995). Because *pmk1* mutants develop deformed germ tubes in response to cAMP on hydrophilic surfaces, it has been proposed that *PMK1* acts downstream of a cAMP signal for appressorium formation (Xu and Hamer 1996).

Recently, *PMK1* homologues have been characterized in several phytopathogenic fungi. In all the appressorium-forming fungi examined, including *M. grisea*, *Colletotrichum lagenarium*, *Cochliobolus heterostrophus*, and *Pyrenophora teres*, *PMK1* and its homologues were found to be essential for appressorium formation and to play an important role in plant infection (Lev et al. 1999; Ruiz-Roldan et al. 2001; Takano et al. 2000; Xu and Hamer 1996). Even in fungal pathogens that do not form appressoria for penetration, such as the root pathogen *Fusarium oxysporum* and the necrotrophic pathogen *Botrytis cinerea*, *PMK1* homologues are required for pathogenicity (Di Pietro et al. 2001; Zheng et al. 2000). It is likely that, in many fungi, the *PMK1* pathway is conserved for appressorium formation and other plant infection processes. However, although *PMK1* homologues have been identified in several fungi, it is not clear how *PMK1* is activated or what kinds of genes are regulated by this MAP kinase pathway for plant infection.

In *S. cerevisiae*, the pheromone response pathway involves the MAP kinase cascade, consisting of the MAP kinase kinase *STE11*, the MAP kinase kinase *STE7*, and two partially redundant MAP kinases, *FUS3* and *KSS1* (Gustin et al. 1998). In response to the mating pheromone, the activated Fus3 phosphorylates several substrates, including *STE12*, *DIG1*, and *DIG2* (Bardwell et al. 1998b; Elion et al. 1993; Sprague and Thorner 1992; Tedford et al. 1997). *DIG1* and *DIG2* are two

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The nucleotide sequence data reported are available in the GenBank database under the accession numbers: AF432913 for *Magnaporthe grisea* *MST12*, A33540 for *Saccharomyces cerevisiae* *STE12*, AAC31206 for *Aspergillus nidulans* *steA*, AAK21854 for *Penicillium marneffei* *StlA*, AAK14814 for *Neurospora crassa* *NcSTE12*, and AAG49739 and AF012924 for *Cryptococcus neoformans* *CnSTE12a* and *CnSTE12α*, respectively.

negative regulators of *STE12* that directly interact with *STE12*, *FUS3*, and *KSS1*, alone or in combination (Cook et al. 1996; Tedford et al. 1997). *STE12* is the major transcription factor that binds to the pheromone response element found in the upstream region of many pheromone-responsive genes (Dolan et al. 1989). Yeast *ste12* mutants are sterile and defective in the mating reaction (Fields and Herskowitz 1985). In addition, *STE12* is involved in filamentous growth in *S. cerevisiae* (Dolan and Fields 1990). Yeast filamentous growth represents invasive growth in haploid cells and pseudohyphal development in diploid cells (Kron and Gow 1995) and requires kinases *STE11*, *STE7*, and *KSS1* and transcription factors

STE12 and *TEC1* (Mosch et al. 1996; O'Rourke and Herskowitz 1998; Roberts et al. 1997). Repression and transcriptional activation of *STE12* are known to be regulated by *KSS1* for filamentous growth in yeast (Bardwell et al. 1998a).

Recently, *STE12* homologues have also been characterized in several other fungi, including *Candida albicans*, *Candida lusitanae*, *Cryptococcus neoformans*, *Aspergillus nidulans*, and *Penicillium marneffei*. In *Cryptococcus neoformans*, both CnSte12a and CnSte12 α are important for virulence and haploid fruiting in serotype D strains (Chang et al. 2000, 2001). The filamentous growth and virulence of *Candida albicans* also require either the MAP kinase pathway mediated by CPH1

A

<i>steA</i>	59	IRRFLLPTGD	.YISCVLW	SN	LFHISGTDIV	RCLAFRFQAF	GRPVKNSKKF	EEGIFSDLRN
<i>ShA</i>	58	IRRFLLPTGD	.YISCVLW	NN	LFHISGTDIV	RCLSFRRQAF	GRPVKNSKKF	EEGIFSDLRN
<i>MST12</i>	61	IRRFLLPTGE	.YVSCVLW	NN	LFHISGTDIV	RCLSFRRQAF	GRPVKNSKKF	EEGIFSDLRN
<i>NeSTE12</i>	60	IRRFLLPTGE	.YVSCVLW	NN	LFHISGTDIV	RCLSFRRQAF	GRPVKNSKKF	EEGIFSDLRN
<i>CnSTE12a</i>	95	LVKFFLP	PRGE	.QVSCVLW	NN	LFHITGTDIV	RALSFRFQAF	SRPDKRSRKK
<i>CnSTE12α</i>	94	LVKFFLP	NGE	.YVACVFW	NN	LYHITGTDII	RALSFRFQAF	SRPVRNMKKF
<i>STE12</i>	60	IRRYLLNSGQ	GFVSCVF	WNN	LYYITGTDIV	KCLYRMQKF	GREVVQKKK	EEGIFSDLRN

<i>steA</i>	118	LKAGTDATLE	EPKSPFLD	DFL	YKNNCIRTQK	KQKVFYWYSV	PHDRLFLDAL	ERDLKREKMG
<i>ShA</i>	117	LKSGTDASLE	EPKSPFLD	DFL	YKNNCIRTQK	KQKVFYWYSV	PHDRLFLDAL	ERDLKREKMG
<i>MST12</i>	120	LKSGTDASLE	EPKSPFLD	DFL	YKNNCIRTQK	KQKVFYWYSV	PHDRLFLDAL	ERDLKREKMG
<i>NeSTE12</i>	119	LKAGSDASLE	EPKSPFLD	DFL	YKNNCIRTQK	KQKVFYWYSV	PHDRLFLDAL	ERDLKREKMG
<i>CnSTE12a</i>	154	LKAGVDATLE	EPKSPFLD	LYL	FRNGCIRTQK	KQKVFYWFVS	PHDRLFLDAL	ERDLKREKMG
<i>CnSTE12α</i>	153	LKFGTDACLE	EPKSPFLD	LL	FRNGCIRTQK	KQKVFYWFVS	PHDRLFLDAL	ERDLKREKMG
<i>STE12</i>	120	LKCGIDATLE	QPKSEFLS	SFL	FRNMCIRTQK	KQKVFYWFVS	AHDKLFADAL	ERDLKRESLN

