The signalling mucin Msb2 regulates surface sensing and host penetration via BMP1 MAP kinase signalling in *Botrytis cinerea*

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SUMMARY

Botrytis cinerea is a necrotrophic fungus that infects a wide range of fruit, vegetable and flower crops. Penetration of the host cuticle occurs via infection structures that are formed in response to appropriate plant surface signals. The differentiation of these structures requires a highly conserved mitogen-activated protein (MAP) kinase cascade including the MAP kinase BMP1. In yeast and several plant-pathogenic fungi, the signalling mucin Msb2 has been shown to be involved in surface recognition and MAP kinase activation. In this study, a *B. cinerea* *msb2* mutant was generated and characterized. The mutant showed normal growth, sporulation, sclerotia formation and stress resistance. In the absence of nutrients, abnormal germination with multiple germ tubes was observed. In the presence of sugars, normal germination occurred, but *msb2* germlings were almost unable to form appressoria or infection cushions on hard surfaces. Nevertheless, the *msb2* mutant showed only a moderate delay in lesion formation on different host plants, and formed expanding lesions similar to the wild-type. Although the wild-type showed increasing BMP1 phosphorylation during the first hours of germination on hard surfaces, the phosphorylation levels in the *msb2* mutant were strongly reduced. Several genes encoding secreted proteins were found to be co-regulated by BMP1 and Msb2 during germination. Taken together, *B. cinerea* Msb2 is likely to represent a hard surface sensor of germlings and hyphae that triggers infection structure formation via the activation of the BMP1 MAP kinase pathway.

Keywords: MAPK, signalling, surface sensing, virulence.

INTRODUCTION

Although plant-pathogenic fungi have evolved diverse strategies of infection, the early stages of host invasion are often similar, involving the formation of appressoria and the use of osmotic force and lytic enzymes to penetrate host cell walls. The appropriate timing of germination and location of the penetration site is achieved by the perception of various host-derived signals. Both

physical signals, such as the hardness, topography and hydrophobicity of the plant surface, and chemical signals, including cuticular waxes, cutin monomers and various nutrients, have been described to trigger germination, appressorium formation and host invasion (Doehlemann *et al.*, 2006; Mendgen *et al.*, 1996; Tucker and Talbot, 2001). How these stimuli are perceived by the spores and the germ tubes of the fungi is still poorly understood. Much better characterized are the signal transduction pathways that control pathogenic differentiation after the perception of host stimuli. An essential component for the pathogenesis of all fungi investigated so far is a conserved mitogen-activated protein kinase (MAPK) cascade homologous to the filamentous growth pathway Kss1 MAPK cascade in yeast. Mutants in this pathway have either lost or are severely hampered in pathogenesis (Zhao *et al.*, 2007).

Botrytis cinerea is a ubiquitous necrotroph that causes grey mould rot in a large number of plants, leading to severe damage on fruits and vegetables (Dean *et al.*, 2012). Several components of the *B. cinerea* MAPK pathway have been analysed for their role in different stages of pathogenesis. Mutants in genes encoding the MAPK BMP1, the MAPK kinase (MAPKK) Ste7, the MAPKK kinase (MAPKKK) Ste11 and the putative MAPK adapter protein Ste50 were all non-pathogenic and showed defects in germination, surface sensing of germ tubes, vegetative growth and sclerotia formation (Doehlemann *et al.*, 2006; Schamber *et al.*, 2010; Zheng *et al.*, 2000). In addition, the transcription factor Ste12, a putative downstream component of the BMP1 MAPK cascade, was analysed. Similar to its role in other plant-pathogenic fungi, Ste12 was found to be required for appressoria-mediated penetration (Schamber *et al.*, 2010; Wong Sak Hoi and Dumas, 2010). In addition to the MAPK pathway, other signal transduction components, including the G-protein subunits G α 1, G α 3 and G β 1, the cyclic adenosine monophosphate (cAMP)-dependent pathway and the Ca²⁺/calciurein calcium-dependent pathway, are also required for the pathogenic differentiation of *B. cinerea* (Harren *et al.*, 2012; Schumacher *et al.*, 2008).

The germination of *B. cinerea* conidia on glass surfaces coated with plant surface waxes is accompanied by major shifts in gene expression (Leroch *et al.*, 2013). The greatest change in the transcriptome profile is observed in the pre-germination stage, within the first hour of incubation. A high proportion of genes induced during germination and appressorium differentiation encode secreted proteins, including lytic enzymes. Interestingly,

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many germination-induced genes are not activated in germlings of the *bmp1* mutant, which fails to form appressoria. The defect of the *bmp1* mutant in infection-related morphogenesis and gene expression appears to be linked to impaired host surface sensing. In *Saccharomyces cerevisiae*, activation of the filamentous growth MAPK cascade and the capability for invasive growth are mediated by the signalling mucins Msb2 and Hkr1, two plasma membrane-anchored proteins containing large, extracellular, heavily O-glycosylated domains. Furthermore, Msb2 and the plasma membrane-spanning protein Sho1 have been shown to interact as stress sensors that regulate signalling cascades involved in the response to osmotic stress (Chen and Thorner, 2007). In the plant pathogens *Ustilago maydis*, *Magnaporthe oryzae* and *Fusarium oxysporum*, homologues of yeast Msb2 and Sho1 have been identified as putative sensor proteins involved in plant surface recognition, MAPK activation and pathogenesis (Fernández-Alvarez *et al.*, 2012; Lanver *et al.*, 2010; Liu *et al.*, 2011; Perez-Nadales and Di Pietro, 2011). In this study, we provide evidence that, in *B. cinerea*, Msb2 acts as a surface sensor, which is required for germ tubes to differentiate appressoria on hard surfaces. Msb2 seems to act via the BMP1 MAPK, but the role of O-glycosylation for its function remains unclear.

