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Independent genetic mechanisms mediate turgor generation and penetration peg formation during plant infection in the rice blast fungus

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Summary

The first barrier to infection encountered by foliar pathogens is the host cuticle. To traverse this obstacle, many fungi produce specialized infection cells called appressoria. *MST12* is essential for appressorium-mediated penetration and infectious growth by the rice pathogen *Magnaporthe grisea*. In this study, we have characterized in detail the penetration defects of an *mst12* deletion mutant. Appressoria formed by the *mst12* mutant developed normal turgor pressure and ultrastructure but failed to form penetration pegs either on cellophane membranes or on plant epidermal cells. Deletion and site-directed mutagenesis analyses indicated that both the homeodomain and zinc finger domains, but not the middle region, of *MST12* are essential for appressorial penetration and plant infection. The *mst12* mutant appeared to be defective in microtubule reorganization associated with penetration peg formation. In mature appressoria, the mutant lacked vertical microtubules observed in the wild type. The *mst12* mutant also failed to elicit localized host defence responses, including papilla formation and autofluorescence. Our data indicate that generation of appressorium turgor pressure and formation of the penetration peg are two independent processes. *MST12* may play important roles in regulating penetration peg formation and directing the physical forces exerted by the appressorium turgor in mature appressoria.

Introduction

Rice blast, caused by the filamentous fungus *Magnaporthe grisea*, is one of the most destructive diseases on cultivated rice, the major food crop for half of the world's population (Ford *et al*., 1994). The blast fungus also infects a wide range of economically important grass species, and it has emerged as a model system to study fungal–plant interactions (Valent, 1990). *M. grisea* infects rice plants in a manner typical of many foliar pathogens. Germ tubes produced from conidia differentiate into specialized infection structures called appressoria. The fungus generates enormous turgor pressure in melanized appressoria that adhere tightly to the plant surface with appressorium mucilage (Howard *et al*., 1991; de Jong *et al*., 1997). Blocking melanin synthesis by mutation or inhibitors prevents penetration because the melanin layer is essential for turgor generation (Chumley and Valent, 1990). Mature appressoria develop thin penetration pegs from the centre of appressorium pores to pierce the host surface and enter cells of the leaf epidermis. At early stages of penetration, the penetration peg contains high concentrations of actin filaments and lacks cytoplasmic organelles (Bourett and Howard, 1992). This zone-of-exclusion includes the area of the appressorium where the penetration peg emerges. Apical vesicles, however, are observed in elongating penetration pegs during later stages of penetration (Bourett and Howard, 1992). During penetration, actin and cytoskeletal elements may be involved in the re-establishment of polarized growth by determining the penetration site and in stabilizing the tip of the penetration peg (Howard and Valent, 1996). After penetration, the peg differentiates into bulbous, lobed infectious hyphae that grow inter- and intracellularly (Heath *et al*., 1990; 1992) and result in development of blast lesions (Tucker and Talbot, 2001).

Signal transduction pathways regulating infection-related morphogenesis have been extensively studied in *M. grisea* during the past few years (Dean, 1997; Xu, 2000; Tucker and Talbot, 2001). Similar to other fungal pathogens (Kronstad *et al*., 1998; D′Souza and Heitman, 2001), both the cAMP signalling pathway and MAP kinase *PMK1* (homologue of yeast *FUS3* and *KSS1*) have been shown to play important roles in appressorium development and infectious growth in *M. grisea* (Dean, 1997; Xu, 2000; Tucker and Talbot, 2001). While the *pmk1* mutant fails to form appressoria (Xu and Hamer, 1996), the *cpkA* mutant deleted of the catalytic subunit of PKA still forms

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Table 1. Glycogen and lipid staining in developing appressoria.

	Glycogen		Lipid	
	Guy11	MK23	Guy11	MK23
6 h	94.16 ± 0.95[a]	88.52 ± 2.43	82.89 ± 10.21	74.34 ± 8.34
12 h	40.46 ± 6.52	40.71 ± 6.59	88.69 ± 5.64	94.53 ± 3.73
24 h	13.86 ± 2.85	16.79 ± 4.67	56.64 ± 4.06	52.13 ± 4.94
48 h	15.68 ± 4.67	11.27 ± 0.31	11.53 ± 5.72	9.73 ± 6.30

a. Percentage of appressoria staining for glycogen or lipid bodies. Mean and standard deviations were calculated from three independent experiments.

melanized appressoria (Mitchell and Dean, 1995; Xu *et al*., 1997). The *cpkA* mutant is likely to be defective in appressorium turgor generation because of its defects in the mobilization of glycogen and lipid (Thines *et al*., 2000). Other *M. grisea* mutants known to form melanized appressoria but fail to penetrate plant cells include the *pls1* (Clergeot *et al*., 2001) and *mps1* MAP kinase deletion mutants (Xu *et al*., 1998). The penetration defects in the *pls1* and *mps1* mutants have not been determined, but the *mps1* mutant appears to have weaker cell walls (Xu *et al*., 1998). Although appressoria formed by the *cpkA* and *mps1* mutants fail to penetrate underlying plant cells, they still elicit plant defence responses, including autofluorescence and papilla formation (Xu *et al*., 1997; 1998).

We previously reported that *MST12*, a homologue of the *Saccharomyces cerevisiae STE12*, is also essential for appressorial penetration and infectious hyphae growth in *M. grisea* (Park *et al*., 2002). In *S. cerevisiae*, *STE12* is a transcription factor that functions in the pheromone response pathway, pseudohyphal development, and invasive growth (Gustin *et al*., 1998; Dohlman and Thorner, 2001). *STE12* homologues have been shown to be important for fungal virulence in *Candida albicans* (Liu *et al*., 1994), *C. glabrata* (Calcagno *et al*. 2003), *Colletotrichum lagenarium* (Tsuji *et al*., 2003), and *Cryptococcus neoformans* (Wickes *et al*., 1997; Chang *et al*., 2000; 2001). In this study, we found that the *mst12* mutant produced appressoria with normal turgor pressure and ultrastructure but failed to develop penetration pegs or elicit localized host defence responses. The homeodomain and zinc finger domains that are well conserved in filamentous fungi, including *Aspergillus nidulans* (Vallim *et al*., 2000) and *Penicillium marneffei* (Borneman *et al*., 2001), are both essential for the function of *MST12*. Appressoria formed by the *mst12* mutant appeared to be defective in microtubule reorganization associated with penetration peg formation in mature appressoria. Our data indicate that generation of appressorium turgor pressure is not sufficient for successful penetration in *M. grisea* and *MST12* plays important roles in penetration peg formation possibly by regulating cytoskeleton reorganization associated with late stages of appressorium formation.

