The response regulator BcSkn7 is required for vegetative differentiation and adaptation to oxidative and osmotic stresses in *Botrytis cinerea*

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SUMMARY

The high-osmolarity glycerol pathway plays an important role in the responses of fungi to various environmental stresses. *Saccharomyces cerevisiae* Skn7 is a response regulator in the high-osmolarity glycerol pathway, which regulates the oxidative stress response, cell cycle and cell wall biosynthesis. In this study, we characterized an Skn7 orthologue BcSkn7 in *Botrytis cinerea*. BcSKN7 can partly restore the growth defects of *S. cerevisiae* SKN7 mutant and vice versa. The BcSKN7 mutant (Δ BcSkn7-1) revealed increased sensitivity to ionic osmotic and oxidative stresses and to ergosterol biosynthesis inhibitors. In addition, Δ BcSkn7-1 was also impaired dramatically in conidiation and sclerotial formation. Western blot analysis showed that BcSkn7 positively regulated the phosphorylation of BcSak1 (the orthologue of *S. cerevisiae* Hog1) under osmotic stress, indicating that BcSkn7 is associated with the high-osmolarity glycerol pathway in *B. cinerea*. In contrast with BcSak1, BcSkn7 is not involved in the regulation of *B. cinerea* virulence. All of the phenotypic defects of Δ BcSkn7-1 are restored by genetic complementation of the mutant with the wild-type BcSKN7. The results of this study indicate that BcSkn7 plays an important role in the regulation of vegetative differentiation and in the response to various stresses in *B. cinerea*.

Keywords: *Botrytis cinerea*, high-osmolarity glycerol pathway, oxidative and osmotic stresses, Skn7, vegetative differentiation.

INTRODUCTION

Botrytis cinerea, a necrotrophic plant pathogen, can cause grey mould on more than 200 plant species (Williamson *et al.*, 2007). The financial impact of grey mould is considerable as *B. cinerea* causes both pre- and post-harvest losses (Dean *et al.*, 2012). Sensing of the environment and ensuring appropriate cellular

responses are essential for this pathogen to infect a wide range of host plants, and to survive under various environmental conditions. Previous studies have shown that the two-component signal (TCS) transduction pathway is one of the key signalling pathways in bacteria, yeasts, plants and filamentous fungi, enabling organisms to adapt to various environmental stresses, including osmotic and oxidative stresses (Barrett and Hoch, 1998; Koretke *et al.*, 2000; Santos and Shiozaki, 2001). To date, the high-osmolarity glycerol (HOG) pathway in *Saccharomyces cerevisiae* is one of the best characterized TCS pathways (Maeda *et al.*, 1995; Posas and Saito, 1997). The HOG pathway in *S. cerevisiae* consists of two upstream branches (Sln1 and Sho1) that converge on the mitogen-activated protein kinase kinase (MAPKK) Pbs2 (Hohmann, 2002). The Sln1 branch consists of the sensor histidine kinase Sln1, the phosphotransfer protein Ypd1 and two response regulators Ssk1 and Skn7. Under low osmotic conditions, Sln1 is autophosphorylated at a conserved histidine (His) residue. The phosphate is transferred to the aspartic acid (Asp) and His residues within Ypd1 and subsequently to Skn7 and Ssk1. Phosphorylation of Skn7 activates the transcription of the genes that are associated with low osmolarity (Posas *et al.*, 1996). In contrast, the phosphorylated Ssk1 is inactive and unable to regulate the HOG MAPK. Hyperosmotic shock deactivates Sln1, leading to enhanced levels of dephosphorylated Ssk1, which activates the downstream MAPKKs Ssk2/Ssk22, the MAPKK Pbs2 and the MAPK Hog1 by sequential phosphorylation (Krantz *et al.*, 2006; Posas *et al.*, 1996). In the Sho1 branch, signal transduction from Sho1 to Pbs2 requires Cdc42, Ste20, Ste50 and Ste11 (O'Rourke and Herskowitz, 1998; Posas and Saito, 1997).