RESULTS

Identification of an *msb2*-like gene in *B. cinerea* and construction of *msb2* mutant strains

A BLASTP search in the National Center for Biotechnology Information (NCBI) protein database with the *S. cerevisiae* Msb2 protein as a query revealed low levels of homology with a 1620-amino-acid protein from *B. cinerea* strain B05.10, encoded by a gene (B0510_3063) containing no intron. This protein shares typical structural features of a signalling mucin, including a signal peptide, an extended extracellular serine/threonine-rich region and a single transmembrane domain close to the C-terminus. Alignment with other fungal Msb2 sequences revealed homology in the C-terminal region, with highest levels of identity at the C-terminus. The mucin domain of *B. cinerea* Msb2 is longer than that of other fungi, and lacks repeats that are found in *M. oryzae* and *F. oxysporum* Msb2 proteins (Fig. 1).

To investigate the function of Msb2, a mutant lacking the *msb2* coding region was constructed (Fig. S1, see Supporting Information). To confirm that the observed phenotypes are caused by the loss of *msb2*, the *msb2* mutant was re-transformed with a full-length wild-type *msb2* copy, resulting in the complemented strain *msb2*-C. Furthermore, an *msb2* mutant strain expressing the enhanced green fluorescent protein (eGFP), named *msb2*-GFP, was generated, which showed a phenotype identical to the *msb2* mutant (not shown). Because Msb2 has been shown to be located upstream of the filamentous MAPK pathway in yeast and other

fungi, the *bmp1* MAPK mutant was included in some experiments for comparison. The *B. cinerea* strains used in this study are listed in Table 1.

The *msb2* mutant shows normal growth and stress responses

Mycelial growth of the wild-type, *msb2* mutant and *bmp1* mutant was compared on agar plates containing different media. The growth rates of the wild-type and *msb2* mutant were not significantly different on full or minimal medium, and in the presence of 0.02% sodium dodecylsulfate (SDS), 10 mM H₂O₂, 1 M sorbitol, 1 M NaCl or the cell wall inhibitors Calcofluor white (100 µg/mL) and Congo red (250 µg/mL) (Fig. S2, see Supporting Information). In contrast, growth of the *bmp1* mutant was significantly retarded on all media.

The *msb2* mutant shows normal germination kinetics, but is defective in the formation of penetration structures

The germination of conidia was tested on different artificial surfaces. On a hydrophilic glass surface and a surface coated with either plant cuticular wax or the cutin monomer trihydroxypalmitic acid (THPA), in the presence of fructose, germination of the *msb2* mutant was slightly retarded during the first hours of incubation, but germination rates resumed wild-type levels after longer incubation (Fig. 2). As shown previously, plant waxes lead to an accelerated germination (Leroch *et al.*, 2013). This effect was also observed for the *msb2* mutant, which indicates that Msb2 is not involved in the sensing of these plant lipids. On hydrophobic surfaces in pure water, the germination rate of the *msb2* mutant was somewhat lower than that of the wild-type after 18 h (Fig. S3, see Supporting Information). As described previously, the *bmp1* mutant showed a significant delay in germination under these conditions, and failed to germinate on hydrophobic surfaces in the absence of a carbon source (Doehlemann *et al.*, 2006; Leroch *et al.*, 2013).

Despite almost normal kinetics of germination, *msb2* germlings revealed an aberrant phenotype in pure water without nutrients. After overnight incubation, they formed up to four, or sometimes even five, germ tubes, whereas only a single germ tube was observed for most wild-type germlings (Figs 3A and S4, see Supporting Information). Video imaging revealed that the *msb2* germ tubes emerged one after the other (not shown), indicating that the first germ tube was less able to inhibit the emergence of secondary germ tubes. Multiple germ tube formation was suppressed when *msb2* mutant conidia were germinated in the presence of fructose or glucose. In sugar-containing germination medium, wild-type germ tubes formed appressoria-like thickenings within the first few hours (Leroch *et al.*, 2013). In contrast, germlings of the *msb2* mutant continued to elongate and usually failed to form

Fig. 1 Homology regions and structure of Msb2 proteins of *Botrytis cinerea* (Bc; B0510_3063), *Fusarium oxysporum* (Fo; FOXG_09254.3) and *Magnaporthe oryzae* (Mo; MGG_06033). (A) Alignment of the conserved C-terminal regions. (B) Domain structure of the three Msb2 proteins. Pro, proline. (C) Prediction of O-glycosylation sites (NetOGly 3.1). The black bars show the O-glycosylation potential for each Ser- and Thr residue in the sequence. The horizontal dotted line indicates the threshold of significance.

Table 1 *Botrytis cinerea* strains used in this study. All strains are transformants derived from the wild-type strain B05.10.

Strain	Description	Reference
<i>msb2</i>	<i>msb2</i> deletion mutant	This work
<i>msb2</i> -C	Complemented <i>msb2</i> mutant	This work
<i>msb2</i> -GFP	<i>msb2</i> deletion mutant expressing <i>oliC</i> -GFP	This work; Leroch <i>et al.</i> (2011)
Bcche-1	Strain expressing <i>mCherry</i>	Leroch <i>et al.</i> (2011)
<i>bmp1</i>	<i>bmp1</i> deletion mutant	Doehlemann <i>et al.</i> (2006)
<i>pmt1</i>	<i>pmt1</i> deletion mutant	González <i>et al.</i> (2013)
<i>pmt2</i>	<i>pmt2</i> deletion mutant	González <i>et al.</i> (2013)
<i>pmt4</i>	<i>pmt4</i> deletion mutant	González <i>et al.</i> (2013)

appressoria. The same behaviour was observed on the cuticle of a host plant (Fig. 3B). Quantitative examination revealed a very low rate of appressorium formation by *msb2* germings (Table S1, see Supporting Information). This provides a likely explanation for the delayed penetration of intact leaf tissue by the *msb2* mutant (see below). In addition to simple appressoria formed by germ tubes,

B. cinerea can form alternative penetration structures originating from hyphae, called complex appressoria or infection cushions (Choquer *et al.*, 2007). In contrast with the wild-type, the *msb2* and *bmp1* mutants were unable to form infection cushions (Fig. 3C; not shown). Collectively, these data demonstrate that the *msb2* mutant shows almost normal germination, but is defective in hard surface recognition and the formation of penetration structures by the germ tubes.

