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Os2 MAP kinase-mediated osmostress tolerance in *Penicillium digitatum* is associated with its positive regulation on glycerol synthesis and negative regulation on ergosterol synthesis

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ABSTRACT

High osmolarity glycerol (HOG) pathway is ubiquitously distributed among eukaryotic organisms and plays an important role in adaptation to changes in the environment. In this study, the Hog1 ortholog in *Penicillium digitatum*, designated Pdos2, was identified and characterized using a gene knock-out strategy. The ΔPdos2 mutant showed a considerably increased sensitivity to salt stress and cell wall-disturbing agents and a slightly increased resistance to fungicides iprodione and fludioxonil, indicating that Pdos2 is involved in response to hyperosmotic stress, regulation of cell wall integrity and sensitivity to fungicides iprodione and fludioxonil. Surprisingly, the mutant was not affected in response to oxidative stress caused by H_2O_2. The average lesion size in citrus fruits caused by ΔPdos2 mutant was smaller (approximately 25.0% reduction) than that caused by the wild-type strain of *P. digitatum* at 4 days post inoculation, which suggests that Pdos2 is needed for full virulence of *P. digitatum*. Interestingly, in the presence of 0.7 M NaCl, the glycerol content was remarkably increased and the ergosterol was decreased in mycelia of the wide-type *P. digitatum*, whereas the glycerol content was only slightly increased and the ergosterol content remained stable in the ΔPdos2 mutant, suggesting that Pdos2-mediated osmotic adaption is associated with its positive regulation on glycerol synthesis and negative regulation on ergosterol synthesis.

1. Introduction

The high osmolarity glycerol (HOG) pathway plays an important role in eukaryotic organisms in the adaptation to the changing environmental conditions. In *Saccharomyces cerevisiae*, the HOG pathway has been intensively characterized. It is involved in the response to high osmotic as well as oxidative, heavy mental, citric acid and heat stresses by controlling the expression of an array of stress responsive genes (Bilsland et al., 2004; de Nadal et al., 2002; Hohmann, 2002; Lawrence et al., 2004; Sotelo and Rodriguez-Gabriel, 2006; Winkler et al., 2002). The mitogen-activated protein kinase (MAPK) Hog1 is a crucial kinase that can be activated by two discrete branches in *S. cerevisiae*. One branch involves a 'two-component' osmosensor, which consists of the Sln1-Ypd1-Ssk1-Ssk2/Ssk22 proteins, and the other is composed of the plasma membrane-localized sensor Sho1, the mucin-like protein Msb2, the small G protein Cdc42, the adaptor protein Ste50, the PAK kinase Ste20, and the MAPK kinase kinase Ste11 (Cullen et al., 2004; Hohmann, 2002; Posas et al., 1998). Both activated branches lead to the phosphorylation of the MAPK kinase Pbs2, which subsequently phosphorylates Hog1. The phosphorylated Hog1 activates the transcription of genes related to the stress response, leading to an increased synthesis of polyols, such as glycerol. It also modulates the activity of the Nha1 Na^+/H^+ antiporter and the Tok1 potassium channel in the cell membrane, leading to a decrease in the intracellular concentration of ions in fungal cells, thereby counteracting the extracellular osmotic pressure (de Nadal et al., 2002; Hohmann, 2002; Proft and Struhl, 2004; Westfall et al., 2004; Zhang et al., 2002).

Ergosterol is an important component of the fungal cell membrane, and it is crucial for fungi to maintain the fluidity and permeability of their cell membranes (Espenshade and Hughes, 2007). A recent study has shown that during the response to osmotic stress the ergosterol content was reduced in *S. cerevisiae*, and during this process Hog1 acted as an indirect transcription repressor by inhibiting the expression of several ergosterol biosynthetic genes (*ERGs*) (Montañés et al., 2011). However, whether this mechanism exists in filamentous fungi as they are challenged by osmotic stress is not known yet.