In *S. cerevisiae*, in addition to responding to osmotic stress, the HOG pathway is also associated with the oxidative stress response. However, the role of Skn7 in the oxidative stress response appears to be independent of Sln1-mediated phosphorylation (Li *et al.*, 1998; Morgan *et al.*, 1997). Under oxidative stress, Skn7 undergoes phosphorylation at serine and forms a complex with the stress-responsive transcription regulator Yap1 (He and Fassler, 2005; Lu *et al.*, 2003). Interaction between Skn7 and Yap1 transcriptionally activates the oxidative responsive genes, including *TRX2*, *TRR1*, *GPX2* and *CCP1* (He

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and Fassler, 2005; Morgan *et al.*, 1997; Wormley *et al.*, 2005). In *S. cerevisiae*, approximately one-half of the genes up-regulated by oxidative stress depend on Yap1 (Delaunay *et al.*, 2000; Kuge *et al.*, 2001), whereas the other half are regulated by both Yap1 and Skn7. In contrast, only a few genes (*DNM1* and *OLA1*) have been reported to be dependent strictly on Skn7 (Lee *et al.*, 1999). As a stress-responsive regulator, fungal Skn7 proteins have a uniform architecture, consisting of an N-terminal DNA-binding domain and a C-terminal receiver domain. The receiver domain confers Skn7 activity by His–Asp phosphorelay signalling via the phosphorylation of a conserved aspartate in Skn7 (Brown *et al.*, 1993, 1994; Krems *et al.*, 1996; Morgan *et al.*, 1995).

To date, orthologues of *S. cerevisiae* Skn7 have been characterized in several fungal pathogens, including *Aspergillus fumigatus* (AfSkn7), *Candida lusitanae* (ClSkn7), *Candida albicans* (CaSkn7), *Cochliobolus heterostrophus* (ChSkn7), *Magnaporthe oryzae* (MoSkn7) and *Penicillium marneffei* (PmSkn7) (reviewed by Fassler and West, 2011). In general, Skn7 orthologues have been documented to be associated with the adaptation to various stress conditions, including oxidative and osmotic stresses, and cell wall damage agents (Bouquin *et al.*, 1999; Brown *et al.*, 1993, 1994; Cao *et al.*, 2009; Chen *et al.*, 2012; Hagiwara *et al.*, 2007; Morgan *et al.*, 1997; Motoyama *et al.*, 2008; Oide *et al.*, 2010; Vargas-Pérez *et al.*, 2007; Wormley *et al.*, 2005). However, the exact functions of Skn7 proteins vary significantly in different fungi.

In silico search for HOG pathway-related genes in the *B. cinerea* genome showed that this fungus contains several putative HOG components, including the osmosensor histidine kinase BcOs1, the histidine phosphotransfer protein BcHpt1, two response regulators BcRrg1 and BcSkn7, the MAPKKK BcOs4, the MAPKK BcOs5 and the MAPK BcSak1. To date, most of these core elements have been well characterized. The BcOs1 deletion mutants of *B. cinerea* are resistant to the dicarboximide fungicide iprodione and the phenylpyrrole fungicide fludioxonil, which have been shown to target the HOG pathway (Liu *et al.*, 2008; Motoyama *et al.*, 2005; Viaud *et al.*, 2006). In contrast, BcOs4, BcOs5, BcSak1 (also named BcOs2) and BcRrg1 deletion mutants are highly sensitive to iprodione and fludioxonil (Segmüller *et al.*, 2007; Yan *et al.*, 2010, 2011; Yang *et al.*, 2012). Interestingly, in other filamentous fungi, such as *Fusarium graminearum* and *Neurospora crassa*, all deletion mutants of Os1, Rrg1, Os4, Os5 and Os2 orthologues are highly resistant to these compounds (Fujimura *et al.*, 2003; Jiang *et al.*, 2011; Ochiai *et al.*, 2007; Yoshimi *et al.*, 2004; Zhang *et al.*, 2002), indicating that the roles of HOG components in *B. cinerea* may be different from those in other filamentous fungi. Thus, in order to further explore the functions of the HOG pathway, we sought to determine the role of the Skn7 orthologue (BcSkn7) in *B. cinerea* by the construction and characterization of *BcSKN7* mutants in this study.