<i>steA</i>	178	QEATTVAVSE	PALSFEFDSS	..QSLYEQ
<i>ShA</i>	177	QEATTVAVSE	PALSFEFDSS	..QSLYEQ
<i>MST12</i>	180	QEATTMAVSE	PALSFOYDSS	..QSLYEQ
<i>NeSTE12</i>	179	QEATTVAVSE	PALSFOYDSS	..QSLYEQ
<i>CnSTE12a</i>	214	REPTTSVIQGE	PARSFRYDPR	..KALYEQ
<i>CnSTE12α</i>	213	LEPTTIVVGE	PARSFRYDPR	..RSLFEQ
<i>STE12</i>	180	QPSSTTKPVNE	PALSFSYDSS	SDKPLVYDQ

B

<i>steA</i>	300	HQRHSSMPTF	.VEYSPAPSF	VSSQYEDYSN	RGLSFEPTVP	PQHSSHIGPE	PAYIANEDTG
<i>ShA</i>	289	HQRHASMPST	.MEYSPAPSF	VSSQYEDYSN	RGLSFEPTVP	PQPM.LGAE	PAYIANEETG
<i>MST12</i>	305	HQRHASMPAY	GLEYSAPSF	VSSHVDDYGN	RGLSFEPLTP	PQAMGMGAE	PAYIANEETG
<i>NeSTE12</i>	322	HQRHTSMPTF	GLECSAPSF	VSSHVDDYSQ	RGLSFEPLTP	PQAMGMVIGE	PAYIANEETG
<i>CnSTE12a</i>	270						
<i>CnSTE12α</i>	277						
<i>STE12</i>	319	TEENAFDPFP	PQAFTPAAPS	MPISYDNVNE	RDSPVNSLL	NRYVYQLSVA	PTFPVPPSSS

<i>steA</i>	359	LYTAIPE.MS	TGSGFNPMH	LPPSNLASAH	FF.....	..TPARTFH	SNVYSVLEGS
<i>ShA</i>	347	LYTAIPD.VG	S.AAYHPMMQ	LPPSNLANPL	GA.....	..T.SRHFQ	ANVFSVIEGS
<i>MST12</i>	365	LYTAIPDHMM	GVNGLNGMTQ	LPPSNLAGAQ	FTRSYGSNNV		..YSVIEGS
<i>NeSTE12</i>	382	LYTAIPDHLN	.AGALNGMMQ	LPPSNLAAPS	YPRSYASSSI	SQDHARGYSP	MAAYTVIEGS
<i>CnSTE12a</i>	272						
<i>CnSTE12α</i>	300	IVSTPPSPEN	ILNNTDQLR	C.LTTASSVL	FPRLKLGSP	AYRQGRRKAL	RD.....
<i>STE12</i>	379	RQHFMNTNRD	YSSNNNKEKL	VSPSD.....	.PFSVMKYDE	PVMDFDESRR	NENCTNAKSH

<i>steA</i>	407	PTYKQRRRRS	SIPPGAANPV	TGTGTHS...P	APALSYAAHK	PSDLRRSVSS	SVAP.GDTRD
<i>ShA</i>	393	PTYKQRRRRS	SIPPGITNAI	AAAQVSGAQ	QPT.SYAAAYR	PSDLRRSISN	SVGPVTEITD
<i>MST12</i>	412	PTYKQRRRRS	SIPPG.MSAI	AAAT.....	..AAATAAHR	PSDLRRSVSA	SVGPVAGGDE
<i>NeSTE12</i>	441	PTYKQRRRRS	SIPPSVLSAT	TAIA.....	..AGTPGVYK	PSDLRRSVSA	SVGPVAGGDE
<i>CnSTE12a</i>	297	..NKSSISQAS	RHLVGTAD..		EFSE	CG.VRSKGSE	SSVLRPTIYFN
<i>CnSTE12α</i>	351	..NKQSTTRQD	SVTISDCDTG	DDESGSESR	AGRVERNNGFE	RD.LALTSQS	SAPTLLNSBD
<i>STE12</i>	433	NSGQQTQKHQ	LYSNNFQQSY	PNGMVPGYYF	KMPYNPMGGD	PL.LDQAFYG	ADDFFPPEEG

<i>steA</i>	463	SRHESLHRSV	NSTYTATL..	PQKNLMHEMS	RNGTP.LSS
<i>ShA</i>	452	SNQSAAGSL	QSDYTSTVA.	SQKNMLHEVS	RTGTP.LPS
<i>MST12</i>	463	SDNNSPPGLI	YSNQPMSSAN	HQREAMEQMS	RHGTP.LST
<i>NeSTE12</i>	493	SADNSPPGLT	YSNPAINMAR	QHHHEMFLS	RHGTP.LST
<i>CnSTE12a</i>	346	SYLNSSTHLL	ATAARVHIPS	LGSPDDRKC	SSLTP.S.
<i>CnSTE12α</i>	408	SHATMAITAS	ESTRYISFPS	LQSTSGTTL	SHELP.SQM
<i>STE12</i>	492	CDNNMLYPQT	ATSWNVLPQ	AMQPAPTYVG	RPVTEYRS