Conidial anastomosis tubes (CATs) are observed in several fungi as fusion events between germinating spores. In *Neurospora crassa*, the filamentous growth MAPK has been shown to be required for CAT formation (Read *et al.*, 2009). In the *B. cinerea* *msb2* mutant, CAT formation was found to be reduced compared with the wild-type, and the *bmp1* mutant was unable to form any CATs. Interestingly, the frequency of CAT formation by the *ste12* mutant, which is deleted in the BMP1-dependent transcription factor Ste12 (Schamber *et al.*, 2010), was about two-fold higher than in the wild-type (Fig. S5, see Supporting Information).

Fig. 2 Germination rates of conidia of *Botrytis cinerea* wild-type (WT), *msb2* and *bmp1* mutants in GB5 medium containing 10 mM fructose. (A) Hydrophilic glass surface. (B) Glass coated with apple wax (AW). (C) Glass coated with the cutin monomer trihydroxypalmitic acid (THPA). Asterisks indicate significant differences between values obtained for the mutants compared with the WT: **P* = 0.05; ***P* = 0.01; ****P* = 0.001.

Botrytis cinerea *bmp1* mutants are unable to form sclerotia and therefore are female sterile (Doehlemann *et al.*, 2006). When the *msb2* mutant was grown on HA agar medium in the dark for 16 days, it formed sclerotia in similar numbers to the wild-type and the complemented *msb2* mutant (data not shown).

The *msb2* mutant shows delayed, but otherwise normal, host infection

Infection tests were performed on detached tomato leaves and *Gerbera* flower petals, and on wounded apples. On tomato leaves and *Gerbera* petals, inoculation with the wild-type and the complemented *msb2*-C strain resulted in the formation of primary lesions within 24 h, and secondary expanding lesions after 48 and 72 h (Fig. 4A, D). In contrast, the *msb2* mutant caused only weak or no symptoms after 24 h, but primary and sometimes starting secondary lesions after 48 h. Despite this delay, *msb2* mutant-induced lesions expanded at a similar rate to wild-type-induced lesions. Wounding or increasing the conidia concentration of the *msb2* mutant abolished the delay in lesion formation (Fig. 4B, E; data not shown). As shown previously (Doehlemann *et al.*, 2006; Schamber *et al.*, 2010), inoculation with the *bmp1* mutant caused only scattered necrotic spots under the inoculation droplets several days after inoculation. Using the histological stain for H₂O₂, diaminobenzidine (DAB), the time course of reactive oxygen formation was investigated in infected tomato leaves. Strong DAB staining was observed after 24 h in lesions caused by the wild-type and the *msb2*-C strain (Fig. 4C). Similar staining intensities were observed with a delay of 1 day in *msb2*- and 2 days in *bmp1*-inoculated leaves. During lesion expansion, the DAB staining moved towards the margins of the colonized areas, but faded to lower intensity within the necrotic area. In the small lesion caused by the *bmp1* mutant, the DAB staining appeared to be maintained at higher levels (Fig. 4C). On *Gerbera* flower petals, infection by the *msb2* mutant was also significantly delayed compared with the wild-type, and the *bmp1* mutant only occasionally induced necrotic spots after several days (Fig. 4D; not shown). On wounded apples, lesion formation by the wild-type and the *msb2*

mutant occurred with similar kinetics, whereas lesion development induced by the *bmp1* mutant was strongly retarded (Fig. 4E; not shown).

Msb2 is involved in hard surface-induced phosphorylation of BMP1

In yeast and the plant pathogens *U. maydis*, *M. oryzae* and *F. oxysporum*, *Msb2* has been identified as a putative surface sensor that activates the filamentous pathway MAPK cascade after perception of appropriate stimuli (Lanver *et al.*, 2010; Liu *et al.*, 2011; Perez-Nadales and Di Pietro, 2011). To test this regulatory connection for *B. cinerea*, phosphorylation of BMP1 was analysed in response to hard surface stimulation of germinating conidia. *Botrytis cinerea* conidia were germinated on Petri dishes coated with apple wax in fructose-containing medium, because appressorium formation occurred most rapidly and synchronously under these conditions. The detection of unphosphorylated BMP1 protein failed, although three different anti-44/42 MAPK antibodies were tested (Fig. 5; data not shown). In dormant wild-type conidia, phosphorylation of BMP1 was weak, but increased rapidly within 12 h. In contrast, only a delayed and slight increase in phosphorylation was observed in the *msb2* mutant. As expected, no BMP1 protein was detected in the *bmp1* mutant, which was included as a control (Fig. 5). In contrast with BMP1, the high osmolarity response MAPK Sak1 showed high phosphorylation levels already in dormant conidia, which decreased or remained unchanged during germination on a hard surface in the wild-type and *msb2* mutant (Fig. S6, see Supporting Information).