RESULTS

Sequence analysis of *BcSKN7* in *B. cinerea*

Based on the amino acid similarity to *S. cerevisiae* Skn7, *BcSKN7* (XP_001554269.1) was retrieved from the genome of *B. cinerea*. The coding region of *BcSKN7* was 2339 bp in length and was predicted to have four introns of 255, 50, 57 and 65 bp located after 57, 514, 1473 and 1729 nucleotides, respectively. The existence of the introns was verified by reverse transcription-polymerase chain reaction (RT-PCR). The primer pair YES2-Skn7-F and YES2-Skn7-R (Table S1, see Supporting Information) generated 1912-bp and 2339-bp fragments from cDNA and genomic DNA, respectively. Sequencing of the 1912-bp fragment obtained from cDNA verified the predicted positions and sizes of the introns. The deduced 618-amino-acid protein contains two conserved domains: an N-terminal DNA-binding domain similar to that of the heat shock transcription factor (HSF) and a C-terminal receiver domain (Fig. S1, see Supporting Information).

Construction of *BcSKN7* deletion and complemented mutants

To investigate the biological function of *BcSKN7* in *B. cinerea*, we generated deletion mutants of *BcSKN7* using a homologous recombination strategy (Fig. S2A, see Supporting Information). Three deletion mutants were identified from 35 hygromycin-resistant transformants by PCR analysis with the primer pair BcSkn7-out-F and BcSkn7-out-R (Fig. S2B and Table S1). All three deletion mutants showed identical phenotypic characters. As shown in Fig. S2C, Southern hybridization patterns confirmed that Δ BcSkn7-1 resulted from the anticipated homologous recombination events at the *BcSKN7* locus. The wild-type *BcSKN7* was ectopically integrated into the genome of the complemented strain (Δ BcSkn7-1-C) (Fig. S2C).

Involvement of *BcSKN7* in conidiation and sclerotial formation

Although the mycelial growth rate of Δ BcSkn7-1 was similar to that of the wild-type progenitor 38B1 on either potato dextrose agar (PDA) or minimal medium (MM) (data not shown), Δ BcSkn7-1 was unable to produce any conidia after 10 days of incubation on PDA (Fig. 1B). As *B. cinerea* produces conidia easily on cucumber, we also examined the conidiation of the mutant on sterilized cucumber fragments. After incubation on autoclaved cucumber fragments for 10 days, 38B1 and Δ BcSkn7-1-C strains produced extensive aerial mycelia covered with a dense layer of conidia, whereas Δ BcSkn7-1 failed to produce detectable conidia (Fig. 1A).

Sclerotial formation is an important survival mechanism of *B. cinerea* in nature (Williamson *et al.*, 2007). Thus, we

Fig. 1 Impact of *BcSKN7* deletion on conidial and sclerotial formation. (A) Comparison of conidiation among 38B1, Δ BcSkn7-1 and Δ BcSkn7-1-C after 10 days of incubation on sterilized cucumber fragments. (B) The number of conidia produced by each strain on potato dextrose agar (PDA) plates (diameter, 9 cm). Bars in each column denote the standard errors of three experiments. Values on the bars followed by the same letter are not significantly different at $P = 0.05$. (C) Comparison of sclerotial formation among 38B1, Δ BcSkn7-1 and Δ BcSkn7-1-C after 4 weeks of incubation on PDA and minimal medium (MM) at 25 °C in darkness. (D) The number of sclerotia was measured on both PDA and MM. Bars denote standard errors from three experiments. Values on the bars followed by the same letter are not significantly different at $P = 0.05$.

investigated the effect of *BcSKN7* deletion on sclerotial formation. After 4 weeks of incubation in the dark, Δ BcSkn7-1 produced significantly fewer sclerotia than 38B1 and Δ BcSkn7-1-C (Fig. 1C, D), indicating that *BcSkn7* is also involved in the regulation of sclerotial development in *B. cinerea*.

Effects of *BcSKN7* deletion on sensitivity of *B. cinerea* to oxidative and osmotic stresses, and to fungicides

As described earlier, *SKN7* mutation in several fungal species resulted in increased sensitivity to oxidative stress (reviewed by Fassler and West, 2011). We therefore examined the sensitivity of the *BcSKN7* mutant to oxidative stress. As shown in Fig. 2A, B, Δ BcSkn7-1 exhibited increased sensitivity to hydrogen peroxide (H_2O_2), but not to paraquat. To a lesser extent, the *BcRRG1* (homologue of *S. cerevisiae* *SSK1*) mutant also showed increased sensitivity to H_2O_2 , but not to paraquat. Significantly, however, the *BcSAK1* mutant exhibited increased sensitivity to both H_2O_2 and paraquat.