C

<i>steA</i>	566	CPIPSCGRLF	KRLEHLKRE	V	RTHTQERPYP	CPYCNKAF.S	RSDNLAQH..	.RRIH
<i>ShA</i>	555	CPIPSCGRLF	KRLEHLKRE	V	RTHTQERPYP	CPYCNKAF.S	RSDNLAQH..	.RRTH
<i>MST12</i>	566	CPIPSCGRLF	KRLEHLKRE	V	RTHTQERPYP	CPYCNKAF.S	RSDNLAQH..	.APYL
<i>NeSTE12</i>	593	CPIPSCGRVF	KRLEHLKRE	V	RTHTQERPYP	CPYCNKAF.S	RSDNLAQH..	.KRTH
<i>CnSTE12a</i>	396	CPYDFCKRP	KRFEHLRRE	V	RCHTDDRP	CDICHRTE.T	RSDNLAQH..	.AKTH
<i>CnSTE12α</i>	511	CHLLSCGRLE	KRLEHLKRE	V	RTHTQERPYP	CSRCAARE.S	RSDNLAQH..	.YKTH

Fig. 1. Alignment of the predicted amino acid sequences of *MST12* to *STE12* homologues from other fungi in the **A**, homeodomain (residues 61 to 205), **B**, middle region (residues 305 to 501), and **C**, C₂H₂-Zn²⁺ finger domains (residues 566 to 611). Identical residues are shown in black boxes. Similar residues are shaded. Dotted underlines indicate the positions of degenerate primers used to clone *MST12*.

(an *STE12* homologue) or a MAP kinase-independent pathway including EFG1 (Chang et al. 2001). In the filamentous fungi *A. nidulans* and *P. marneffei*, the *STE12* homologues are dispensable for both hyphal growth and asexual reproduction, but the *A. nidulans* *steA* deletion mutant is blocked in sexual reproduction (Bornemann et al. 2001; Vallim et al., 2000).

To further characterize the *PMK1* MAP kinase pathway, we isolated and functionally characterized the *STE12* homologue gene *MST12* from *M. grisea*. *MST12* is dispensable for vegetative growth, conidiation, conidium germination, and appressorium formation. However, similar to *pmk1* mutants, gene replacement mutants of *MST12* were defective in infectious growth and appressorial penetration. *MST12* may be a major transcription factor regulated by *PMK1* for controlling genes essential for appressorial penetration and infectious growth.

RESULTS

The *MST12* gene is homologous to yeast *STE12*.

A polymerase chain reaction (PCR)-based strategy was used to isolate the *STE12* homologue from *M. grisea*. PCR clone pLZ14, containing a 139-bp insert homologous to *S. cerevisiae* *STE12*, was used to screen Guy11 cDNA and genomic libraries. Eight cosmid clones and one cDNA clone were identified. Sequence analysis revealed an open reading frame (ORF) interrupted with two introns. This ORF was predicted to encode a 685-amino acid protein that has the highest homology with *Neurospora crassa* *NcSTE12* (59.4% identity). Similar to all fungal *STE12* homologues, the *MST12* (*M. grisea* *STE12*

homologue) gene has a homeodomain at the N-terminal region (residues 61 to 205). The homeodomain of *MST12* is 53, 65, 70, 92, 94, and 94% identical with homeodomains of *STE12*, *CnSTE12a*, *CnSTE12*, *steA*, *StlA*, and *NcSTE12*, respectively (Fig. 1). Residues 150 to 160 are believed to be essential for DNA binding and are highly conserved among these six fungal genes showing 70 to 100% identity (Vallim et al. 2000). In addition, *MST12* contains two tandem C_2H_2 -Zn²⁺ finger domains near the C-terminus (residues 566 to 611) that are conserved only in *STE12* homologues from filamentous fungi. In the C_2H_2 -Zn²⁺ finger domains, *MST12* shares 46, 72, 87, 93, and 93% identity with the same regions of *CnSTE12a*, *CnSTE12a*, *NcSTE12*, *StlA*, and *steA*, respectively (Fig. 1).

In *S. cerevisiae*, *STE12* has an interacting region (residues 298 to 473) downstream from the homeodomain that interacts with the MAP kinase *KSS1* (Bardwell et al. 1998a). Amino acid residues 303 to 313 in this area are well conserved among *S. cerevisiae*, *Candida albicans*, and *K. lactis* and are critical for activation by the MAP kinase (Bardwell et al. 1998a; Liu et al. 1994; Pi et al. 1997). However, *MST12* and *STE12* homologues from other filamentous fungi, such as *P. marneffei*, *A. nidulans*, and *N. crassa*, lack similarity with *STE12* in this area. Interestingly, the amino acid residues (residues 305 to 501 in *MST12*) between the homeodomain and the C_2H_2 -Zn²⁺ finger domains are well conserved between *MST12* and *STE12* homologues from other filamentous fungi (Fig. 1). In this region, *MST12* is 38, 38, and 60% identical to *StlA*, *steA*, and *NcSTE12*, respectively. There are a putative cAMP- and cGMP-dependent protein kinase phosphorylation site (RRRS; residues 418 to 421), a putative protein kinase C phosphorylation site (TYK; residues 413 to 415), three putative casein kinase II phosphorylation sites, and four putative N-myristoylation sites in this region, predicted by computer programs Pfam and PROSITE.

MST12 is not essential

for vegetative growth and conidiation.

The gene replacement construct pMSK1 was linearized with *Pst*I and transformed into the wild-type strain Guy11. Around 150 hygromycin-resistant transformants were isolated and purified by single-spore isolation. All these transformants were screened by PCR with primers MK3N and H856F and with primers MK1F and MK2R. In four transformants, primers MK3N and H856F amplified a 1.0-kb PCR fragment, indicating that homologous recombination between pMSK1 and the *MST12* genomic sequence had occurred (data not shown). In addition, primers MK1F and MK2R amplified a 0.6-kb fragment in Guy11, but not in these four putative *mst12* gene replacement mutants (data not shown).