Hog1-homolog MAPKs have been characterized in several phytopathogenic fungi. Osm1 in *Magnaporthe oryzae* was the first Hog1-type MAPK studied in phytopathogenic fungi. The Δ*osm1*

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mutants showed a remarkable reduction in the content of arabitol in the mycelium and their morphology was defective under hyperosmotic conditions. However, the *osm1* deleted mutants produced functional appressoria and were fully pathogenic (Dixon et al., 1999). In *Botrytis cinerea*, the Hog1 homolog BcSak1 was involved in the response to osmotic stress, resistance to iprodione and oxidative stress reaction caused by H_2O_2. The Δ*Bcsak1* mutants were blocked in conidiogenesis and were completely nonpathogenic in unwounded plant tissue (Segmueller et al., 2007). A recent study showed that BcSak1 also participated in cell wall integrity and melanin biosynthesis (Liu et al., 2011). In *Fusarium graminearum*, Hog1-type MAPK FgOs2 mediated in planta production of deoxynivalenol and zearalenone, as well as the transcription and activity of fungal catalases. *Fgos2*-mutated strains were hypersensitive to osmotic pressure; the germination of conidia of Δ*Fgos2* was severely impaired when exposed to osmotic conditions. Deletion of *Fgos2* renders this fungus more resistant to fludioxonil and dramatically reduced the pathogenicity toward maize and wheat (Nguyen et al., 2012). In *Aspergillus nidulans*, Hog1-type MAPK SakA was necessary for conidiospore viability and repression of sexual development. Significantly, growth of Δ*AnsakA* mutants was not affected under high osmolarity conditions, though Δ*AnsakA* mutants were sensitive to heat shock and H_2O_2 stress (Kawasaki et al., 2002). So, it is important to realize that despite its evolutionary conservation, there are some differences in the roles that the Hog1-type MAPKs can play in physiological processes among diverse fungi.

Penicillium digitatum, the causal agent of green mold in citrus fruit, is the most important postharvest pathogen of citrus worldwide (Kanetis et al., 2007; Macarisin et al., 2007). In spite of its economic importance, there are few studies aimed to understand the physiological bases of its adaptation to the changing environment and to unravel the mechanisms of pathogenicity deployed by this fungus (Sun et al., 2011; Zhang et al., 2013a,b). Recently, the genomic sequence and *Agrobacterium tumefaciens*-mediated genetic transformation have been made available for *P. digitatum* (Marcet-Houben et al., 2012; Sun et al., 2013; Wang and Li, 2008). These studies have paved the way to uncover these mechanisms and to develop new strategies for disease control. In this study we have identified the Hog1 MAP kinase gene *Pdos2* in *P. digitatum* and characterized its functions using a gene deletion and complementation strategy.

2. Materials and methods

2.1. Strains and culture conditions

The wild-type strain Pd01 of *P. digitatum* was isolated from an infected citrus fruit from Zhejiang, China (Zhu et al., 2006). It was deposited in the CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands with accession number CBS130525. The wild-type and mutant strains (conidial suspensions) were stored in 20% glycerol solutions at −80 °C until used. Conidia were inoculated either on solid PDA (extract of 200-g potato-boiled water, 20 g dextrose, and 13 g agar per liter) and incubated at 25 °C or in liquid PDB (potato dextrose broth) and incubated on a rotary shaker set at 160 rpm.