Results

Appressorium turgor generation is normal in the mst12 *mutant*

To determine whether the *mst12* mutant has defects in turgor generation, we first examined the mobilization and degradation of glycogen bodies and lipid droplets during appressorium formation. Glycogen bodies were detectable in both conidia and young appressoria by 6 h but were more intensely stained in appressoria by 6 h, and only faintly stained in appressoria after 12 h in Guy11 and the *mst12* deletion mutant MK23 (Fig. 1A, Table 1). No significant difference in glycogen staining was observed between MK23 and Guy11 during appressorium formation (Fig. 1A, Table 1). In lipid staining assays, Guy11 and the *mst12* mutant MK23 also were similar in the number of appressoria stained and intensity of fluorescence after incubation at room temperature for 6, 12, 24, or 48 h (Fig. 1A, Table 1). The majority of lipid droplets in MK23 and Guy11 had moved into developing appressoria by 6 h and were degraded by 48 h (Fig. 1B). In control experiments with the *cpkA* mutant deleted of the catalytic subunit of PKA (Xu *et al*., 1997), the movement and degradation of lipid droplets were delayed (data not shown; Thines *et al*., 2000). These results indicated that the *mst12* mutant had no defect in mobilization and degradation of lipid and glycogen bodies.

Appressorium turgor can be measured in *M. grisea* by calculating the rate of cellular collapse (cytorrhysis) when appressoria are incubated in hyperosmotic solutions (Howard *et al*., 1991). In both Guy11 and MK23, cytorrhysis was observed in less than 10% of the appressoria treated with 30% PEG, but over 50% of the appressoria underwent cytorrhysis when treated with 40% PEG (Table 2). Guy11 and MK23 were not significantly different in the percentage of appressoria that underwent cytorrhysis (Table 2, $P > 0.1$), suggesting that appressoria of Guy11 and the *mst12* mutant had similar turgor pressures.

The mst12 *mutant fails to develop penetration pegs*

Appressoria formed by the *mst12* mutant were examined by SEM and TEM for possible ultrastructural defects. When examined by SEM, Guy11 and MK23 exhibited

Table 2. Cytorrhysis assay of the appressorium turgor.

	Guy11	MK23
30% PEG	6.7 ± 3.2%[a]	6.8 ± 5.4%
40% PEG	66.0 ± 4.7%	58.2 ± 9.3%

a. Percentage of appressoria that underwent cytorrhysis. Mean and standard deviations were calculated from four replicates. At least 100 appressoria were examined in each repetition.

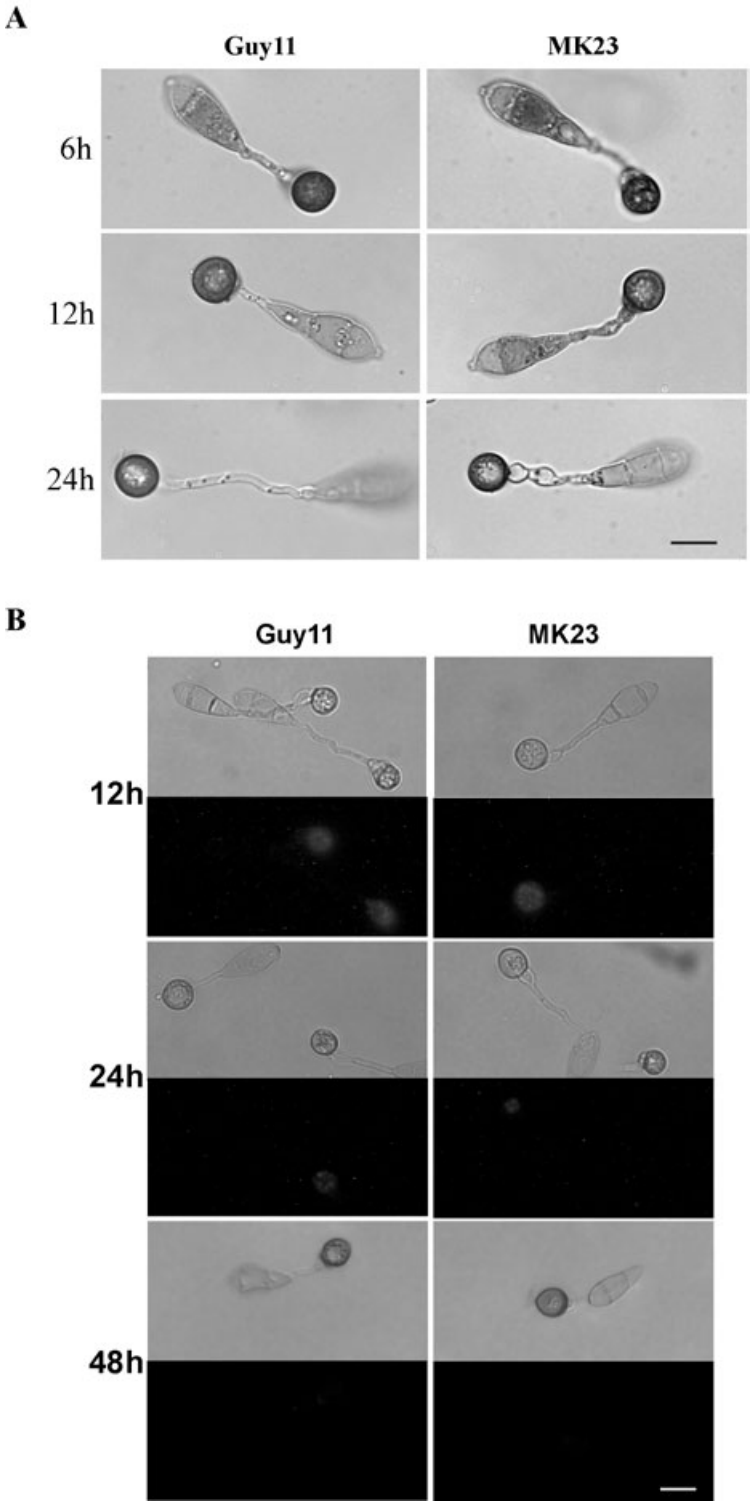

Fig. 1. *MST12* is dispensable for mobilization and degradation of glycogen and lipid storages. Conidia of the wild-type strain Guy11 and the *mst12* deletion mutant MK23 were germinated on glass coverslips and removed at the indicated times.
A. Glycogen was stained with iodine and examined by epifluorescence microscopy. In both Guy11 and MK23, glycogen in conidium cells was transferred to and degraded in appressoria.
B. Lipid droplets were stained with Nile Red and visualized by epifluorescence microscopy (lower images). The upper images were the same field examined under differential interference contrast (DIC) microscopy. Bar = 10 μm.

indistinguishable appressorium morphology and surface texture (Fig. 2A). When cross sections of appressoria formed on cellophane membrane were examined by TEM, appressoria formed by the *mst12* mutant had typical cell walls, a distinct melanin layer, appressorium mucilage, and appressorium pore (Fig. 2B). In the isogenic wild-type strain Guy11, however, many appressoria developed penetration pegs and penetrated the underlying cellophane membrane by 48 h (Fig. 2B). However, in MK23, none of the over 35 appressoria examined by serial sections had

