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The osmosensing signal transduction pathway from *Botrytis cinerea* regulates cell wall integrity and MAP kinase pathways control melanin biosynthesis with influence of light

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ABSTRACT

Mitogen activated protein kinase (MAPK) signal transduction pathways are ubiquitous among eukaryotic organisms with evolutionary conserved modules. Although generally classified as osmotic and cell wall integrity pathways, functional divergences have been observed for HOG1- and SLT2-related MAPK pathways. Here we show that the osmotic signal transduction cascade is involved in cell wall integrity in the phytopathogenic ascomycete *Botrytis cinerea*. The deletion mutants of the upstream histidine kinase Bos1 and of the MAPK Sak1 showed modified tolerance to cell wall degrading enzymes and cell wall interfering agents, as well as increased staining of β1-3 glucan and chitin compared to the wild-type. The Sak1 MAPK was phosphorylated upon cell wall challenging. Sak1 interfered with the phosphorylation status of the SLT2 type MAPK Bmp3 hinting to cross talk between both MAPK pathways. All signal transduction components interfered with the expression of melanin biosynthesis genes in dark and bright, suggesting a coordinated control of melanin biosynthesis.

1. Introduction

Fungi perceive and respond to a variety of changing signals through ubiquitous and evolutionarily conserved MAP kinase signaling pathways. As a typical eukaryotic model, the budding yeast *Saccharomyces cerevisiae* has five MAP kinase (MAPK) pathways, governed by Hog1, Slt2, Fus3, Kss1, and Smk1 respectively. These MAPK pathways have distinct functions (reviewed in Gustin et al., 1998; Hunter and Plowman, 1997; Tamas and Hohmann, 2003). Hog1 is required for osmoregulation, whereas Slt2 is essential for cell wall integrity. Fus3 and Kss1 show closely related sequences and have partially redundant functions in sexual and asexual reproduction, and in the morphological switch. The less studied MAPK is Smk1 that seems to be involved in ascospore morphogenesis.

In contrast to *S. cerevisiae*, filamentous fungi have conserved MAP kinase modules to regulate various biological processes, but generally they lack an Smk1 homolog (Miskei et al., 2009; Rispail et al., 2009; Xu, 2000). As in the case of *S. cerevisiae*, the HOG1-related MAP kinase pathways are essential for osmoregulation in filamentous fungi, with the exception of *Aspergillus nidulans* in which the HogA/SakA MAP kinase only plays a partial role (Kawasaki et al., 2002). Recent studies indicate that HOG1-related MAP kinases are also involved in fungal morphology and development such as pigmentation or cleistothecia formation and resistance of conidiospores (Igbaria et al., 2008; Jones et al., 2007; Zhang et al., 2002). In addition the HOG1-related MAP kinase pathways confer sensitivity to dicarboximide and phenylpyrrole fungicides in *Neurospora crassa*, *Cochliobolus heterostrophus*, and other filamentous fungi (Kojima et al., 2004; Schumacher et al., 1997; Yoshimi et al., 2005).

Slt2 orthologs are responsible for cell wall integrity in the filamentous fungi *Magnaporthe grisea*, *Fusarium graminearum*, *Mycosphaerella graminicola*, *A. nidulans* and *Aspergillus fumigatus* (Fujioka et al., 2007; Hou et al., 2002; Mehrabi et al., 2006; Valiante et al., 2008; Xu et al., 1998), but not in *Colletotrichum lagenarium* (Kojima et al., 2002). In filamentous phytopathogens SLT2-related MAP kinases have additional roles in development and infection processes. *C. lagenarium* MAF1 is required for the early differentiation phase of appressoria formation (Kojima et al., 2002), whereas *M. grisea* MPS1 is essential for appressoria penetration (Xu et al., 1998). At the developmental level, *M. grisea* MPS1 and *C. lagenarium*

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MAF1 are required for normal conidiation. In addition, *M. grisea* MPS1 and *F. graminearum* MGV1 are essential for female fertility (Hou et al., 2002; Kojima et al., 2002; Mehrabi et al., 2006; Xu et al., 1998).