In general, *Skn7* does not confer an osmotic stress response, whereas mutations of *Ssk1* are typically strikingly sensitive

(reviewed by Fassler and West, 2011). In a previous study, we found that the deletion of *BcRRG1* in *B. cinerea* leads to increased sensitivity to both osmotic and oxidative stresses (Yan *et al.*, 2011). Thus, we also examined the sensitivity of Δ BcSkn7-1 to osmotic stress. As shown in Fig. 3A, B, Δ BcSkn7-1 exhibited a strongly increased sensitivity to osmotic stress mediated by 1 M NaCl and 1 M KCl, but not by 1 M sorbitol. These results indicate that *BcSkn7* is involved in the response to ionic osmotic stress.

The phenylpyrrole fungicide fludioxonil activates the HOG pathway in filamentous fungi (Kojima *et al.*, 2004, 2006; Zhang *et al.*, 2002). In fungicide sensitivity tests, unexpectedly, we observed that Δ BcSkn7-1 showed increased sensitivity to the ergosterol biosynthesis inhibitors (EBIs) triadimefon, propiconazole and tebuconazole, but not to fludioxonil (Fig. 3, B).

In addition to oxidative and osmotic stresses, *Skn7* has also been documented to be associated with the cell wall damage stress response (reviewed by Fassler and West, 2011). Sensitivity testing of Δ BcSkn7-1 to cell wall damage agents was also performed. However, Δ BcSkn7-1 did not show remarkable changes in sensitivity to Congo red and calcofluor white relative to that of the

Fig. 2 Comparison of sensitivity to oxidative stresses among 38B1, Δ BcSkn7-1, Δ BcSkn7-1-C, the *BcSAK1* deletion mutant Δ BcSak1 and the *BcRRG1* deletion mutant Δ BcRrg1. (A) Comparisons were made on potato dextrose agar (PDA) plates amended with H₂O₂, paraquat and menadione at the indicated concentrations. The images were taken after the plates had been incubated at 25 °C for 3 days. (B) Inhibition of mycelial growth compared with non-treatment was examined after each strain had been incubated for 3 days on PDA amended with the indicated compounds. Bars denote the standard errors from three experiments. Values on the bars in each treatment followed by the same letter are not significantly different at $P = 0.05$.

wild-type strain (data not shown), indicating that BcSkn7 is not involved in the response to cell wall damaging agents in *B. cinerea*.

As the deletion of BcSkn7 leads to increased sensitivity to osmotic and oxidative stresses, we also examined the transcriptional levels of *BcSKN7* under these stresses. As shown in Fig. 4, the expression of *BcSKN7* was up-regulated 20.6-, 5.4- and 3.8-fold by 0.5 M NaCl, 20 mM H₂O₂ and 2.5 μ g/mL triadimefon, respectively, indicating that BcSkn7 is associated with multi-stress responses in *B. cinerea*.

Effects of BcSkn7 deletion on phosphorylation of BcSak1

Previous studies have shown that the BcOs4-BcOs5-BcSak1 MAPK cassette is responsible for the adaptation of *B. cinerea* to osmotic

and oxidative stresses. Thus, we tested whether BcSkn7 affects the phosphorylation of BcSak1. In the wild-type, BcSak1 phosphorylation was dramatically increased after treatment of the fungus with 0.5 M NaCl or 20 mM H₂O₂ for 2 h (Fig. 5A, B). Compared with the wild-type, Δ BcSkn7-1 revealed a reduced phosphorylation level of BcSak1 under NaCl, but not under H₂O₂ treatment (Fig. 5B).