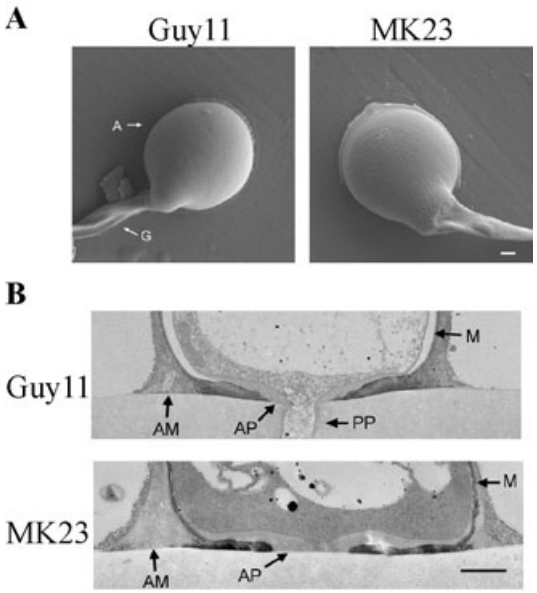

Fig. 2. Appressoria formed by the wild-type strain Guy11 and the *mst12* mutant MK23 were examined by scanning electron microscopy (A) and transmission electron microscopy (B). Bars represent 1 μm. Appressoria formed by MK23 had normal morphology and ultrastructure, including the melanin layer (M), appressorium pore (AP), and appressorium mucilage (AM), but the penetration peg (PP) was observed only in Guy11. Bar = 1 μm.

developed penetration pegs under the same conditions (Fig. 2B). We also examined over 300 appressoria formed by MK23 on onion or barley or rice sheath epidermal cells but never observed penetration pegs in repeated experiments (Fig. 3). Under the same conditions, about 80% of appressoria formed by Guy11 penetrated underlying plant cells and developed infectious hyphae (Fig. 3). These data indicated that appressoria formed by the *mst12* mutant were normal in morphology but defective in penetration peg formation. Penetration peg formation is likely to be a process regulated by *MST12* and appears to be independent of appressorium turgor generation.

Appressoria of the mst12 *mutant fail to elicit papilla formation in underlying plant cells*

On onion epidermal cells, appressoria formed by Guy11

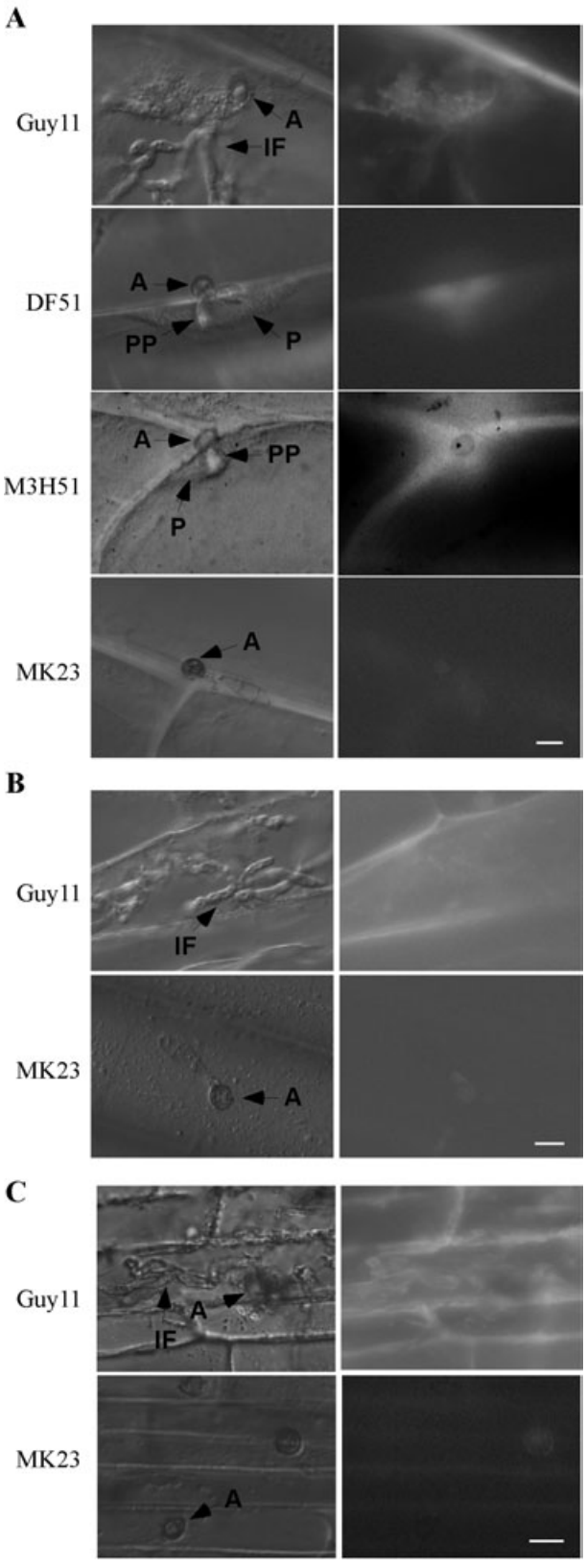

Fig. 3. Penetration assays with onion, barley, and rice epidermal cells.
A. Onion epidermal strips were inoculated with conidia of the wild type (Guy11), *mst12* mutant (MK23), *cpka* mutant (DF51), and *mps1* mutant (M3H51).
B. Barley leaf epidermis inoculated with Guy11 and MK23.
C. Rice leaf sheath epidermal cells inoculated Guy11 and MK23. Photographs were taken 48 h after inoculation. Panels on the left and right were the same field examined under DIC and epifluorescence microscopy, respectively. A, appressorium; P, papilla; PP, penetration peg; IF, infectious hypha. Bar = 10 μm.

penetrated plant cell walls and differentiated into infectious hyphae by 48 h (Fig. 3A). Autofluorescence was observed at the initial penetration sites (Fig. 3A). Under the same conditions, *mst12* appressoria failed to penetrate and develop infectious hyphae in onion epidermal cells. No papilla or autofluorescence was observed in plant cells beneath appressoria formed by the *mst12* mutant (Fig. 3A). In the control experiments with the *cpkA* mutant DF51 (Xu *et al*., 1997) and mutant M3H51 deleted of the *MPS1* MAP kinase gene (Xu *et al*., 1998), penetration and infectious hyphae were not observed. However, penetration pegs, papillae formation, and autofluorescence were observed in underlying onion epidermal cells (Fig. 3A), indicating that the *cpkA* and *mps1* mutants were different from the *mst12* mutant in triggering plant defence responses.

Consistent results were obtained in penetration assays with the epidermal cells of barley leaf and rice leaf sheath. The *mst12* mutant formed appressoria but failed to penetrate and elicit papilla formation and autofluorescence in epidermal cells of barley leaves (Fig. 3B) or rice leaf sheaths (Fig. 3C) after 48 h. No autofluorescence was observed in barley or rice epidermal cells around appressoria formed by MK23 even after prolonged incubation up to 72 h (data not shown). Under the same conditions, Guy11 produced infectious hyphae and elicited autofluorescence in barley leaf and rice sheath epidermal cells (Fig. 3B). Thus, appressoria formed by the *mst12* mutant, different from those of *cpkA* and *mps1* mutants, failed to elicit papilla formation and autofluorescence in onion, barley, or rice epidermal cells, suggesting that penetration peg formation is essential for plant cells to initiate localized defence responses in *M. grisea*. It is likely that the *cpkA* and *mps1* mutants form penetration pegs but are defective in either overcoming localized plant defence responses or differentiation of infectious hyphae.