Many germination-induced genes encoding secreted proteins are co-regulated by BMP1 and *Msb2*

In a previous study, we have identified genes that are specifically up-regulated during conidial germination (Leroch *et al.*, 2013). A large proportion of these genes, in particular those encoding proteins with predicted N-terminal leader peptides, are only weakly expressed in the *bmp1* mutant (Leroch *et al.*, 2013). Based on microarray data, the majority of these genes also appeared to be

Fig. 3 Differentiation of *Botrytis cinerea* germlings on different surfaces (24 h). (A) Morphology of wild-type (WT), *msb2* and *bmp1* germlings on glass, polyethylene (PE), apple wax-coated glass (AW) and trihydroxypalmitic acid-coated glass (THPA). Arrows indicate appressorial swellings. Conidia were incubated in GB5 medium containing 10 mM fructose, except on PE (in water). Bars, 20 μm. (B) Scanning electron micrographs of germlings on PE in water and on a bean leaf surface in GB5 medium containing 10 mM fructose (24 h). Bars, 10 μm. (C) Formation of infection cushions by the WT, but not the *msb2* mutant. Conidia of the WT (labelled with mCherry) and *msb2* mutant [labelled with green fluorescent protein (GFP)] were mixed and incubated on a glass slide for 48 h.

down-regulated in the *msb2* mutant during germination (Table S2, see Supporting Information). To confirm this observation, the expression of six BMP1-regulated genes was analysed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

in the wild-type and *msb2* mutant. Five of these genes were also found to be down-regulated in the *msb2* mutant (Fig. 6). These data confirm that a significant number of germination-induced genes require both Msb2 and BMP1 for their expression. To test

Fig. 4 Infection of tomato leaves, *Gerbera* flowers and wounded apples by *Botrytis cinerea* wild-type (WT) and mutant strains. (A, B, D, E) Lesion development after inoculation of spore suspensions on unwounded (A) or wounded (B) tomato leaves, on *Gerbera* petals (D) and on wounded apples (E). (C) Time course of lesion development (top) and H₂O₂ formation (bottom), visualised by brown diaminobenzidine (DAB) staining, on unwounded tomato leaves.

Fig. 5 BMP1 mitogen-activated protein (MAP) kinase phosphorylation response to germination on a hard surface. No BMP1-specific signal was obtained with the 44/42 MAPK antibody. On the membrane probed with the p-44/42 MAPK antibody, the arrow indicates the BMP1-P-specific band, which is missing in the *bmp1* mutant. Numbers indicate the incubation time (h) of conidia on an apple wax-coated Petri dish. Similar results were obtained in three biological replicates. WT, wild-type.

whether the failure of the two mutants to induce many genes during germination may be a result of their inability to form appressoria, gene expression was analysed in wild-type conidia germinated on a soft agarose surface which does not induce appressorium formation. Three of the six genes tested were induced in the wild-type under these conditions, whereas another three genes were not induced. Of the latter group, one gene (B0510_10108) was up-regulated in the *msb2* mutant on a hard surface (Fig. 6).

A large number of genes that are induced during germination in a BMP1- and *Msb2*-dependent manner encode lytic enzymes that are potentially involved in the degradation of plant cell wall polymers, such as endoglucanases, pectate lyase and cutinases (Leroch *et al.*, 2013). Therefore, it was tested whether the *msb2* and *bmp1* mutants might be defective in the use of these polymers. When *B. cinerea* strains were grown in medium containing polygalacturonic acid, pectin, xylan, bovine serum albumin or carboxymethyl cellulose, no difference in growth between the wild-type and *msb2* mutant was observed. In contrast, radial growth of the *bmp1* mutant on most of these media was strongly inhibited (Fig. S7, see Supporting Information).

PMT1 and PMT4 play no obvious role in the activation of the BMP1 MAPK cascade

The first step of the protein *O*-mannosylation pathway is catalysed by protein *O*-mannosyltransferases (PMTs) which add the first mannose to hydroxyl groups of serine and threonine residues in target proteins in the endoplasmic reticulum. In fungi, three PMT subfamilies exist, called PMT1, PMT2 and PMT4. *O*-mannosylation is crucial for fungi, and no mutants defective in all *pmt* genes have

Fig. 6 Transcript levels of six germination-induced, BMP1-regulated genes in *Botrytis cinerea* wild-type (WT) and *msb2* mutant, determined by reverse transcription-polymerase chain reaction (RT-PCR). Conidia were germinated on wax-coated glass, or on wax-coated 0.5% agarose (soft), for the indicated times (h). Transcript levels are shown in logarithmic scale, relative to the levels in resting WT conidia, which were set to unity. Accession numbers for the genes are shown.

been isolated. In *U. maydis*, PMT4 has been found to be essential for pathogenesis (Fernández-Alvarez *et al.*, 2009). Further studies in this fungus have shown that Msb2 is a target of PMT4, and that *O*-glycosylation is required for Msb2 activity (Fernández-Alvarez *et al.*, 2012). In *B. cinerea*, all three *pmt* genes are required for normal growth and plant infection. Although the *pmt2* mutant showed very poor growth and failed to sporulate and to infect plant tissues, the *pmt1* and *pmt4* mutants displayed different degrees of reduced virulence (González *et al.*, 2013). *Botrytis cinerea* Msb2 contains a large number of serine and threonine residues, many of which could represent *O*-glycosylation sites, as predicted by NetOGlyc 3.1 (Fig. 1C). To reveal a possible functional connection between PMTs, Msb2 and the BMP1 MAPK cascade, a phenotypic analysis of the *pmt1* and *pmt4* mutants was performed. First, we analysed the ability of their spores to form infection structures on different surfaces (Fig. 7). In contrast with germlings of the wild-type and the *pmt1* mutant, which formed terminal appressorial thickenings on a glass surface after 24 h, the *pmt4* mutant formed multiple swollen structures, which have been described by González *et al.* (2013). On 0.5% agarose, on which wild-type and *pmt1* germlings failed to form appressoria because of the lack of a hard surface contact, the *pmt4* mutant showed a lower frequency of swellings. This finding suggested to us that the swollen structures of the *pmt4* mutant could represent malformed appressoria which are strongly suppressed on a soft surface. On glass surfaces after 48 h, both the wild-type and *pmt1* mutant formed infection cushions, although those of the *pmt1* mutant appeared with a slight delay. The *pmt4* mutant also formed infection cushion-like structures, but these showed an aberrant morphology. The *pmt4* mutant has been found by González *et al.* (2013) to be strongly impaired in penetration and host invasion