Involvement of BcSkn7 in the regulation of the transcription levels of the genes associated with ergosterol biosynthesis and the oxidative stress response

Deletion of BcSkn7 led to increased sensitivity to the EBIs, indicating that BcSkn7 might be associated with ergosterol biosynthesis in *B. cinerea*. In order to confirm this hypothesis, we

Fig. 3 Sensitivity of the wild-type 38B1, Δ BcSkn7-1 and Δ BcSkn7-1-C to osmotic stresses (NaCl, KCl and sorbitol) and fungicides (fludioxonil, triadimefon, propiconazole and tebuconazole). (A) Comparisons were made on potato dextrose agar (PDA) plates amended with the above compounds at the indicated concentrations. The images were taken after the plates had been incubated at 25 °C for 3 days. (B) Inhibition of mycelial growth compared with non-treatment was examined after each strain had been incubated for 3 days on PDA amended with the indicated compounds. Bars denote standard errors from three experiments. Values on the bars in each treatment followed by the same letter are not significantly different at $P = 0.05$.

Fig. 4 Expression levels of *BcSKN7* in the wild-type 38B1. The expression level of *BcSKN7* under each stress condition is relative to that in the untreated sample (NT). RNA samples were extracted from fresh mycelia untreated or treated with 0.5 M NaCl, 20 mM H_2O_2 or 2.5 $\mu\text{g/mL}$ triadimefon for 2 h. Bars denote standard errors from three experiments. Values on the bars followed by the same letter are not significantly different at $P = 0.05$.

Fig. 5 Phosphorylation of BcSak1 in 38B1 and $\Delta\text{BcSkn7-1}$. (A) Total proteins were extracted from fresh mycelia of each strain treated with 0.5 M NaCl or 20 mM H_2O_2 for 2 h. The cultures without any treatment were used as controls (NT). The BcSak1 and phosphorylated BcSak1 proteins were detected using the yeast anti-Hog1p (C-terminal anti-Hog1) and phosphorylated p38 (Thr180/Tyr182) antibodies, respectively. (B) The intensity of phosphorylated BcSak1 relative to the amount of BcSak1 in each sample. Bars denote standard errors from four repeated experiments. Values on the bars in each treatment followed by the same letter are not significantly different at $P = 0.05$.

analysed the expression levels of the ergosterol biosynthesis genes, *BcERG2*, *BcERG3* and *BcERG11*, in the wild-type and BcSkn7 deletion mutant. As shown in Fig. 6A, the transcription levels of *BcERG2*, *BcERG3* and *BcERG11* were significantly up-regulated by triadimefon treatment in the wild-type strain.

Fig. 6 (A) Relative expression of *BcERG2*, *BcERG3* and *BcERG11* in 38B1 and $\Delta\text{BcSkn7-1}$. RNA samples were extracted from fresh mycelia untreated or treated with 2.5 $\mu\text{g/mL}$ triadimefon for 2 h. The expression level of each gene in $\Delta\text{BcSkn7-1}$ or each treated sample is relative to that in the untreated wild-type strain. Bars denote standard errors from three experiments. Values on the bars for each gene followed by the same letter are not significantly different at $P = 0.05$. (B) Expression analyses of oxidative stress-response genes in the wild-type 38B1, ΔBcSak1 and $\Delta\text{BcSkn7-1}$. Oxidative stress was induced by the addition of 20 mM H_2O_2 for 2 h. The expression level of each gene in $\Delta\text{BcSkn7-1}$ or each treated sample is relative to that in the untreated wild-type strain. Bars denote standard errors from three experiments. Values on the bars for each gene followed by the same letter are not significantly different at $P = 0.05$.

However, the transcription levels of *BcERG3* and *BcERG11* in $\Delta\text{BcSkn7-1}$ were dramatically lower than those in the wild-type strain under triadimefon treatment (Fig. 6A). The results are in agreement with the observation that $\Delta\text{BcSkn7-1}$ shows increased sensitivity to EBIs.

A previous study has shown that the transcription of a number of oxidative stress response genes is dependent on BcSak1 (a Hog1 orthologue) (Heller *et al.*, 2012). In *S. cerevisiae*, however, the role of Skn7 in the oxidative stress response appears to be independent of Hog1 (Li *et al.*, 1998; Morgan *et al.*, 1997). As both ΔBcSak1 and ΔBcSkn7 showed increased sensitivity to H_2O_2 (Fig. 2), we were interested in determining whether BcSak1-dependent genes responding to oxidative stress are also regulated transcriptionally by BcSkn7 in *B. cinerea* (Heller *et al.*, 2012). As shown in Fig. 6B, the transcriptional levels of three BcSak1-dependent genes [BC1G_09259.1 (encoding a mannitol dehydrogenase), BC1G_11680.1 (*BcREG1*) and BC1G_03070.1 (*BcAP8*, a family A1 protease)] were significantly up-regulated in

Δ BcSkn7-1 in response to H_2O_2 . These results indicate that the role of BcSkn7 in the oxidative stress response may be independent of BcSak1.