Fus3/Kss1 orthologs were generally found to be essential for appressoria formation and pathogenicity in the fungal pathogens *M. grisea*, *C. lagenarium*, *C. heterostrophus*, *Fusarium oxysporum*, and *Ustilago maydis* (Di Pietro et al., 2001; Lev et al., 1999; Mayorga and Gold, 1999; Müller et al., 1999; Takano et al., 2000; Xu and Hamer, 1996). In addition to plant infection, these MAP kinase pathways have diverse functions in hyphal growth, conidiation, mating and conidia germination in *C. lagenarium* (Takano et al., 2000), *C. heterostrophus* (Lev et al., 1999), and in *U. maydis* (Müller et al., 1999). Contrastingly, *M. grisea* PMK1 is not required for vegetative growth, nor sexual or asexual reproduction, or conidial germination (Xu and Hamer, 1996).

Among the control elements upstream of the MAPKKK–MAPKK–MAPK modules, two-component phosphorelay systems are common to fungi. They are composed of a sensor histidine kinase (HK), a phosphotransfer protein and a response regulator. Among the 11 major groups of histidine kinases classified by Catlett et al. (2003), families III and VI are principally involved in fungal HOG1-related osmoregulation pathways (reviewed in Bahn, 2008). Sln1, the sole histidine kinase in *S. cerevisiae* – a representative of family-VI – is essential and acts as sensor for the osmotic environment of the cell (Maeda et al., 1994; Posas et al., 1996). Disruption of the family-III histidine kinases, such as *N. crassa os-1* (Alex et al., 1996), *M. grisea hik1* (Motoyama et al., 2005), and *A. nidulans nikA* (Hagiwara et al., 2007), results in impaired growth under high osmolarity compared to the wild-type, consistent with the idea that family-III histidine kinases generally function in the osmotic response in euascomycetes. These disruption mutants also show resistance to dicarboximide, phenylpyrrole and aromatic hydrocarbon fungicides. Together, the data thus imply a universal role including osmosensitivity and fungicide resistance for family-III histidine kinases in filamentous fungi. The functions of other fungal histidine kinases, however, remain to be elucidated.

Botrytis cinerea is an important necrotrophic pathogen that causes gray mold on various plants, leading to seriously losses of global crop harvests worldwide. Osmo-adaptation, which is one important aspect for the development of appressorial infection structures in plant-pathogenic fungi (de Jong et al., 1997), is potentially involved in the *B. cinerea* infection process (Segmüller et al., 2007; Viaud et al., 2006). Tolerance to oxidative burst, characterized by a strong accumulation of reactive oxygen species (ROS), has been considered to be another important element of a successful infection strategy of this necrotrophic pathogen (Govrin and Levine, 2000; Kuźniak and Skłodowska, 2005; Tiedemann, 1997). In addition cell wall integrity is required for *B. cinerea* virulence. For example, the chitin synthase mutants *chs1* and *chs3a* exhibit substantially reduced chitin content in cell wall composition leading to cell wall weakening and, ultimately, to reduced virulence (Arbelet et al., 2010; Soulié et al., 2006, 2003). Melanin synthesis was also shown to be important for virulence in several phytopathogenic and human pathogenic fungi (Bell and Wheeler, 1986; Casadevall et al., 2000; Howard and Valent, 1996; Kihara et al., 2004a,b; Langfelder et al., 2003). Its role in *B. cinerea* virulence is yet unknown.