after inoculation with mycelium on agar discs. For better comparison with the *msb2* and *bmp1* mutants, we re-investigated the mutants using spore suspensions for the penetration and infection tests. Although the wild-type and *pmt1* mutant showed similar multiple appressorial penetrations into onion epidermal cells, the penetration frequency of the *pmt4* mutant was strongly reduced, but some successful penetrations were also observed. After penetration, the *pmt4* hyphae appeared to grow normally and lacked the swellings observed with germlings (Fig. 7). However, on detached tomato leaves, infection by the *pmt1* mutant was strongly retarded, whereas only a slight delay in lesion development was observed with the *pmt4* mutant (Fig. S8A, see Supporting Information). Because this result was different to that reported by González *et al.* (2013), we confirmed the genetic identity of the two mutants by RT-PCR analysis (Fig. S8B). In summary, the phenotypes of the *pmt* mutants showed distinct differences from those of the *msb2* and *bmp1* mutants. The formation of multiple swollen structures on glass surfaces, which might represent malformed appressoria, indicated the possibility of hyperactivation of the BMP1 MAPK cascade, similar to the situation in *S. cerevisiae*, in which defective glycosylation of Msb2 caused by disruption of the gene encoding PMT4 leads to the overactivation of the Kss1 MAPK (Yang *et al.*, 2009). However, BMP1 phosphorylation studies performed with spores germinated on a hard surface revealed no obvious differences between the wild-type and the *pmt1* and *pmt4* mutants (Fig. S9, see Supporting Information). Taken together, in contrast with the situation in other fungi, these results provide no evidence for a role of PMT1 and PMT4 in the surface sensing ability of Msb2 and the activation of the BMP1 MAPK pathway during appressorium formation in *B. cinerea*.

Fig. 7 Differentiation of *Botrytis cinerea* wild-type (WT), *pmt1* and *pmt4* conidia on different surfaces. Appressorium formation was analysed on glass slides and (soft) 0.5% agarose after 24 h, and infection cushion formation on glass slides after 48 h. Penetration of heat-killed onion epidermal cells was investigated after 16 h. External fungal structures are stained dark with trypan blue, whereas penetrated hyphae remain unstained. Scale bars, 20 µm.

DISCUSSION

We have identified a structural and functional homologue of the sensor-like mucin Msb2 in *B. cinerea*. The homology between fungal Msb2 proteins is restricted to the C-terminal region, including a part of the extracellular domain, the transmembrane region and the C-terminal end. The large, extracellular, serine/threonine-rich region, which is predicted to be highly *O*-glycosylated, is more than 1000 amino acids in size and much longer in *B. cinerea* Msb2 than in other fungal Msb2 proteins described so far, and lacks repeats that are present in Msb2 of yeast, *U. maydis* and *F. oxysporum*. An *msb2* deletion mutant was constructed and used for phenotypic studies, together with a complemented strain. Because all phenotypic defects of the *msb2* mutant were restored in the complemented *msb2*-C strain, they could be attributed to the absence of Msb2. Compared with the wild-type, the *msb2* mutant showed similar growth characteristics, including the formation of aerial mycelium, macroconidia and sclerotia. This is in contrast with *msb2* mutants of *F. oxysporum* and *U. maydis*, which both showed a slight reduction in vegetative growth (Liu *et al.*, 2011; Perez-Nadales and Di Pietro, 2011). Incubation on media containing different toxic compounds resulted in growth inhibition similar to the wild-type, indicating that Msb2 plays no role in stress adaptation. This is similar to *msb2* mutants of *M. oryzae* and *U. maydis*, but different from yeast and *F. oxysporum* *msb2* mutants, which have been described to be hypersensitive to osmotic stress and cell wall targeting inhibitors, respectively (Cullen *et al.*, 2004; Lanver *et al.*, 2010; Liu *et al.*, 2011; Perez-Nadales and Di Pietro, 2011). In contrast with the *msb2* mutant, growth of the *bmp1* mutant was significantly retarded. Therefore, Msb2 is not involved in the growth regulation

via the MAPK cascade. CAT formation was found to be slightly reduced in the *msb2* mutant. In contrast, it was completely abolished in the *bmp1* mutant. This is similar to the situation in *F. oxysporum*, in which the Fmk1 MAPK was found to be required, but Msb2 was dispensable, for vegetative hyphal fusion. Therefore, the filamentous growth MAPK module is essential, but Msb2 does not appear to be a major component of the hyphal fusion machinery (Read *et al.*, 2009).

Germination of *B. cinerea* conidia is stimulated by host plant lipids, such as cuticular waxes (Leroch *et al.*, 2013). In this study, we confirmed a significant stimulation of germination in the presence of apple wax and the cutin monomer THPA. Irrespective of the presence of plant lipids, the germination of *msb2* conidia was almost as efficient as the germination of wild-type conidia. Therefore, with regard to the stimulation of spore germination by surface hardness, hydrophobicity or plant lipids, Msb2 does not play an important role. The sensors for these signals remain to be identified. However, Msb2-mediated sensing starts after germ tube emergence, and plays a role in germ tube 'dominance', i.e. the ability of the first germ tube to suppress the formation of other germ tubes. The lack of germ tube dominance in *msb2* mutant germlings is only observed in the absence of sugars on hydrophobic surfaces.