Host cell actin reorganization is not triggered by appressoria of the mst12 *mutant*

Actin cytoskeletal reorganization and cytoplasmic aggregation also are components of the early cellular response to penetration in plant cells (Schmelzer, 2002). In onion epidermal cells inoculated with Guy11 or the *cpkA* mutant DF51, actin filaments stained with rhodamine-phalloidin were reorganized towards penetration sites and had formed an array of radial bundles directed towards appressoria by 24 h (Fig. 4). Furthermore, cytoplasmic aggregation and cytoplasmic streaming towards the appressorium attachment site were often observed in plant cells. In contrast, actin filaments were dispersed and mainly axially oriented in onion epidermal cells inoculated with MK23, in the same manner as those observed in mock-inoculation experiments with water (Fig. 4). Actin cytoskeletal rearrangement in response to appressoria of MK23 was not observed in underlying plant cells even after 72 h (data not shown), indicating that the *mst12* mutant is different from the *cpkA* and *mps1* mutants in triggering cytoplasmic aggregation and cytoskeletal reorganization. These results indicate that *MST12* and the emergence of the penetration peg are essential for *M. grisea* to trigger plant cytoskeletal rearrangement and other localized cellular defence responses.

Penetration peg formation is not required for triggering lesion mimic responses

The *mst12* mutant is non-pathogenic on compatible rice plants (Park *et al*., 2002). To test whether the *mst12* mutant still triggers lesion mimic responses (disease-like lesions) in the absence of penetration peg formation, seedlings of cultivar IR68 and the lesion mimic mutant *spl11* (Yin *et al*., 2000) were inoculated with conidial suspensions of Guy11 and MK23 at the same concentration. IR68 carries the *pi-9* resistance gene and is resistant to Guy11 (Bastiaans and Roumen, 1993). Whereas no lesions were observed on IR68 leaves sprayed with MK23 (Fig. 5A), many brown specks appeared on leaves sprayed with Guy11. On *spl11* leaves sprayed with conidia from Guy11 or MK23, lesion-mimic lesions were observed by 7 days post-inoculation (Fig. 5A), indicating that *MST12* and penetration peg formation are dispensable for causing lesion mimic reactions. However, more lesion-mimic lesions developed on *spl11* leaves sprayed with Guy11 than on those sprayed with MK23 (Fig. 5A), suggesting that the biosynthesis or secretion of the chemical factors eliciting lesion mimic reactions are reduced in the *mst12* deletion mutant.

To determine whether the *mst12* mutant still induces the expression of plant defence genes, we isolated RNAs from CO-39 seedlings infected with Guy11 and MK23. Northern blot analyses indicated that the *mst12* mutant still induced the expression of *PR1* and *PBZ1* (Yin *et al*., 2000) at the level similar to that induced by Guy11 in inoculated rice plants (Fig. 5B), suggesting that induced expression of defence related genes, such as *PR1* and *PBZ1* (Yin *et al*., 2000), is not triggered by appressorial penetration. In contrast to localized cellular defence responses triggered by penetration pegs in cells directly below appressoria formed by the *mst12* mutant, the induction of plant defence genes and lesion mimic reactions in rice leaves sprayed with conidia may be elicited by chemical or enzymatic factors that are still produced by *mst12* germlings.

Both the homeodomain and zinc finger domains are essential for the function of MST12

MST12 has an N-terminal homeodomain that is con-

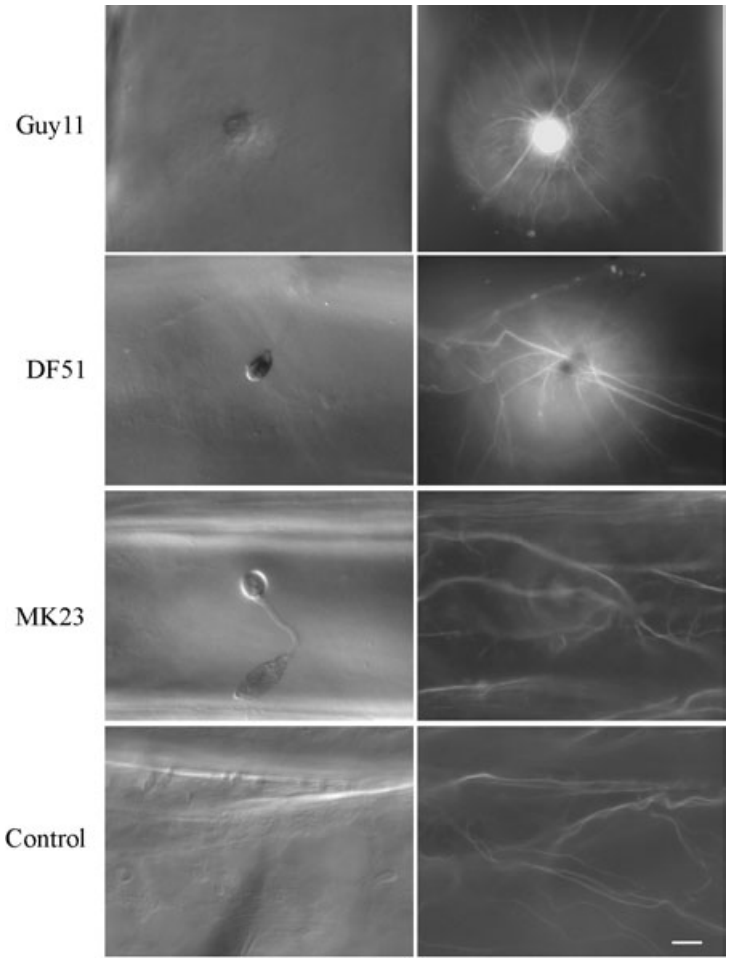

Fig. 4. Host actin cytoskeleton rearrangements in response to appressorial penetration. Actin filaments in onion epidermal cells inoculated with the wild type (Guy11), *cpka* mutant (DF51), and *mst12* mutant (MK23) after 24 h incubation were stained with rhodamine-phalloidin and examined under DIC (left panels) and epifluorescence microscopy (right panels). Appressoria formed by Guy11 and DF51 caused the alignment of actin filaments towards the penetration sites. There was no obvious actin cytoskeleton change in cells inoculated with MK23 or sterile distilled water. Bar = 10 μm.

served among all fungal *STE12* homologues and two zinc finger domains at the C-terminus that are unique to filamentous fungi (Park *et al*., 2002). To determine the functions of these two regions, *MST12* constructs pGP66 and pGP68 deleted of the homeodomain and zinc finger domains, respectively, were generated and transformed into the *mst12* mutant MK23. None of the five transformants harbouring pGP66 or pGP68, including transformants HD3 and ZD7 (Table 3), were able to penetrate and infect rice seedlings (Fig. 6). All of these transformants were phenotypically similar to the original *mst12* mutant MK23 and failed to penetrate onion epidermal cells (Fig. 6). However, in the control experiment, MK23 transformants harbouring pGP3 (wild-type *MST12*) were rescued in appressorial penetration and plant infection (Fig. 6). These data indicate that both the homeodomain

Table 3. Wild-type strains and mutants of *Magnaporthe grisea* used in this study.