All four putative *mst12* knockout mutants were further confirmed to have the correct gene replacement event by Southern blot analysis (Fig. 2B). When genomic DNAs were digested with *Eco*RI and hybridized with the 0.8-kb *Eco*RI fragment

Fig. 2. *MST12* gene replacement. **A**, Physical map of *MST12* genomic region and gene replacement vector pMSK1. Arrows indicate orientations of the *MST12* and *hph* genes. The restriction enzymes are Bg, *Bgl*III; B, *Bss*HII; C, *Cla*I; E, *Eco*RI; and S, *Sph*I. The *Eco*RI site derived from cloning vector pAhd5 is marked with an asterisk. MK3N, MSF15, MK1F, MK2R, MSR16, and H856F are primers used for generating and screening *mst12* knock-out mutants. **B**, Southern blot of wild-type (Guy11), ectopically inserted transformant (E175) and *mst12* gene replacement transformant (MK23). All DNA samples were digested with *Eco*RI. The blot was hybridized with the *Eco*RI 0.8-kb fragment from pMG3.2 (left), and then stripped and rehybridized with the *hph* gene (right).

Fig. 3. Germination and appressorium formation in the *Magnaporthe grisea* wild-type strain (Guy11) and *mst12* gene replacement mutant (MK23). Photographs were taken under bright field microscopy after 2 or 24 h of incubation on plastic coverslips. Bar = 10 μ m.

from pMG3.2 (Fig. 2A), the *mst12* knockout mutant MK23 had a 1.7-kb fragment instead of the wild-type 0.8-kb fragment (Fig. 2B). Similar hybridization results were observed in the other three putative *mst12* knockout transformants, MK8, MK31, and MK111 (data not shown). A randomly selected transformant, E175, contained both the wild-type 0.8-kb *EcoRI* fragment and the 1.7-kb *EcoRI* fragment that is diagnostic for the gene replacement cassette, indicating that the gene disruption cassette integrated ectopically in the fungal genome (Fig. 2B). All putative *mst12* knockout mutants and E175, but not Guy11, had the 1.7-kb *EcoRI* band that hybridized to the hygromycin phosphotransferase (*hph*) gene (Fig. 2B).

The *mst12* knockout mutants had the typical, grayish growth and colony morphology of the wild-type Guy11 colonies on oatmeal agar. The growth rate of *mst12* mutants was measured in oatmeal agar race tube cultures (Xu and Hamer 1996) and was the same as that of Guy11 and E175 after incubation at room temperature for 3 weeks (data not shown). All *mst12* mutants produced normal vegetative hyphae and abundant three-celled conidia (Fig. 3). No obvious difference in conidiation and conidial morphology was observed among Guy11, E175, and *MST12* replacement mutants (data not shown). These data indicate that *MST12* is not essential for vegetative growth and conidiation in *M. grisea*.

***MST12* is dispensable for appressorium formation.**

To test if *MST12* is the major downstream transcription factor regulated by *PMK1* for appressorium formation, we assayed appressorium formation in the *mst12* mutants. Conidia from the *mst12* mutants had no defect in surface attachment and germination. After 2 hr of incubation at room temperature, over 94% of conidia germinated (Table 1) and produced normal germ tubes (Fig. 3). Similar to those of Guy11 and E175, the vast majority of *mst12* germ tubes formed appressoria after incubation at room temperature for 24 hr (Table 1). *mst12* Mutants formed typical, dome-shaped and melanized appressoria (Fig. 3). No obvious difference in appressorium morphology and melanization was observed between *mst12* mutants and Guy11. It is clear from these data that *MST12* is dispensable for conidium germination and appressorium formation and, most likely, the regulation of appressorium formation by *PMK1* is mediated by transcription factors other than *MST12*.

***mst12* Knockout mutants are nonpathogenic.**

When leaves of the susceptible rice cultivar CO39 were sprayed with conidia from *mst12* mutants, no lesions developed up to 7 days postinoculation (Fig. 4A). Rice leaves sprayed with Guy11 and the ectopic transformant E175 developed typical rice blast lesions under the same conditions (Fig. 4A). We repeated spray infection assays with all four *mst12* mutants more than three times and did not observe any blast lesions on the inoculated seedlings. We also repeated the spray infection assays on barley seedlings. While barley leaves sprayed with Guy11 showed many lesions after 5 to 7 days postinoculation, no lesions were observed on barley leaves

sprayed with conidia from *mst12* mutants (Fig. 4B). These results indicate that *mst12* mutants are nonpathogenic and *MST12* is essential for plant infection.

Appressoria formed by *mst12* mutants are nonfunctional for plant penetration.

Because *mst12* mutants form appressoria, we examined appressorial penetration by the *mst12* mutants. On onion epidermal cells, conidia from *mst12* mutants formed normal melanized appressoria (Fig. 5). However, while the majority of the appressoria of Guy11 and E175 penetrated and produced typical bulbous infectious hyphae in the onion epidermal cells, no penetration or infectious hyphae were developed from appressoria formed by *mst12* mutants on onion epidermal cells (Fig. 5). In at least three experiments and during examination of more than 500 appressoria formed by *mst12* mutants, we did

Fig. 4. Infection assays of *Magnaporthe grisea* strains. **A**, Typical infected leaf areas of rice seedlings inoculated with either wild-type (Guy11), ectopic transformant (E175), or *mst12* gene replacement mutant (MK23). **B**, Typical infected leaf areas of barley seedlings inoculated with Guy11 and MK23. Two-week-old rice cv. CO39 and 8-day-old barley cv. Golden Promise seedlings were sprayed with conidia suspensions (5×10^5 conidia per ml).

Table 1. Conidial germination and appressorium formation

Time	Conidial germination (%)			Appressorium formation (%)		
	Guy11	E175	MK23	Guy11	E175	MK23
30 min	53.8 $\pm$ 5.8 ^a	31.4 $\pm$ 3.7	42.1 $\pm$ 4.8	0	0	0
2 h	96.3 $\pm$ 2.9	94.1 $\pm$ 2.4	94.7 $\pm$ 1.1	0	0	0
12 h	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0	99.5 $\pm$ 0.8	88.9 $\pm$ 14.1	98.1 $\pm$ 1.7	96.8 $\pm$ 2.3
24 h	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0	100.0 $\pm$ 0.8	97.8 $\pm$ 0.8	99.2 $\pm$ 0.9	99.1 $\pm$ 1.0

^a Mean and standard deviation were calculated from three replicate experiments.

not observe the formation of infectious hyphae. No obvious plant defense responses, such as papilla formation and auto-fluorescence, were observed in the onion epidermal cells underlying the appressoria formed by *mst12* mutants (data not shown), indicating that *mst12* mutants may be defective in either appressorial turgor pressure generation or penetration peg formation, or both.