On hard surfaces, *B. cinerea* wild-type germ tubes cease elongation soon after emergence and develop terminal thickenings that function as appressoria. In contrast, *msb2* and *bmp1* germlings largely ignore the surface; they continue to elongate and show only a very low rate of appressorium formation. Neither apple nor the cutin monomer THPA increased the rate of appressorium formation in the *msb2* and *bmp1* mutants. Therefore, none of the physical and chemical surface signals can be

recognized by these mutants. The sensing defect of the *msb2* mutant also includes hyphae, which are defective in their ability to form infection cushions. Nevertheless, the mutant is still able to infect intact tissue after mycelium inoculation; therefore, a low rate of infection cushion formation seems to occur (data not shown). We suggest that Msb2 of *B. cinerea* serves as a sensor of surface hardness in germ tubes and hyphae for the initiation of penetration. In *M. oryzae* and *U. maydis*, signals required for appressorium formation include surface hydrophobicity and the presence of plant lipids (Mendoza-Mendoza *et al.*, 2009; Tucker and Talbot, 2001). In both fungi, Msb2 was found to be required for the sensing of surface hydrophobicity and, in *M. oryzae*, it was also involved in the perception of plant waxes (Lanver *et al.*, 2010; Liu *et al.*, 2011).

Despite the dramatically reduced appressorium formation, the infection ability of the *msb2* mutant was only moderately impaired. Compared with the wild-type, lesion formation on intact leaf or flower tissue by the mutant was delayed by 1 day, but occurred with the same kinetics on wounded tissue. Thus, the small number of appressoria is sufficient for penetration of the host surface. Increasing the spore concentration in the infection droplet abolished the delay in infection, probably by increasing the number of penetrating germlings. Remarkably, secondary lesion formation by the *msb2* mutant was similar to that of the wild-type. Thus, the delayed invasion does not increase the host plant's capability to build up a more effective defence against the mutant.

The role of Msb2 as a sensor that acts upstream of the BMP1 MAPK cascade was investigated by BMP1 phosphorylation studies. Weak phosphorylation of BMP1 was observed already in resting wild-type conidia, indicating a low basal activation of the MAPK pathway. The amount of phosphorylated BMP1 was elevated after 6 h and increased to higher levels after 12 h. In the *msb2* mutant, only a small increase in BMP1 phosphorylation during this period was observed. These data indicate that, in the absence of Msb2, signalling through the BMP1 MAPK is strongly impaired, but not completely blocked, and that an Msb2-independent pathway for background BMP1 phosphorylation is active in germlings. This is similar to the *msb2* mutants of *M. oryzae* and *F. oxysporum*, which also showed reduced but clearly detectable MAPK phosphorylation. The equally severe defects of the *msb2* and *bmp1* mutants in appressorium formation indicate that, for the activation of this Msb2-triggered, BMP1-dependent differentiation process, a stable threshold phosphorylation of BMP1 is required. In *M. oryzae*, *msb2* expression is down-regulated in mutants of the filamentous growth MAPK cascade (Liu *et al.*, 2011). Microarray and qRT-PCR data with the *B. cinerea* *bmp1* mutant, however, indicated that *msb2* expression levels were similar to those in the wild-type (data not shown). In contrast with BMP1, the high osmolarity response MAPK Sak1 was phosphorylated independently of germination in

both the wild-type and *msb2* mutant. In *F. oxysporum*, the MAPK Hog1 showed a transiently increased phosphorylation on transfer of a submerged culture to solid medium, which was not observed in the *msb2* mutant (Perez-Nadales and Di Pietro, 2011). Altogether, these data indicate significant differences in the involvement of Msb2 and the two MAPK pathways in the sensing of physical and chemical plant surface qualities in different plant pathogens.

In addition to the surface sensing defect, the *msb2* mutant displayed major changes in germination-associated gene expression. Several germination-induced genes encoding secreted proteins were down-regulated in both the *msb2* and *bmp1* mutant. When wild-type germlings were germinated on non-appressoria-inducing agarose surfaces, three of the six genes were not induced, indicating that their expression is dependent on appressorium differentiation. Although many of the germination-induced genes encode lytic enzymes potentially involved in host cell wall degradation, growth of the wild-type and *msb2* mutant on minimal medium containing several plant cell wall polymers was found to be similar. These data indicate that, during vegetative growth, expression of the major plant cell wall-degrading enzymes is similar in the wild-type and mutants. Alternatively, genes that are down-regulated in the *msb2* mutant are not required for growth on the substrates tested in this study.

In *S. cerevisiae* and *U. maydis*, PMT4-mediated O-glycosylation has been shown to be essential for correct functioning of Msb2. Although, in yeast, Msb2 was found to be hyperactivated in an O-glycosylation-defective mutant, the opposite, namely loss of Msb2 function and concomitant loss in virulence, was observed in a *pmt4* mutation of *U. maydis* (Fernández-Alvarez *et al.*, 2012; Yang *et al.*, 2009). It was therefore assumed that, in *B. cinerea*, PMT4 may also be involved in Msb2 function. The swollen hyphae in the germ tube stage of the *pmt4* mutant, which is also observed in other fungi, indicates a cell wall defect, which can be partially rescued by the addition of osmotic stabilizers (González *et al.*, 2013; Mouyna *et al.*, 2010). Nevertheless, the mutant appears to recognize a hard surface and is able to penetrate onion epidermis with moderate efficiency, in contrast with the *msb2* and *bmp1* mutants. Because the *pmt2* mutant grows very poorly and fails to produce spores, it could not be tested for its surface sensing phenotype. As another difference from the *msb2* mutant, the *pmt1* and *pmt4* mutants had no effect on BMP1 phosphorylation in germlings developing on a hard surface. A recent comparative secretome analysis of the *B. cinerea* wild-type and the three *pmt* mutants surprisingly revealed no dramatic differences in their glyco-secretome composition (González *et al.*, 2014). Only a few proteins were clearly absent in one or more mutants, and therefore were candidate substrates of the corresponding PMTs. Therefore, it is possible that O-glycosylation of Msb2 still occurs in the *pmt* mutants. In summary, the role of O-glycosylation for the function of Msb2 remains unclear.