Strain	Genotype/brief description	Reference
Guy11	wild-type, *MAT1-2*	Xue *et al*. (2002)
nn78	*pmk1* deletion mutant of Guy11	Xu and Hamer (1996)
M3H51	*mps1* deletion mutant of Guy11	Xu *et al*. (1998)
DF51	*cpkA* deletion mutant of 4091-5-8	Xu *et al*. (1997)
70–15	wild-type, *MAT1-1*	Xue *et al*. (2002)
MK23	*mst12* deletion mutant of Guy11	Park *et al*. (2002)
WT5	MK23 transformed with pGP3 (wild-type *MST12*)	This study
HD3	MK23 transformed with pGP66 (Δ homeodomain)	This study
ZD7	MK23 transformed with pGP68 (Δ zinc fingers)	This study
MD4	MK23 transformed with pGP14 (Δ middle region)	This study
MPK8	MK23 transformed with pGP37 (*mst12*^{T343V})	This study
CPK3	MK23 transformed with pGP39 (*mst12*^{S421G})	This study

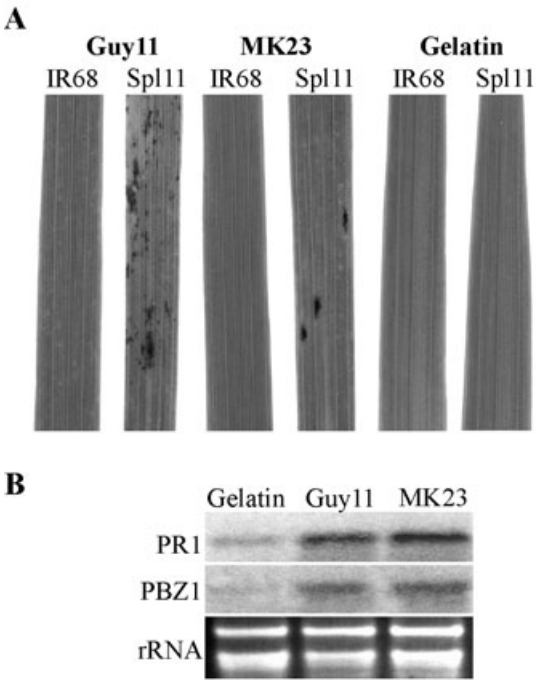

Fig. 5. Rice infection assays.
A. Leaves of rice cultivar IR68 and lesion mimic mutant *spl11* inoculated with Guy11 or MK23. MK23 failed to cause lesions on IR68 and produced fewer lesion-mimic lesions than Guy11 on *spl11* leaves.
B. Northern blot analyses of the expression of rice *PR1* and *PBZ1* genes in CO-39 seedlings inoculated with Guy11 or MK23. RNAs were isolated from rice leaves collected at 48 h post-inoculation. The same blot was hybridized with *PR1* then stripped and re-hybridized with *PBZ1*.

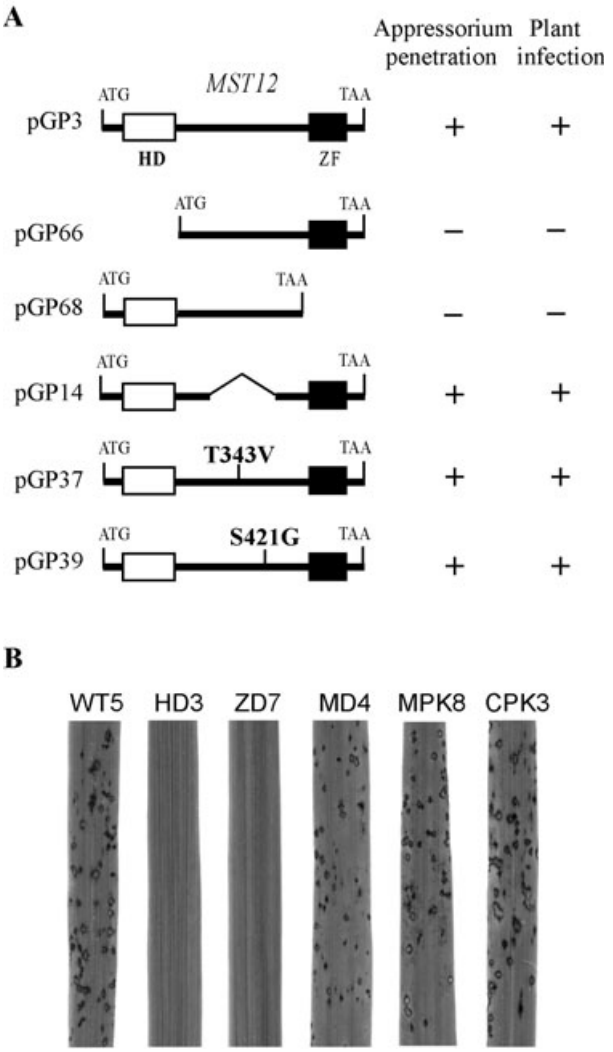

Fig. 6. Functional characterization of different regions of the *MST12* gene.
A. *MST12 has* one N-terminal homeodomain (HD) and two C-terminal zinc finger domains (ZF). Amino acid residues 2–205, 306–461, and 566–685 containing the HD, middle region, and ZF, respectively, were deleted in the *MST12* deletion constructs pGP66, pGP14, and pGP68. Site-directed mutagenesis was used to create the *mst12*^{T343V} and *mst12*^{S421G} mutant construct pGP37 and pGP39. All these *MST12* deletion or site-specific mutant constructs had the same native *MST12* promoter and terminator sequences (present on pGP3) and were transformed in to the *mst12* mutant MK23. Appressorial penetration of resulting transformants was assayed on onion epidermal cells. '–' indicates that no penetration was observed in over 100 appressoria examined for any of the transformants. For rice infection assays with CO-39 seedlings, '–' indicates that no lesion was observed on any of the infected plants in three independent infection assays with each transformant.
B. Rice leaves sprayed with conidia from transformants of MK23 expressing the wild-type *MST12* allele or mutant *MST12* alleles carried by pGP66 (HD3), pGP68 (ZD7), pGP14 (MD4), pGP37 (MPK8), and pGP39 (CPK3). Photos were taken 7 days after inoculation.

and the zinc finger domains are essential for the function of *MST12* in *M. grisea*.