Complementation of Δ BcSkn7-1 with the yeast *SKN7* and the yeast *SKN7* mutant with *BcSKN7*

In order to further explore the functions of BcSkn7, we tested whether *BcSKN7* would complement the yeast *SKN7* mutant and vice versa. An expression vector pYES2 containing full-length *BcSKN7* cDNA was transformed into the yeast *SKN7* mutant BY4741 Δ SKN7. As a control, the mutant was also transformed with the empty pYES2 vector. As shown in Fig. 7A, BY4741 Δ SKN7 exhibited dramatically decreased resistance to H_2O_2 , but increased resistance to triadimefon and tebuconazole. These phenotypic defects were partly restored by genetic complementation of the yeast BY4741 Δ SKN7 mutant with *B. cinerea* *BcSKN7* (Fig. 7A). Furthermore, we also tested whether the yeast *SKN7* (*ScSKN7*) would restore the growth defects of Δ BcSkn7-1. The full cDNA fragment of the yeast *SKN7* gene driven by the promoter of *B. cinerea* *BcSKN7* was transformed into Δ BcSkn7-1. As shown in Fig. 7B, Δ BcSkn7-1 exhibited strongly increased sensitivity to H_2O_2 , NaCl and triadimefon, and these phenotypic defects were

also partly restored in Δ BcSkn7 bearing *ScSKN7*. The results indicate that Skn7 orthologues are conserved in the budding yeast and the filamentous fungus *B. cinerea*.

BcSkn7 is not involved in the virulence of *B. cinerea*

Three days after inoculation, Δ BcSkn7-1 and two other *BcSKN7* deletion mutants were able to infect tomato and cucumber leaves. The diameter of the disease lesion caused by Δ BcSkn7-1 was not dramatically different from that caused by the wild-type 38B1 (Fig. S3, see Supporting Information). In addition, onion penetration assay revealed that Δ BcSkn7-1 did not show any defects in the penetration of onion cells when compared with the wild-type 38B1 (data not shown). These results suggest that BcSkn7 is not involved in the virulence of *B. cinerea*.

Generally, it is believed that the oxidative burst, a transient and rapid accumulation of reactive oxygen species (ROS), is a common defence mechanism of higher plants against pathogen attack (Heller and Tudzynski, 2011). In this study, however, Δ BcSkn7-1 did not show a significant change in virulence on tomato and cucumber leaves, although the mutant exhibited increased sensitivity to oxidative stress (Fig. 2). Thus, we were interested in determining ROS accumulation in plant tissue inoculated with the

Fig. 7 Complementation of Δ BcSkn7-1 with the yeast *SKN7* and of the yeast *SKN7* mutant with *BcSKN7*. (A) Complementation of the *Saccharomyces cerevisiae* *SKN7* mutant with *Botrytis cinerea* *BcSKN7*. The yeast *SKN7* mutant was transformed with *BcSKN7* cDNA to generate the strain BY4741 Δ SKN7+pYES2-BcSKN7. The wild-type BY4741 and *SKN7* mutant BY4741 Δ SKN7 transformed with the empty pYES2 vector were used as controls. Serial dilutions of cell suspensions of each strain were spotted onto YPRG (1% yeast extract, 2% peptone, 1% galactose, 1% raffinose, 2% agar) plates containing no inhibitor (CK), 4 mM H_2O_2 , 0.5 μ g/mL triadimefon and 0.5 μ g/mL tebuconazole. After yeast cells had been incubated at 30 °C for 4 days, the growth of each strain on the plates was examined. (B) Complementation of Δ BcSkn7-1 with the yeast *SKN7*. Δ BcSkn7-1 was transformed with the yeast *SKN7* cDNA driven by the promoter of *BcSKN7* to generate the strain Δ BcSkn7-1-ScSkn7. Sensitivity of 38B1, Δ BcSkn7-1, Δ BcSkn7-1-C and Δ BcSkn7-1-ScSkn7 to 20 mM H_2O_2 , 1 M NaCl and 2.5 μ g/mL triadimefon was tested. Comparisons were made on potato dextrose agar (PDA) plates amended with the above compounds at the indicated concentrations. The images were taken after the plates had been incubated at 25 °C for 3 days.