Our work did not address the mechanism of putative surface sensing by Msb2 and its method of interaction with the BMP1 MAPK cascade. The morphogenetic defects of *msb2* germlings indicate that cell polarity establishment may be affected by Msb2. In yeast, the small G protein Cdc42 has been shown to be involved in cell polarity establishment and filamentous growth, and interacts with the Kss1 MAPK pathway (Cullen *et al.*, 2004). In *B. cinerea*, *cdc42* mutants show a pleiotropic growth phenotype and defects in nuclear division (Kokkelink *et al.*, 2011). By yeast two-hybrid screening, we did not observe an interaction of Msb2 with Cdc42, or with any other fungal polarity factors (N. Mueller, unpublished data). The activation of yeast Msb2 is achieved via proteolytic processing, which leads to shedding of the inhibitory mucin domain from the cells and generation of the active form of Msb2 (Vadaie *et al.*, 2008). Shedding of Msb2 was also observed in *F. oxysporum* (Perez-Nadales and Di Pietro, 2011). In yeast, *U. maydis* and *F. oxysporum*, Msb2 and Sho1 interact with each other in the activation of filamentous growth and, only in the case of yeast and *U. maydis*, in the high osmolarity response MAPK pathways (Cullen *et al.*, 2004; Lanver *et al.*, 2010). In *U. maydis*, *M. oryzae* and *F. oxysporum*, *msb2* and *sho1* mutants were defective in host invasion, and *msb2 sho1* double mutants of *U. maydis* and *M. oryzae* were almost completely nonpathogenic (Lanver *et al.*, 2010; Liu *et al.*, 2011; Perez-Nadales and Di Pietro, 2014). These data clearly indicate a complementary role of Msb2 and Sho1 in surface perception of these fungi. In *B. cinerea*, however, the phenotype of *sho1* mutants indicates no major role in appressorium formation and MAPK regulation (M. Leroch, unpublished data). Further studies are required for a better understanding of the activation of Msb2 by hard surface contact, and to reveal the regulatory pathway towards and beyond the BMP1 MAPK cascade.

EXPERIMENTAL PROCEDURES

Construction of transformants

For the generation of the *B. cinerea msb2* mutant, flanking gene primers were used for amplification of 5' (554 bp) and 3' (572 bp) regions of the *msb2* coding region (5': Msb2_KO1/2; 3': Msb2_KO3/4). The 5' region was cloned via *XhoI/EcoRI* sites and the 3' region via *EcoRI/BamHI* sites into pBSKS. The hygromycin resistance cassette was amplified with the primers Hyg_EcoRI_fw/rev and integrated between both flanking regions. After amplification of the knockout construct with the primers Msb2_KO1/KO4, the construct was transformed into *B. cinerea* wild-type strain B05.10. For the generation of the *msb2* complementation strain, yeast recombinational cloning was used (Schumacher, 2012). For cloning, 1562 bp of the *msb2* promoter region was amplified using primers Msb2_1fw/rev, and the entire *msb2* coding region was amplified in two overlapping fragments using primers Msb2_2fw/rev and Msb2_3fw/rev. The three PCR fragments were transformed together with the *SpeI/NotI*-digested vector pNANOGG into the yeast strain InvSC1. The complementation construct was amplified with primers pNA_fw/rev and used for the

transformation of the *B. cinerea msb2* mutant and homologous integration into the *niiA* gene.

For the analysis of infection cushions, the *Botrytis* strain Bcche-1, expressing the fluorescent protein mCherry, was used (Leroch *et al.*, 2011). For the generation of a GFP-expressing *msb2* mutant, a PCR fragment for transformation into the *msb2* mutant was generated on the basis of the pNANOGG vector using the primers pNA_fw/rev. This fragment consists of an entire nourseothricin resistance cassette, a constitutively expressed codon-optimized GFP-encoding gene and the flanking regions of *niiA* for homologous recombination into the *niiA* locus (Leroch *et al.*, 2011; Schumacher, 2012).

All primers used in this study are listed in Table S3 (see Supporting Information).

Cultivation and tests for growth, differentiation and plant infection

Botrytis cinerea strains were routinely cultivated on HA plates at 20 °C until sporulation, and conidia were harvested as described by Leroch *et al.* (2013). Growth tests were performed on HA agar or Gamborg B5 minimal medium (Rui and Hahn, 2007). For germination tests, 20- μ L droplets containing 2000 conidia (in minimal medium with 10 mM fructose) were incubated on 24 $\times$ 50-mm² cover slips (Roth, Karlsruhe, Germany) for the indicated times. Coating of the cover slips with apple wax or THPA (Sigma, Taufkirchen, Germany) was performed as described by Leroch *et al.* (2013). Germination without a carbon source on a hydrophobic polyethylene membrane, penetration and plant infection tests were performed as described by Doehlemann *et al.* (2006). For the infection of wounded apples, 6-mm holes created with a cork borer were inoculated with 20 μ L of spore suspension (10⁵/mL). Infection cushion formation was analysed in 30- μ L droplets containing 750 conidia (in minimal medium with 25 mM glucose) on cover slips, which were incubated for 48 h at room temperature in the dark before microscopic analysis. For the analysis of CAT fusions, 300 μ L of a suspension with 10⁶ conidia/mL were spread on a thin 9-cm minimal medium agar plate. After incubation for 16 h in the dark, the rate of CAT fusion formation was determined microscopically. The microscope and settings for light and fluorescence microscopy have been described by Leroch *et al.* (2013). Low-temperature scanning electron microscopy was performed as described by Kucheryava *et al.* (2008). For the analysis of gene expression on a soft surface, wild-type conidia were incubated on apple wax-coated 0.5% agar containing GB5 medium with 10 mM fructose for 4 h.