Amino acid sequences between the homeodomain and zinc finger domains also are well conserved among *MST12* homologues from filamentous ascomycetes (Vallim *et al*., 2000; Borneman *et al*., 2001; Park *et al*., 2002). T343 and S421 were the putative MAP kinase and PKA phosphorylation sites identified in the middle region of *MST12* and its homologues from *A. nidulans* and *P. marneffei*. To determine the function of these two putative phosphorylation sites, we generated *mst12*^{T343V} and *mst12*^{S421G} mutant alleles and transformed them into MK23. We examined over eight transformants each expressing the *mst12*^{T343V} or *mst12*^{S421G} mutant allele, including transformants MPK8 and CPK3 (Table 3). None of them exhibited obvious defects in appressorial penetration and plant infection (Fig. 6), indicating that these two putative phosphorylation sites are not involved in the activation of *MST12*. We also generated a *MST12* mutant construct (pGP14) deleted of amino acid residues 306–461 and transformed it into MK23. All six transformants harbouring pGP14 (represented by MD4, Table 3) were normal in appressorial penetration and plant infection. No obvious difference in virulence was

observed between transformants MD4 and WT5 expressing the wild-type *MST12*. These data indicate that deletion of this middle region has no effect on the function of *MST12*.

The mst12 *mutant is defective in microtubule reorganization in mature appressoria*

To examine microtubule structures during appressorium formation, a β-tubulin-GFP fusion construct pMF309 was transformed into Guy11 and MK23. GT4 and MT6 were transformants derived from Guy11 and MK23, respectively, and confirmed to have a single copy of pMF309 by Southern blot analysis (data not shown). GFP-labelled microtubules were observed in vegetative hyphae, conidia, and germ tubes of GT4 and MT6 (Fig. 7A). Treatment with benomyl, a fungicide that inhibits microtubule polymerization, destabilized the fluorescent microtubules (Fig. 7A), indicating that β-tubulin-GFP fusion proteins were incorporated into microtubules. Both GT4 and MT6 were normal in growth and conidiation, and GT4 had no defect in plant infection (data not shown). Therefore, expression of the β-tubulin-GFP construct had no adverse effect on growth and pathogenesis in *M. grisea*.

Cortical microtubules were observed in both GT4 and MT6 during the early stages of appressorium development. Similar networks of microtubules sensitive to benomyl treatment were present in young appressoria (6 h) formed on heat-killed onion epidermal cells in both GT4 and MT6 (Fig. 7B). However, differences in microtubule structures were observed between mature appressoria formed by GT4 and MT6. In GT4, the majority of appressoria (57.3 ± 4.2%) examined at 24 h contained normal microtubule arrays, but they also developed a few microtubule bundles that were noticeably resistant to benomyl treatment (Fig. 7B). In MT6 at 24 h, over 50% (56.1 ± 0.8%) of appressoria developed fluorescent spherical bodies that lacked normal microtubules (Fig. 7B). These spherical bodies were insensitive to benomyl treatment and were not observed in appressoria formed by GT4 (Fig. 7B and C). When mature appressoria of GT4 were examined by confocal laser-scanning microscopy, individual microtubules were often observed in consecutive optical sections, suggesting that they were vertically oriented in appressoria. A three-dimensional reconstruction of these images also indicated the presence of vertical microtubules (Fig. 7C). Similar vertical microtubule arrays were not observed in mature appressoria formed by MT6. These data indicate that deletion of *MST12* disturbed microtubule dynamics in mature appressoria and possibly caused defects in cytoskeletal reorganization associated with penetration peg formation.

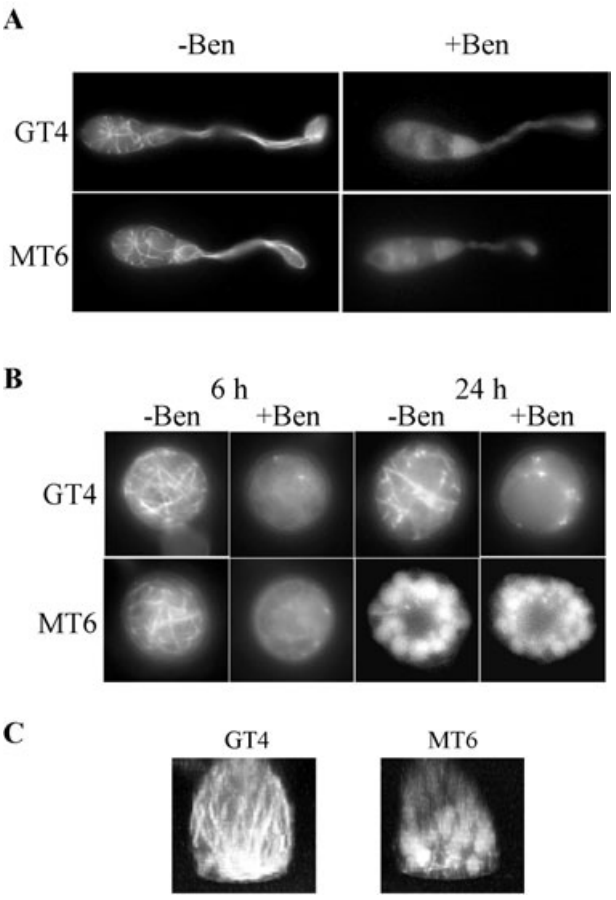

Fig. 7. Microtubule structures during appressorium formation.
A. Conidia from transformants of Guy11 (GT4) and MK23 (MT6) expressing a β-tubulin-GFP fusion construct were germinated on glass coverslips for 2 h. Green fluorescent microtubules were observed in conidia and germ tubes without benomyl treatment (–ben) but not in samples treated with benomyl (+ben).
B. Fluorescent microtubules sensitive to benomyl were observed in most young (6 h) or mature (24 h) appressoria in GT4. In MT6, fluorescent microtubule arrays were present in young appressoria (6 h) but significantly reduced in mature appressoria (24 h). GFP signals were located primarily in spherical fluorescent bodies unique to MT6 in the majority of mature appressoria.
C. Mature appressoria (24 h) formed by GT4 and MT6 were examined by confocal microscopy. The y-z views of the middle sections of confocal images of the appressorium formed by GT4 or MT6 were analysed with the MetaMorph software. Fluorescent vertical microtubules and spherical bodies were visible in GT4 and MT6, respectively.

Discussion

Many plant pathogenic fungi develop appressoria that function to penetrate plant cells. In *M. grisea*, the elevated osmotic pressure within appressoria is used to puncture leaf cuticles and plant cell walls (Howard *et al*., 1991; de Jong *et al*., 1997). The *mst12* deletion mutant forms normal appressoria but is unable to penetrate rice plants or invade through wounds (Park *et al*., 2002). However, several lines of evidence from this study indicate that *MST12* is not essential for generation of appressorium turgor pressure. First, the *mst12* mutant, in contrast to the *cpkA*

mutant that is defective in turgor generation (Thines *et al*., 2000), was able to mobilize and degrade glycogen and lipid bodies for turgor generation (Fig. 1). Appressoria formed by the *mst12* mutant also had normal morphology and ultrastructure (Fig. 2). In addition, the appressorium turgor pressure in MK23 estimated by the cytorrhysis assay was similar to that in Guy11. Thus, the *mst12* mutant is capable of generating normal turgor but appressorium turgor pressure apparently is not sufficient for penetration. Several *M. grisea* mutants, including *mps1* (Xu *et al*., 1998) and *pls1* (Clergeot *et al*., 2001), are known to be normal in appressorium formation and maturation but fail to penetrate plant cells. The *cst1* mutant of *Colletotrichum lagenarium* is also defective in penetration, although it has faster lipid metabolism in appressoria compared to the wild type (Tsuji *et al*., 2003).