mutant. The accumulation of H_2O_2 and superoxide anions (O_2^-) was detected by staining with 3,3'-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT), respectively (Doke, 1983; Thordal-Christensen *et al.*, 1997). As shown in Fig. S4 (see Supporting Information), the levels of H_2O_2 and O_2^- generated in tomato leaves at the inoculation site of $\Delta\text{BcSkn7-1}$ were similar to those of the wild-type 38B1 and the complemented strain $\Delta\text{BcSkn7-1-C}$, indicating that ROS may not play an important role in the resistance of tomato leaves to *B. cinerea* infection.

DISCUSSION

A previous study has shown that the *SKN7* mutant of *Cryptococcus neoformans* exhibits enhanced mating (Bahn *et al.*, 2006). In contrast, the *Schizosaccharomyces pombe SKN7* mutant is defective in mating (Nakamichi *et al.*, 2003; Ohmiya *et al.*, 2000). Interestingly, the *Aspergillus nidulans SKN7* mutant shows normal sexual reproduction, but exhibits a striking defect in asexual sporulation (Hagiwara *et al.*, 2007; Vargas-Pérez *et al.*, 2007). Likewise, in *Alternaria alternata*, conidia produced by the *SKN7* mutant are smaller and have fewer transverse septa than those produced by the wild-type strain (Chen *et al.*, 2012). In this study, we observed that the *BcSKN7* mutant did not produce any conidia. These results indicate that Skn7 is involved in the regulation of sexual and asexual development in a species-specific manner.

The osmosensitivity attributable to Skn7 mutations has been noted in several fungi, including *C. heterostrophus* (Izumitsu *et al.*, 2007, 2009), *As. nidulans* (Hagiwara *et al.*, 2007; Vargas-Pérez *et al.*, 2007) and *A. alternata* (Chen *et al.*, 2012). In the current study, we observed that the *BcSKN7* mutant exhibited increased sensitivity to osmotic stress mediated by NaCl and KCl, but not by sorbitol. In addition, BcSkn7 positively regulated the phosphorylation of BcSak1 under NaCl treatment. Similarly, the mutants of *BcRRG1* and *BcSAK1* also showed increased sensitivity to 1 M NaCl and 1 M KCl, but not to sorbitol (Liu *et al.*, 2008; Yan *et al.*, 2011). These results strongly suggest that the HOG pathway is associated with the ionic, but not neutral, stress in *B. cinerea*, and the adaptation of *B. cinerea* to ionic and neutral stresses is regulated by different pathways. This finding is consistent with the previous observations from *C. heterostrophus* and *As. nidulans*, in which both the Ssk1 and Skn7 orthologues are involved in the high-osmolarity response (Hagiwara *et al.*, 2007; Izumitsu *et al.*, 2007; Vargas-Pérez *et al.*, 2007). In contrast, in *N. crassa* and *M. oryzae*, the Ssk1 orthologue is a major contributor, but Skn7 is only a minor contributor, to the high-osmolarity response (Banno *et al.*, 2007; Jones *et al.*, 2007; Motoyama *et al.*, 2008). Thus, the contribution of Skn7 orthologues to the high-osmolarity response is also species dependent.

In general, Skn7 orthologues are transcription factors contributing to the oxidative stress response (reviewed by Fassler and