Phosphorylation assay

MAPK phosphorylation assays were performed with 2.5×10^7 conidia incubated in 25 mL of minimal medium with 10 mM fructose in apple wax-coated Petri dishes. Germlings were harvested from the surface with a cell scraper and centrifuged at 2.000 *g* for 5 min at 4 °C. After washing of the germlings with 20 mL of ice-cold H₂O, the supernatants were completely removed and the pellets were lyophilized. Dried germlings (150 mg) were suspended in 250 μ L of protein extraction buffer [20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.05% Triton X100, 1 mM phenylmethylsulfonylfluoride (PMSF)]. After the addition of 300 μ L of glass beads (ϕ = 0.75–1 mm), the tissue was homogenized in a

FastPrep™ FP120 homogenizer (Thermo Scientific, Schwerte, Germany) for 3 × 15 s at 4.5 m/s. After centrifugation (14 000 *g*, 5 min), the protein content of the supernatant was determined, and 30 µg of total proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. For the detection of MAPKs, the following antibodies (from Cell Signaling, Boston, MA, USA) were used: BMP1, p-44/42 MAPK antibody (no. 4695); BMP1p, phospho-p-44/42 MAPK antibody (no. 4370); SAK1, p-38 MAPK antibody (no. 9212); phosphorylated SAK1, phospho-p-38 MAPK antibody (no. 9211). Anti-rabbit immunoglobulin G (IgG), alkaline phosphatase (AP)-linked antibody (no. 7054), which was used as secondary antibody, was detected by staining with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate.

Gene expression analysis

RNA extraction from germinating conidia, RT-PCR and microarray analysis were performed as described by Leroch *et al.* (2013).

Statistics

Tests for phenotypic characterization were performed at least three times with three replicates each. To identify significant differences, Student's *t*-test was performed.

Accession number

msb2: B0510_3063 (http://www.broadinstitute.org/annotation/genome/botrytis_cinerea/GeneDetails.html?sp=S700000757751614).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Cloning strategy for deletion of *msb2* in *Botrytis cinerea*. (A) Scheme of the *msb2* gene with the primers used for amplification of the flanking regions F1 and F2 for homologous recombination. H, *Hind*III; X, *Xho*I; E, *Eco*RI; P, *Pvu*II; B, *Bam*HI restriction sites. (B) Knockout construct. (C) Southern blot analysis of the *msb2* mutants. Genomic DNA was digested with *Hind*III and *Pvu*II, which resulted in a 3542-bp fragment for the wild-type and a 931-bp fragment for *msb2* deletion mutants. WT, wild-type; ect., strain with ectopic insertion of the knockout construct.

Fig. S2 Vegetative growth of *Botrytis cinerea* wild-type (WT), *msb2* and *bmp1* mutants on HA and Gamborg B5 medium with 5 or 50 mM glucose (Glc), in the presence or absence of stress-inducing chemicals. Inhibitors were added to Gamborg medium with 5 mM Glc, and used at the following concentrations: 1 M sorbitol; 1 M NaCl; 100 µg/mL Calcofluor white; 250 µg/mL Congo red. For growth on 0.02% sodium dodecylsulfate (SDS) and 10 mM H₂O₂, Gamborg medium with 50 mM Glc was used.

Fig. S3 Germination of *Botrytis cinerea* wild-type (WT), *bmp1* and *msb2* conidia on a hydrophobic polyethylene foil in water after 18 h.

Fig. S4 Multiple germ tube formation of *Botrytis cinerea* wild-type (WT), *msb2* (three transformants) and *msb2-C* conidia on a hydrophobic polyethylene surface without nutrients.

Fig. S5 Rate of conidial anastomosis tube (CAT) formation by *Botrytis cinerea* wild-type (WT) and various mutants. Asterisks indicate significant differences between values obtained for the mutants compared with the wild-type (***P* = 0.01; ****P* = 0.001).

Fig. S6 Sak1 mitogen-activated protein (MAP) kinase phosphorylation response to germination on a hard surface. The arrows show Sak1-P-specific bands. Numbers indicate the incubation time (h) of conidia. WT, wild-type.

Fig. S7 Mycelial growth on minimal medium with different carbon sources. Colony diameters after 3 days of incubation are shown. The broken line indicates the diameter of the inoculation site. PGA [1% polygalacturonic acid (Sigma) in GB5 medium with 10 mM KH₂PO₄, 2.5% agarose, pH 6.3]. Xylan, bovine serum albumin (BSA) [GB5 medium (0.3% w/v) with 1% xylan from birch wood (Sigma) or 1% BSA, 1.5% agarose]. Pectin, CMC [50 mM Na₂HPO₄, 10 mM citric acid (pH 6.3), 0.1% maltose, 0.1% KNO₃, with 1% citrus pectin (Sigma) or carboxymethyl cellulose (CMC, Sigma), 1.5% agarose]. WT, wild-type.

Fig. S8 (A) Infection of intact tomato leaves by *Botrytis cinerea* wild-type (WT) and *pmt1* and *pmt4* mutant strains. Inoculation was performed with 20 µL of conidia suspension (10⁵/mL); C, medium control. (B) Genotypic verification of the *pmt1* and *pmt4* mutants by reverse transcription-polymerase chain reaction (RT-PCR). The gene encoding *actin* (*act*) served as a positive cDNA control.

Fig. S9 BMP1 mitogen-activated protein (MAP) kinase phosphorylation during germination of *Botrytis cinerea* wild-type (WT) and *pmt* mutants on a hard surface. The arrow indicates the BMP1-P-specific band. Conidia of the WT, *pmt1* and *pmt4* mutants were incubated for the indicated times in GB5 medium containing 10 mM fructose on apple wax-coated Petri dishes.

Table S1 Rate of appressorium formation by *Botrytis cinerea* wild-type, *msb2* and *bmp1* mutant germlings in GB5 medium with 10 mM fructose on different surfaces. Numbers indicate the percentage of germlings with terminal thickenings after 24 h (values are the mean of six replicates, with 100 germlings evaluated for each replicate; with standard deviations).

Table S2 *Botrytis cinerea* genes that are potentially co-regulated by BMP1 and Msb2.

Table S3 Primers used in this study.