***MST12* replacement mutants are nonpathogenic through wounding sites.**

We tested whether *mst12* mutants can infect through wounds by injection inoculation and abrasion infection assays. In leaves inoculated with Guy11, typical spreading blast lesions were observed in and around the injection sites (Fig. 6A). Similar results were obtained for the ectopic transformant E175 (data not shown). While wound sites injected with a 0.5% gelatin solution developed little or no plant cell necrosis, injections with *mst12* mutants resulted in small areas of necrosis at the injection sites (Fig. 6A). However, *mst12* mutants did not form any lesions outside injection sites because of a defect in appressorial penetration (Fig. 6A). Interestingly, the necrotic area caused by injection inoculation with *mst12* mutants was very limited and similar to that formed by *pmk1* mutants but much smaller than that of Guy11. In leaves injected with *buf1* (Chumley and Valent 1990) and *chm1* (J.-R. Xu, unpublished data) mutants that are defective in appressorial penetration but normal in infectious growth, the necrotic lesion usually spreads to a much larger area surrounding the injection site (Fig. 6A). In addition, *mst12* mutants failed to form lesions on the leaves abraded with diatomaceous earth (Fig. 6B). On leaves abraded with the *chm1* mutant and Guy11, blast lesions were visible 5 days after inoculation (Fig. 6B).

To test if *mst12* mutants were still alive in the necrotic area, we excised wound sites from leaves injected with either Guy11 or mutant strains and incubated these excised leaf sections on water agar plates for conidiation. Although wound sites inoculated with Guy11 produced abundant conidia and mycelia, no mycelial growth and conidiation were observed in wound sites inoculated with *mst12* mutants (Fig. 6C) in four separate experiments. We conclude that *MST12*, similar to *PMK1*, is required for infectious growth or viability in plants. The loss of pathogenicity in *mst12* mutants may be caused by defects in both appressorial penetration and infectious growth after penetration.

Because all four independent *mst12* gene replacement transformants generated in this study had the same defects in

appressorial penetration and plant infection, most likely the disruption of *MST12* was directly responsible for these phenotypes. To further confirm that all the phenotypes described above are caused by the disruption in the *MST12* gene, we isolated 33 random ascospore progeny from a cross between MK23 (*MAT1-2*) and 2539 (*MAT1-1*). All 20 progeny resistant to hygromycin failed to infect rice seedlings and were defective in appressorial penetration. On the other hand, all 13 progeny sensitive to hygromycin were fully pathogenic on rice leaves and normal in appressorial penetration. Thus, defects in appressorial penetration and infectious growth in the *mst12*

Fig. 5. Penetration assays on onion epidermal cells. The appressorium of wild-type Guy11 penetrated epidermal cells and produced abundant infectious hyphae (panels on the left), whereas the appressorium of MK23 did not penetrate or form infectious hyphae (panels on the right). Photographs were taken after 60 h of incubation at 25°C. Bars = 10 μ m. AP = appressorium, G = germ tube, IF = infectious hyphae, and S = spore.

Fig. 6. Infection assays by injection and abrasion. **A**, Seedlings from rice cv. CO39 were wound-inoculated by injection with wild type (Guy11), *mst12* gene replacement mutant (MK23), *chm1* gene replacement mutant (MCH3), and 0.5% gelatin solution (gelatin). Typical leaves are shown following 1 week of symptom development. **B**, Leaves from CO39 seedlings abraded with diatomaceous earth and inoculated with Guy11, MK23, MCH3, and gelatin solution. **C**, Lesions from injection-inoculated rice leaves were incubated with light and humidity on 4% water agar for 2 days. Fungal growth and sporulation were observed in the wound sites inoculated with Guy11 (left) but were not observed in the wound sites inoculated with MK23 (right).

mutants cosegregated with the hygromycin resistance marker associated with the gene replacement event.

***MST12* is dispensable for mating in *M. grisea*.**

All the original *mst12* transformants were female sterile but male fertile. However, the ectopic transformant E175 was also female sterile, indicating that the loss of female fertility might be caused by the transformation process. Female fertility was scored in 33 progeny derived from the cross between 2539 and MK23. Five of twenty progeny resistant to hygromycin were female fertile and produced normal perithecia as efficiently as the wild-type strain Guy11 (Fig. 7). Seven of thirteen progeny sensitive to hygromycin were female sterile. We confirmed that these female fertile progeny were indeed *mst12* knockout mutants by Southern blot analysis (data not shown). Perithecia formed by these female fertile *mst12* progeny (both as male and female) contained normal asci and viable ascospores, indicating that *MST12* is dispensable for mating either as male or female in *M. grisea*.

To further confirm that the loss of female fertility is not related to *mst12* gene knockout, the wild-type *MST12* gene was reintroduced into MK23 by cotransformation. After screening over 60 bleomycin resistant transformants, two MK23 transformants with the wild-type *MST12* gene ectopically integrated into the fungal genome were identified and confirmed by Southern blot analysis (data not shown). Both of these complemented *MST12* transformants are still female sterile but are capable of penetrating onion epidermal cells and infecting rice seedlings. Thus, the disruption of the *MST12* gene in MK23 is not responsible for the loss of its female fertility.

DISCUSSION

In *M. grisea* and other fungal pathogens, the *PMK1* MAP kinase and its homologues have been shown to be important for pathogenicity and other functions (Xu 2000). In this study, we isolated and characterized the *STE12* homologue *MST12* as a potential downstream transcription factor of the *PMK1* pathway in *M. grisea*.