One critical step in appressorium-mediated penetration is the development of the penetration peg, which breaches the plant cuticle and then differentiates into infectious hyphae. Although appressoria formed by MK23 on cellophane membranes developed normal appressorium pores, penetration pegs were not observed in TEM examinations (Fig. 2B). The *mst12* mutant also failed to differentiate penetration pegs on onion or barley epidermal cells (Fig. 3), indicating that the defect of the *mst12* mutant in appressorial penetration is related to its inability to develop penetration pegs. Appressorium turgor generation and penetration peg formation are therefore likely to be two independent processes regulated by different genetic mechanisms in *M. grisea*. *MST12* may play critical roles in regulating the emergence of penetration pegs and directing the physical force exerted by appressorium turgor. In *S. cerevisiae*, the pheromone response pathway and the cell polarity-establishment complex are involved in regulating shmoo formation (Madden and Snyder, 1998; Farley *et al*., 1999). Shmoos are partially polarized yeast cells that project towards one another during mating. Many parallels can be drawn between shmoo formation and penetration peg development in *M. grisea*. It is likely that *MST12* functions downstream from the *PMK1* MAP kinase in *M. grisea* (Park *et al*., 2002) for regulating appressorium-mediated penetration.

Appressoria formed by *mst12* failed to elicit localized plant defence responses, including papilla formation, autofluorescence, and actin cytoskeleton reorganization in underlying plant cells (Figs 3 and 4), suggesting that appressorium development is not sufficient for triggering localized plant defence responses in the plant cells directly below the appressoria formed by *M. grisea*. In a previous study, we reported that the *M. grisea mps1* mutant is defective in appressorial penetration but elicits defence responses in underlying plant cells (Xu *et al*., 1997; 1998). Close examinations, however, indicated that appressoria formed by the *mps1* mutant, different from those of the *mst12* mutant, were able to develop penetration pegs and trigger papilla formation (Figs 3 and 4). Thus, the *mst12* mutant appears to be unique in its inability to develop penetration pegs and to trigger localized plant cell defence responses. However, the *mst12* mutant still elicited lesion mimic reactions and the induction of defence response genes. These data suggest that the emergence of the penetration peg is essential for triggering localized plant defence responses in plant cells lying underneath appressoria formed by *M. grisea*. However, chemical or enzymatic factors produced by germlings are responsible for the induction of plant defence genes and lesion mimic reactions in rice leaves, and their effects are likely not limited to the cells directly below appressoria formed by *M. grisea*.

In addition to the N-terminal homeodomain that is highly conserved among all known Ste12 homologues, *MST12* also contains two tandem zinc finger domains near the C-terminus (residues 566–611) that are conserved only in *STE12* homologues from filamentous fungi (Vallim *et al*., 2000; Borneman *et al*., 2001; Park *et al*., 2002; Tsuji *et al*., 2003). While the homeodomain has been shown to be involved in DNA binding in *S. cerevisiae* (Yuan *et al*., 1993), the function of the zinc domains in *MST12* and its homologues is not clear. *STE12* from *S. cerevisiae* and its homologues from other ascomycetous yeasts lack the zinc finger domain. Our data indicate that both the homeodomain and zinc finger domains are essential for the function of *MST12* in appressorial penetration and infectious growth. Zinc finger is a common DNA binding domain (Evans and Hollenberg, 1988; Mackay and Crossley, 1998), but it has also been shown to be involved in RNA-binding and protein–protein interactions (Klug and Rhodes, 1987; Merika and Orkin, 1995; Sun *et al*., 1996). It will be important to further characterize the function of the homeodomain and zinc finger domain in *MST12*. Although *MST12* lacks any sequence homology with *STE12* over the region downstream from the homeodomain that interacts with *KSS1* and other proteins in yeast (Liu *et al*., 1994; Pi *et al*., 1997; Bardwell *et al*., 1998), amino acid sequences between the homeodomain and zinc finger domains are well conserved among *MST12* homologues from filamentous ascomycetes (Park *et al*., 2002). Surprisingly, transformants expressing the *mst12*^{T343V} or *mst12*^{S421G} mutant allele or the *MST12* construct deleted of amino acid residues 306–461 were phenotypically identical to transformants expressing the wild-type *MST12* allele (Fig. 6). It appears that this conserved middle region is either dispensable for the function of *MST12* or plays a negative regulatory role in its activation during appressorium formation and plant infection in *M. grisea*. However, no obvious increase in virulence was observed in transformants expressing pGP37, pGP39, or pGP14.

The development and organization of microtubule cytoskeleton during appressorium formation and penetration processes have not been examined closely in plant pathogenic fungi. During the early stages of appressorium formation by *C. lagenarium*, microtubules were found to be oriented randomly in appressoria expressing a GFP-tagged α-tubulin construct (Takano *et al*., 2000), but appressoria were not examined in later stages of formation or during penetration. We examined microtubule structures in appressoria to determine the possible links between defects in penetration peg formation of the *mst12* mutant and cytoskeleton rearrangements. Although young appressoria of Guy11 and MK23 had a similar network of microtubules (Fig. 7A), mature appressoria displayed different microtubule structures. The wild-type strain developed vertically oriented microtubule arrays that were unique to appressoria (Fig. 7C). These vertical microtubules, distinct from cortical microtubules seen in other stages of development, were formed only in mature appressoria and may be involved in penetration peg formation. Mature appressoria of the *mst12* mutant formed fluorescent spherical bodies (Fig. 7) that were insensitive to benomyl treatment and likely contained pools of β-tubulin monomers or α/β-heterodimers, indicating that *MST12* may control microtubule rearrangements associated at late stages of appressorium formation.

In *M. grisea*, cytoskeletal elements may be involved in the re-establishment of polarized growth and selection of the site for penetration peg formation in mature appressoria. High concentrations of actin filaments have been observed in penetration pegs (Bourett and Howard, 1992). Similar to *STE12* in *S. cerevisiae* (Zeitlinger *et al*. 2003), *MST12* may regulate genes associated with cytoskeletal rearrangements in *M. grisea* during appressorium-mediated penetration. Our preliminary data with *M. grisea* transformants expressing an endogenous actin gene tagged with EGFP indicated that the *mst12* mutant is defective in actin filament aggregation at the site where the penetration peg emerges in the wild-type strain. It will be important to identify genes regulated by *MST12* and further characterize roles of actin and microtubule cytoskeleton rearrangements during appressorium-mediated penetration. *GAS1* and *GAS2* are two genes specifically expressed in appressoria (Xue *et al*., 2002). Our preliminary data indicate that the expression of *GAS1* and *GAS2* is normal in the *mst12* deletion mutant (data not shown), indicating that these two genes are not regulated by *MST12*.