2.2. Isolation of the Hog1 ortholog Pdos2 from P. digitatum

Based on the genome sequence of *P. digitatum*s train Pd1 (Marcet-Houben et al., 2012), a pair of primers, Pdos2F-F/Pdos2F-R (Table 1), was designed and used to amplify a fragment encompassing the full length genomic sequence of *Pdos2* (HQ416719) together with partial 5′ and 3′ untranslated regions from genomic DNA of *P. digitatum*. The amplified fragment was cloned into the pMD18-T vector according to the supplier's recommendations (TaKaRa Biotech. Co., Dalian, China) and verified by sequencing. Total RNA was extracted from mycelia using an AxyPrepTM multisource total RNA miniprep Kit (Axygen, Hangzhou, China). First strand cDNA was synthesized using the RNA PCR (AMV) 3.0 kit (TaKaRa Biotech. Co., Dalian, China). The coding region of *Pdos2* was then amplified from the cDNA template, using primers Pdos2-F/Pdos2-R (Table 1).

2.3. Construction of Pdos2 gene deletion mutants

The *Pdos2* deletion vector pTFCM- *Pdos2*-Del was constructed by inserting two flanking sequences of the *Pdos2* into the left and right sides of the hph gene (hygromycin-resistance gene) in the pTFCM vector (Wang and Li, 2008). Briefly, a 1.0 kb upstream flanking sequence fragment of *Pdos2* was amplified from wild-type strain Pd01 genomic DNA using primer pair Pdos2-up-F/Pdos2-up-R (Table 1), and was inserted into the *Hind*III–*Kpn*I sites of the pTFCM vector to generate plasmid pTFCM–*Pdos2*-up. Subsequently, a 1.0 kb downstream flanking sequence fragment of *Pdos2* amplified from Pd01 genomic DNA with the primers Pdos2-down-F/Pdos2-down-R (Table 1) was inserted into *Spe*I–*Xho*I sites of the pTFCM–*Pdos2*-up vector to generate the deletion plasmid pTFCM-*Pdos2*-Del (Fig. 1A). Then this vector was transformed into *A. tumefaciens* strain AGL-1. *A. tumefaciens*-mediated fungal transformation (ATMT) was performed according to Wang and Li (2008). Putative transformants were selected from PDA medium supplemented with hygromycin (100 mg/L) and subjected to PCR to identify putative deletants, which were further confirmed by Southern blot hybridization using the Detection Starter kit (Roche, Mannheim Germany).

2.4. Genetic complementation of the Pdos2 deletion mutant

To confirm that the phenotypic changes of the *Pdos2* deletion mutant were due to disruption of the gene, the mutant was complemented with the 3.7 kb full length genomic sequence of *Pdos2* from Pd01, including its promoter and terminator. This fragment was amplified by PCR with primers Pdos2-CP-F/Pdos2-CP-R (Table 1), digested with *Kpn*I and *Xba*I and cloned into the vector pA1300-NEO, which was constructed by replacing the hygromycin cassette in pCAMBIA1300 with a neomycin cassette between *Xho*I and *Kpn*I sites (Zhang et al., 2013b), to produce the complementation plasmid pA1300-NEO-*Pdos2*. Transformation of the Δ*Pdos2* mutant with plasmid pA1300-NEO-*Pdos2* was conducted as described above, except that neomycin (100 μg/mL) was used as the selection agent.

2.5. Mycelial growth tests

Growth of the wild-type, Δ*Pdos2* and CP*Pdos2* mutants of *P. digitatum* under different conditions was performed on PDA plates supplemented with NaCl, D-sorbitol, H_2O_2, fludioxonil, iprodione, calcofluor white (CFW), SDS or Congo red (CR) at different concentrations. Each plate was inoculated with a 5-mm mycelia plug taken from a 1-day-old colony prepared as described previously (Zhang et al., 2009). The diameters of the colonies were measured after the plates were incubated at 25 °C for 5 or 6 days. The percentage of the mycelial radial growth inhibition (MRGI) rate was calculated using the formula $MRGI\% = ((C-N)/(C-5)) \times 100\%$, where C is colony diameter of the control (PDA only) and N is that of a treatment. The experiments were repeated three times.

2.6. Infection assays

Citrus fruits (*Citrus reticulata Blanco*) infection assays were performed using the wild-type, *Pdos2*-deleted (Δ*Pdos2*) and