***MST12* has the typical structural features of *STE12* homologues from filamentous fungi.**

The *M. grisea* *MST12* gene encodes a 685-amino acid protein with an N-terminal homeodomain that is 53 to 94% identical to *STE12* and its homologues from other fungi. It has two adjacent C_2H_2 -Zn²⁺ finger domains at the C-terminal that are not present in *STE12* and its homologues from other yeasts.

Fig. 7. The *mst12* knock-out mutants are female fertile. When mated with strains 2359 (right) and 4091-5-8 (left) of the opposite mating type, both the wild-type Guy11 (top) and *mst12* progeny 26 (bottom) can produce perithecia either as the male or female. Fertile perithecia formed by *mst12* progeny 26 as the female are indicated by arrows.

MST12 also lacks significant homology with *S. cerevisiae* *STE12* in the regions that are important for transcriptional activation, *Kss1* binding and pheromone inducibility (residues 298 to 473), and Dig1 binding (residues 309 to 547) in *S. cerevisiae* (Bardwell et al. 1998a; Olson et al. 2000; Song et al. 1991). In general, we found that homology is relatively low between *STE12* homologues from yeasts and filamentous fungi, except in the N-terminal homeodomain region. However, among the *STE12* homologues from filamentous fungi, there is significant homology in the region between the homeodomain and C_2H_2 -Zn²⁺ finger domains. We noticed that stretches of amino acid residues, such as EPAYIANEETGLYTAIP (residues 354 to 370 in *MST12*) and VIEGSPTYKQRRRRSSIPP (residues 407 to 425 in *MST12*), are identical in *MST12*, *steA*, *StlA*, and *NcSTE12* (Fig. 1B). Interestingly, the putative cAMP- and cGMP-dependent protein kinase phosphorylation site (residues 418 to 421, RRRS) is located in one of these highly conserved areas. It is possible that these well-conserved areas facilitate the interaction of *MST12* and its homologues with other genes (such as PKA, MAP kinases, or Dig1/Dig2 counterparts) for their activation or suppression in filamentous fungi.

***MST12* is dispensable for appressorium formation but essential for plant infection.**

We generated four *MST12* gene-disruption mutants by replacing a 0.8-kb fragment of *MST12* containing the homeodomain and part of the middle region (Fig. 2A) with the bacterial *hph* gene. Because the *hph* gene is inserted in the direction opposite to the *MST12* gene, we do not expect the truncated 3' portion of *MST12* remaining in the *mst12* gene replacement mutants to be expressed or functional without a promoter. *MST12* appears to be essential for both appressorial penetration and infectious growth in *M. grisea*. Even though appressoria formed by *mst12* mutants are normal in morphology and melanization, they fail to penetrate and produce infectious hyphae on onion epidermal cells. In all of our infection assays, lesions were never observed in rice and barley leaves sprayed with conidia from *mst12* mutants.

To date, there is no *STE12* homologue that has been characterized in plant pathogenic fungi. However, *STE12* homologues have been characterized in the human pathogens, *Candida albicans* and *Cryptococcus neoformans*. In the asexual diploid *Candida albicans*, the *STE12* homologue *CPH1* is required for hyphal growth and for dimorphic switching on certain solid media and, in combination with *EFG1*, is required for virulence in a mice model (Liu et al. 1994; Lo et al. 1997a). In *Cryptococcus neoformans*, there are mating-type-specific *STE12* homologues, *CnSTE12α* and *CnSTE12β*, in mating type *MATα* and *MATβ* strains, respectively. These homologues have similar functions in mating and are required for haploid fruiting (Chang et al. 2000, 2001). Deletion of *CnSTE12α* or *CnSTE12β* in serotype D strains significantly reduced the capsule size and virulence in mice (Chang et al. 2000, 2001).

***MST12* may function downstream from *PMK1* for regulating infectious growth.**

Two primary functions of *PMK1* in *M. grisea* are to regulate appressorium formation and infectious growth (Xu and Hamer 1996). Similar to *mst12* mutants, *pmk1* mutants are nonpathogenic and fail to colonize rice leaves through wounds (Xu and Hamer 1996). Based on the relationship between *FUS3/KSS1* and *STE12* and similar defects in infectious growth in *pmk1* and *mst12* mutants, we believe *MST12* may be the major transcription factor regulated by *PMK1* for controlling infectious growth in *M. grisea* (Fig. 8). Our preliminary data from yeast two-hybrid assays indicate that the Pmk1 protein may directly interact with the Mst12 protein (G. Park and J.-R. Xu, *unpub-*

lished data). However, the interaction between Pmk1 and Mst12 proteins appears to be relatively weak. In *S. cerevisiae*, the direct interaction between *KSS1* and *STE12* (a K_d of approximately 400 nM) is also considerably weaker than the direct interaction between *KSS1* and *STE7* (a K_d of approximately 5 nM) (Bardwell et al. 1998a). The interaction between *FUS3* and *STE12* is even weaker than that of *KSS1* and *STE12*.

In *M. grisea*, *PMK1* is also essential for appressorium formation. Because *mst12* mutants still produce normal appressoria, a transcription factor or factors other than *MST12* must exist in *M. grisea* and function downstream from *PMK1* to regulate appressorium formation (Fig. 8). However, although we cannot rule out the possibility that *MST12* is involved in regulating appressorium formation, there may be other redundant transcription factors in *M. grisea*. Similarly, it is possible that *PMK1* may also be essential for regulating appressorial penetration, even though *pmk1* mutants do not form appressoria.

***MST12* may be involved in the cAMP signaling pathway to regulate appressorial penetration.**

Appressoria formed by *mst12* mutants are defective in penetration. It is likely that *MST12* regulates the processes involved in a late stage of appressorium development, such as turgor generation and appressorium pore formation. We assayed appressorial turgor using the cytorrhysis methods described by Howard and associates (1991). Preliminary data did not show any differences in cytorrhysis between appressoria formed by Guy11 and *mst12* mutants (data not shown), suggesting that appressoria of *mst12* mutants do not have defects in the generation of turgor pressure.