Experimental procedures

Strains and growth media

The wild-type strain Guy11, *mst12* mutant MK23 (Park *et al*., 2002), *cpka* mutant DF51 (Xu *et al*., 1997), *mps1* mutant M3H51 (Xu *et al*., 1998) and all the transformants (Table 3) were cultured on oatmeal agar plates (Xu and Hamer, 1996). Media were supplemented with 250 μg ml^{-1} hygromycin B or 200 μg ml^{-1} zeocin for selecting hygromycin-resistant and zeocin-resistant transformants, respectively. The rice cultivar IR68 and the *spl11* lesion mimic mutant (Yin *et al*., 2000) were provided by Dr Guoliang Wang at Ohio State University.

Appressorium turgor and penetration assays

Conidia harvested from 2-week-old oatmeal cultures were used for appressorium formation and infection assays as previously described (Park *et al*., 2002). Glycogen bodies and lipid droplets were stained with KI/I_2 and Nile Red, respectively, and examined by epifluorescent microscopy as previously described (Thines *et al*., 2000). Appressorium turgor was examined with the incipient-cytorrhysis technique using 30% and 40% aqueous solutions of PEG-8000 (Howard *et al*., 1991). Appressorial penetration assays with onion and barley epidermal cells were performed as previously described (Xu *et al*., 1997; Clergeot *et al*., 2001). Three-week-old seedlings of rice cultivar CO-39 were used for penetration assays with leaf sheath epidermis as described (Koga, 1994). Infectious hyphal growth, host cell autofluorescence, and papilla formation were examined after 48–72 h of incubation at room temperature. Actin filaments in onion epidermal cells were stained with rhodamine-phalloidin (Molecular Probes) as described previously (Xu *et al*., 1998). For plant infection assays, seedlings of 3-week-old rice and 10-day-old barley were sprayed with conidial suspensions of 10^5 conidia ml^{-1} in 0.2% gelatin solution. Plant incubation, inoculation, and lesion examination were as described (Valent *et al*., 1991; Xu *et al*., 1997). RNA was extracted from infected rice leaves collected at 48 h post-inoculation with the TRIzol reagent (Invitrogen) according to instructions provided by manufacturer. Northern blot hybridizations with the rice *PR1* and *PBZ1* genes were performed as described previously (Yin *et al*., 2000).

Examination of appressoria by scanning and transmission electron microscopy

Appressoria formed on autoclaved cellophane membranes by 48 h were examined by scanning and transmission electron microscopy (SEM and TEM). For SEM, samples were frozen with liquid nitrogen and sputter-coated with gold in the presence of argon in a Hexland CT-1000 cryo-system (Gatan). Appressoria were examined at −140°C in a JEOL JSM-840 scanning electron microscope. For TEM, appressoria were fixed, dehydrated, and embedded as described previously (Bourett and Howard, 1990). Serial thin-sections were removed onto slot grids (Sherman and Krause, 1990) and examined with a Philips/FEI CM-100 transmission electron microscope (FEI Company).

Examination of microtubule structures with GFP-tagged β-tubulins

The plasmid pMF309 containing the *Neurospora crassa* β-tubulin gene fused in-frame to EGFP was provided by Dr

Michael Freitag at University of Oregon. It was introduced into Guy11 and MK23 by cotransformation with pAC905 as described (Park *et al*., 2002). Monoconidial cultures of the resulting transformants expressing β-tubulin-GFP fusion proteins were analysed by Southern blot hybridization. Appressoria formed on glass coverslips or onion epidermal strips were examined with a Nikon E800 epifluorescence microscope and a MRC Bio-Rad 2100 confocal laser scanning microscope (Bio-Rad). Confocal images were analysed with the MetaMorph software (Universal Imaging).

Construction of domain deletion and point mutation alleles of MST12

The full length *MST12* gene and its 2.5 kb upstream and 0.3 kb downstream flanking sequences were cloned as a 5.0 kb *Sma*I fragment from cosmid 10P16 (Park *et al*., 2002) between the *Eco*RV and *Sma*I sites on pYK11 as pGP3. pYK11 was constructed by cloning the bleomycin-resistance gene from pAC905 (Zheng *et al*., 2000) into *Xmn*I site on pBCKS (Stratagene). To delete the homeodomain, pGP3 was digested with *Aat*II and *Bst*PI and ligated with a DNA fragment that was amplified with PCR primers F71A (5′-CTTGACGTC ATGTTGACCAAGGCCCAACAA-3′) and R44B (5′-CGCGG ATTCGTAAACATTGTTTGACCC-3′) and digested with *Aat*II and *Bst*PI. In the resulting construct pGP66, the *MST12* gene was still expressed under its native promoter but deleted of amino acid residues 2–205 (Fig. 6). The *MST12* alleles deleted of the zinc finger domains and the middle region between the homeodomain and zinc finger domains were constructed with similar approaches. In pGP68, the zinc finger domains (residues 566–685) were deleted by cloning a 610 bp fragment amplified with primers F46 (5′-TTTAGCGG ATGGCCTGGA-3′) and R64B (5′-CGCGGATCCTTATCC CTCGGCAACGGGACCGA-3′) between the *Bst*PI and *Bam*HI sites on pGP3. In pGP14, the middle region (residues 306–461) was deleted by cloning a PCR product amplified with primers F43B (5′-ACAGGTCACCACGAGTCGCTCGAC AACT-3′) and R40P (5′-AACCTGCAGTTGTTCAAGGTTGAC ATTGT-3′) between the *Bst*PI and *Sph*I sites on pGP3. The *MST12* site-directed mutagenesis constructs pGP37 and pGP39 were generated in pGP3 with the QuikChange II Site-Directed Mutagenesis kit (Stratagene) following the instructions provided by the manufacturer. In pGP37 (*mst12*^{T343V}), the putative MAP kinase phosphorylation site PLTP (residues 341–344) was changed to PLVP by changing the nucleotide sequence TCTCACA to ACTAGTA (creating a *Spe*I site). In pGP39 (*mst12*^{S421G}), the putative PKA phosphorylation site RRRS (residues 418–421) was changed to RRRG by changing the nucleotide sequence TCATCA to GGATCC (introducing a *Bam*HI site).

All PCR reactions were performed with *pfu* DNA polymerase (Stratagene). The wild-type *MST12* and all deletion or site-specific mutation constructs were confirmed by sequencing and transformed into the *mst12* deletion mutant MK23 (Park *et al*., 2002). Transformants resistant to 200 μg ml^{-1} zeocin were isolated and confirmed by Southern blot analysis. WT5, HD3, ZD7, MD4, MPK8, and CPK3 are randomly selected transformants containing a single copy of pGP3, pGP66, pGP68, pGP14, pGP37, and pGP39, respectively (Table 3).

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