As other filamentous fungi *B. cinerea* has the three types of MAP kinases described above, including HOG1-related Sak1, SLT2-related Bmp3, and FUS3/KSS1-related Bmp1 MAP kinases, which have been characterized by gene replacements. In contrast to other fungal HOG1-related MAP kinases studied so far, the *B. cinerea* Sak1 MAP kinase is involved in appressoria formation, penetration and invasive growth, which may be partially dependent on osmoadaptation and peroxide tolerance (Liu et al., 2008; Segmüller et al., 2007). The two other MAP kinases Bmp1 and Bmp3 in *B. cinerea* are required respectively for conidia germination on hydrophobic surfaces, or (plant) surface sensing, and, ultimately, for plant penetration and infectious growth (Doehlemann et al., 2006; Rui and Hahn, 2007; Zheng et al., 2000). In contrast to most fungal HOG1-related MAP kinases *B. cinerea* Sak1 MAP kinase is not essential for sensitivity to dicarboximide and phenylpyrrole fungicides (Liu et al., 2008), and the Bmp3 MAP kinase is not required for cell wall integrity (Rui and Hahn, 2007). Comparing *sak1* and *bmp3* knockout mutants some common phenotypes are evident, including reduced vegetative growth, strongly impaired conidiation, and increased sensitivity to peroxide (Rui and Hahn, 2007; Segmüller et al., 2007). Both, Sak1 and Bmp3, are involved in sclerotia development in *B. cinerea*. The *sak1* null mutant exhibits enhanced sclerotia development, whereas the *bmp3* null mutant has lost sclerotia production capacity. In addition to the osmoregulating MAP kinase Sak1, Bmp3 is also involved in osmoadaptation, but restricted to low osmolarity (Rui and Hahn, 2007). Taken together, these related phenotypes shown by both *sak1* and *bmp3* mutants reflect possible cross talks between these MAP kinase pathways in *B. cinerea*.

The sole histidine-kinase characterized so far in *B. cinerea* is the family-III HK, Bos1. However, conversely to other characterized family-III histidine kinases of filamentous fungi, Bos1 negatively regulates the phosphorylation status of its downstream MAP kinase Sak1. Moreover, Bos1 regulates certain phenotypes in a Sak1-independent manner, such as tolerance to neutral hyperosmolarity, dicarboximides and phenylpyrroles, suggesting that other Bos1-dependent downstream partners could be responsible for these cellular functions (Liu et al., 2008).

In this paper we report a detailed phenotypical analysis of the *B. cinerea* mutants defective in the HOG1-related MAP kinase Sak1 or its upstream histidine kinase Bos1. Based on the phenotypes observed under cell wall perturbing conditions, we provide evidence implicating the Bos1–Sak1 pathway in cell wall integrity, confirmed by Sak1 phosphorylation upon cell wall challenging. In addition phosphorylation assays of the Sak1 and Bmp3 MAP kinases indicate that the Bos1–Sak1 pathway affects Bmp3 phosphorylation. Moreover, qRT-PCR analyses of the putative melanin biosynthesis genes *pks13*, *brn1* and *scd1* demonstrate that the protein kinases Bos1, Sak1, Bmp1, and Bmp3 regulate melanin production in *B. cinerea*; Sak1 and Bmp1 in opposite light dependent fashions.

2. Materials and methods

2.1. Media and growth conditions

All *B. cinerea* strains listed in Table 1 (Büttner et al., 1994; Doehlemann et al., 2006; Liu et al., 2008; Rui and Hahn, 2007; Segmüller et al., 2007) were grown in the synthetic complete media CM

Table 1
Fungal strains used in this study.

Strain	Genotype	Reference
B05.10	European wild-type strain sequenced by Syngenta	Büttner et al. (1994)
Δ*sak1*	B05.10 Δ*sak1::nar*[a]	Segmüller et al. (2007)
Δ*bos1*	B05.10 Δ*bos1::bar*[b]	Liu et al. (2008)
Δ*bmp1*	B05.10 Δ*bmp1::hph*[c]	Doehlemann et al. (2006)
Δ*bmp3*	B05.10 Δ*bmp3::hph*	Rui and Hahn (2007)

[a] *nar*: Nourseothricine resistance gene (S60706).
[b] *bar*: Bialaphos resistance gene (6762039).
[c] *hph*: Hygromycine resistance gene (AJ439603).