Similar to *mst12* mutants, the *cpkA* mutants also produce melanized appressoria and are defective in appressorial penetration in *M. grisea* (Xu et al. 1997). Thus, it is possible that *MST12* is involved in the cAMP signaling pathway for regulating appressorial penetration (Fig. 8). However, appressoria formed by *cpkA* mutants appear to be reduced in size, whereas *mst12* mutants produced appressoria of similar size to that of the wild type. In addition, *mst12* mutants still respond to cAMP by forming appressoria on hydrophilic surfaces (data not shown). It is likely that there may be a transcription factor or factors other than *MST12* involved in cAMP signaling (Fig. 8).

It has been reported that there is a cooperative regulation of cAMP signaling and the *PMK1* MAP kinase pathway for appressorial turgor generation in *M. grisea* (Gancedo 2001; Kronstad et al. 1998). *PMK1* appears to regulate the mobilization and transport of glycogen and lipid reserves into appressoria, while *CPKA* may control the degradation of these compounds in appressoria to generate the turgor pressure required for appressorial penetration (Thines et al. 2000). *MST12* may regulate genes involved in either the transfer, the degradation, or both of the glycogen and lipid reserves in *M. grisea*.

The other possibility is that *MST12* may regulate the early stages of appressorial penetration, such as development of the penetration peg or the differentiation of infectious hyphae. In *M. grisea*, penetration peg emergence and differentiation of infectious hyphae are processes that involve dramatic cytoskeleton reorganization (Bourett and Howard 1990). In *S. cerevisiae*, *STE12* is involved in differentiation processes such as schmoos formation and pseudohyphal development (Gustin et al. 1998; Lo et al. 1997b). Furthermore, although *MST12* may directly regulate genes involved in appressorial function or infectious growth, an additional transcription factor or factors may exist and coregulate these processes by forming heterodimers with *MST12* (Fig. 8). Multiple transcription factors or heterodimers may act on different sets of genes involved in

different stages of appressorial formation, maturation, and penetration that are controlled by either *PMK1* or cAMP signaling pathways, or both. In *S. cerevisiae*, *STE12* forms heterodimers with *TEC1* for regulating filamentous growth (Madhani and Fink 1997).

***MST12* is dispensable for sexual reproduction in *M. grisea*.**

Even though our original *mst12* knockout mutants are female sterile, further analyses indicated that the loss of female fertility is not a direct result of the disruption of the *MST12* gene. In *M. grisea*, loss of female fertility can be caused by culture conditions and transformation procedures (Xu 2000; Valent 1990). We have recovered five *mst12* female fertile progeny that have all other phenotypes associated with the deletion of *MST12*, indicating that deletion of *MST12* does not affect female fertility in *M. grisea*. This is different from the *ste12*, *cls12*, and *steA* mutants in *S. cerevisiae*, *Candida lusitanae*, and *A. nidulans*, which are sterile (Hartwell 1980; Vallim et al. 2000; Young et al. 2000). The *steA* deletion mutants fail to differentiate ascogenous tissues and fruiting bodies (Vallim et al. 2000). Perhaps additional *STE12*-like transcription factors exist and regulate female fertility in *M. grisea*. In the homothallic ascomycete *F. graminearum*, our preliminary data indicated that gene replacement mutants of a *STE12* homologue still form fertile perithecia (J.-R. Xu, unpublished data). *F. graminearum* is a pyrenomycete that is more closely related to *M. grisea* than to *A. nidulans* or *S. cerevisiae*. In *Cryptococcus neoformans*, both the *cnste12a* deletion and *cnste12 α* deletion mutants are fertile, although with reduced mating frequency. The *cnste12a* deletion mutants still mate with the *cnste12 α* deletion mutants (Chang et al. 2000, 2001; Wickes et al. 1997).

Overall, our study has demonstrated that *MST12* is essential for appressorial penetration and infectious growth but dispensable for appressorium formation in *M. grisea*. *STE12* homologues from other fungal pathogens have also been reported to be involved in fungal morphogenesis and virulence (Chang et al. 2001; Liu et al. 1994). *MST12* may be a major downstream transcription factor of the *PMK1* pathway for regulating infectious hyphae growth, but a transcription factor or factors other than *MST12* may be involved in regulating appressorium formation. It will be interesting to further characterize the middle region that is conserved among *STE12* homologues from fila-

Fig. 8. A model for possible roles of *MST12* in the *PMK1* MAP kinase and cAMP signaling pathways in *Magnaporthe grisea*. *MST12* may function as a downstream transcription factor of *PMK1* for regulating infectious growth. A transcription factor or factors other than *MST12* is regulated by *PMK1* or cAMP signaling for appressorium formation and surface recognition. There may be crosstalk between the cAMP signaling and *PMK1* MAP kinase pathways in the appressorium maturation and penetration processes. *MST12* and an additional transcription factor or factors may form a transcriptional complex that is regulated both by *CPKA* and *PMK1*.

mentous fungi. This region may interact with upstream signaling elements of different pathways and affect the specific activity of *MST12*.

MATERIALS AND METHODS

Strains and growth conditions.

Wild-type strains Guy11 (*MAT1-2*), 2539 (*MAT1-1*), and 4091-5-8 (*MAT1-1*) and all the transformants were cultured on oatmeal agar plates at 25°C under fluorescent light for conidiation and stored on desiccated Whatman #1 filter paper at -20°C as previously described (Romao and Hamer 1992). Mycelia collected from 2-day-old 5× YEG (0.5% yeast extract, 2% glucose) culture shaken at 150 rpm at room temperature were used for extracting fungal DNA. Genetic crosses and progeny isolations were performed as described (Crawford et al. 1986).

Isolating the *MST12* gene.

Two degenerate PCR primers, TF7 (5'AARTTYGARGARGGNAT3') and TR4 (5'TTYTGYTTYTTYTGNGT3'), were designed according to the amino acid residues KFEEGI and TQKKQK that are conserved among the *STE12* homologues from *S. cerevisiae*, *Candida albicans*, and *Kluyveromyces fragilis* (Malathi et al. 1994). Primers TF7 and TR4 were used to amplify *STE12* from Guy11 genomic DNA by a PCR reaction consisting of 30 cycles of 1 min at 94°C, 1 min at 48°C, and 2 min at 72°C, followed by 5 min at 72°C. PCR products amplified with these two primers were cloned in pGEM-T Easy vector (Promega, Madison, WI, U.S.A.) and sequenced. One clone, pLZ14, contained a 139-bp insert that was homologous to *S. cerevisiae STE12*. This clone was used to screen a Guy11 pMOcosX cosmid genomic library and a ZAPII (Stratagene, La Jolla, CA, U.S.A.) cDNA library constructed with RNA isolated from a nitrogen-starved Guy11 culture (Xu and Hamer 1996). *MST12* cDNA and genomic clones were isolated and sequenced with the ABI Prism BigDye Terminator cycle sequencing kit (PE Applied Biosystems, Foster City, CA, U.S.A.). DNA sequences were analyzed with the DNASIS program V3.0 (Hitachi Software, Santa Clara, CA, U.S.A.). Homologues in GenBank were found with the BLAST program (Altschul et al. 1997). Amino acid sequence comparisons and alignment were made with the PILEUP and BOXSHADE programs in the GCG Wisconsin software package (Accelrys, San Diego, CA, U.S.A.).

Constructing the *MST12* gene replacement vector and screening for *mst12* mutants.

To construct the *MST12* gene replacement vector, a 3.2-kb fragment containing the entire *MST12* gene was amplified with primers MSF15 (5'AGTCGGATCCGACGCCAGTGTGCAAC3') and MSR16 (5'AGGTGATATCTGCATCCAGTTGTATGT3') and cloned in the pGEM-T Easy vector (Promega) as pMG3.2 (Fig. 2A). The 1.5-kb *Bss*HII-*Cla*I fragment containing the *hph* gene from pAhd5 (Carroll et al., 1994) was then cloned between the *Bss*HII and *Cla*I sites on pMG3.2. In the resulting *MST12* gene replacement vector pMSK1 (Fig. 2A), the 0.8-kb *Bss*HII-*Cla*I fragment containing part of the *MST12* gene was replaced by the *hph* gene in the opposite orientation of *MST12* (Fig. 2A).

For generating *mst12* disruption mutants, pMSK1 was digested with *Pst*I and transformed into Guy11 as previously described (Sweigard et al. 1992). Transformants resistant to 100 µg of hygromycin B per ml were isolated and purified by single-spore isolation. Genomic DNAs of these transformants were isolated as described (Xu and Leslie 1996) and screened by PCR with primers MK3N (5'TCATTGCTTTGTCTGTGT3') and H856F (5'GTCGATGCGACGCAATCGT3'). Be-

cause the MK3N primer sequence was not present in the gene replacement vector pMSK1 (Fig. 2A), only transformants that had undergone homologous recombination between pMSK1 and the *MST12* genomic sequence would have the 1.0-kb PCR product. All the transformants were also screened by PCR with primers MK1F (5'AAGCCTGGACAAAAGACT3') and MK2R (5'AACCTGCTGAGCGTTGAA3') that were located in the region of *MST12* that was deleted in pMSK1 (Fig. 2A). All putative *mst12* gene replacement mutants were confirmed by Southern blot analysis. For complementation, one cosmid clone (10P16) containing the full-length *MST12* gene was introduced into the *mst12* knockout mutant MK23 by cotransformation with pAC905 (Zheng et al. 2000). Bleomycin-resistant transformants grown on medium containing 75 µg of bleomycin per ml were screened by PCR with primers MK1F and MK2R and confirmed by Southern blot analysis for the presence of the wild-type *MST12*. Standard molecular biology procedures were followed for restriction enzyme digestion and hybridization (Sambrook et al. 1989).

Assaying germination, appressorium formation, and penetration.

Conidia were harvested from 6-day-old oatmeal agar cultures, filtered once through Miracloth (Calbiochem, La Jolla, CA, U.S.A.), and resuspended in sterile distilled water to 3 × 10⁴ conidia per ml. Drops of the conidial suspension (50 µl) were placed on plastic microscope cover slips (Fisher Scientific Co., Pittsburgh, PA, U.S.A.) and incubated in a moist chamber at room temperature. Conidial germination and appressorium formation were examined at 30 min, 2, 12, and 24 h after incubation. Appressorial penetration on onion epidermal strips was assayed as previously described (Xu et al. 1997). Appressorium formation and infectious hyphal growth were examined after 48 to 72 h of incubation at room temperature. Photographs were taken with Kodak Technical-Pan film using an Olympus Vanox-S microscope (Olympus, Melville, NY, U.S.A.).

Plant infection assays.

Conidia were collected from 10-day-old oatmeal agar cultures and resuspended to 5 × 10⁵ conidia per ml in 0.5% gelatin solution. Two-week-old seedlings of the rice cultivar CO39 and 8-day-old seedlings of the barley cultivar Golden Promise were used for infection assays. Plant incubation, inoculation, and lesion examination were performed as described (Valent et al. 1991; Xu et al. 1997). For injection inoculation, conidial suspensions were injected into leaf sheaths of rice seedlings with a 26G_{1/2} needle. Lesion formation and necrosis around the inoculation sites were examined when wounded leaves had unfolded 5 to 7 days after injection. For abrasive inoculation, the second and third leaves of 2-week-old rice seedlings were powdered with diatomaceous earth (Sigma Co., St. Louis, MO, U.S.A.). A drop of conidial suspension (30 µl) was then applied onto each leaf and rubbed gently with diatomaceous earth. For sporulation assays with infected leaves, wound inoculation sites were excised, surface sterilized with 70% ethanol for 1 min, and incubated on 4% water agar with fluorescent light and high humidity as previously described (Xu and Hamer 1996).

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