West, 2011). In *S. cerevisiae*, Skn7 undergoes a post-translational modification by phosphorylation following exposure to an oxidant; as Skn7 phosphorylation was eliminated in strains lacking the Yap1 transcription factor, this procedure most probably cooperates with Yap1 (He *et al.*, 2009). However, the *Cr. neoformans SKN7* mutant is distinguished by having a very subtle defect in its response to oxidants (Bahn *et al.*, 2006). Similarly, the *SKN7* mutant of the rice blast fungus, *M. oryzae*, is reportedly insensitive to oxidant, and oxidative stress (10 mM H_2O_2) did not activate the Osm1 MAP kinase (Hog1 orthologue) in this fungus, suggesting that Osm1 and upstream factors are not required for the oxidative stress response. In contrast with the observations in *M. oryzae*, we found that the *BcSKN7* mutant exhibited dramatically increased sensitivity to oxidative stress mediated by H_2O_2 . However, the phosphorylation levels of BcSak1 in *B. cinerea* under oxidative stress were not affected by the deletion of *BcSKN7* (Fig. 5A, B). Furthermore, the transcription of BcSak1-dependent oxidative stress-response genes was independent of BcSkn7 (Fig. 6B). These results indicate that the oxidative stress response associated with BcSkn7 is independent of BcSak1 in *B. cinerea*.

The roles of Skn7 in the virulence of fungal pathogens are highly diverse among these pathogens. The deletion of Skn7 has no impact on pathogenicity in *As. fumigatus* (Lamarre *et al.*, 2007). Likewise, *MoSKN7* mutants of *M. oryzae* are fully virulent on rice (Motoyama *et al.*, 2008). However, the *A. alternata SKN7* mutants induced significantly fewer necrotic lesions on susceptible citrus hosts, even though they produced host-specific ACT toxin at a level similar to that of the wild-type. The reduced virulence phenotype of the *SKN7* mutants was apparently associated with decreased activities of fungal antioxidants, because effective detoxification of ROS is critical for successful colonization of citrus by *A. alternata* (Chen *et al.*, 2012). However, as a necrotrophic pathogen, *B. cinerea* is not inhibited by ROS during infection, and even stimulates ROS on host plants (Lyon *et al.*, 2007; Tenberge *et al.*, 2002; Tiedemann, 1997). In addition to ROS-generating systems, *B. cinerea* also possesses effective ROS detoxification systems. In this study, we found that, in spite of the peroxide sensitivity, the *BcSKN7* mutant showed no difference in virulence on tomato and cucumber leaves. The accumulation of H_2O_2 and superoxide anions at the inoculation site of $\Delta\text{BcSkn7-1}$ was similar to that of the wild-type strain (Fig. S4). These results are consistent with a previous observation that Bap1 (the orthologue of Yap1) is not required for *B. cinerea* to infect plant hosts (Temme and Tudzynski, 2009). These results further confirm that *B. cinerea* is not restricted by ROS during infection *in planta*.

In *S. cerevisiae*, two structurally related transcription factors, Ecm22 and Upc2, are the main activators of genes encoding enzymes for the late steps of ergosterol biosynthesis, e.g. *ERG2* and *ERG3* (Vik and Rine, 2001). Both transcription factors bind to the sterol regulatory element, which is present in many *ERG* gene

promoters. Ecm22 is the main transcriptional activator of *ERG2* in normal growth conditions. However, Upc2 takes over its role on sterol depletion (Davies *et al.*, 2005). More recently, Montañés *et al.* (2011) found that Ecm22 is negatively regulated by Hog1 and its downstream components Mot3 and Rox1 on salt shock. Oxidative stress also triggers the repression of *ERG2* and *ERG11* transcription, which is largely independent on Mot3/Rox1 and Hog1. In this study, we found that the yeast *SKN7* mutant showed increased resistance to EBIs, suggesting that Skn7 could be involved in repression of the transcription of *ERG* genes. Surprisingly, in contrast with observations from the yeast *SKN7* mutant, we observed that the *BcSKN7* mutant revealed increased sensitivity to EBIs, although BcSkn7 could partially complement the yeast *SKN7* mutant in the sensitivity to these compounds (Fig. 7A). Gene transcription assays further confirmed that BcSkn7 positively regulated the transcription of *ERG3* and *ERG11* (Fig. 6A). In a previous study, we also found that the BcRrg1 mutant increased sensitivity to an EBI fungicide, triadimefon (Yan *et al.*, 2011). Thus, it would be very interesting to investigate the molecular mechanisms of the involvement of BcSkn7 and other HOG components in the regulation of ergosterol biosynthesis in *B. cinerea*.

EXPERIMENTAL PROCEDURES

Fungal strain and culture conditions

Botrytis cinerea strain 38B1, isolated from grape in California, USA, was used as a recipient strain for the transformation experiments. *Botrytis cinerea* strains were grown on PDA (200 g potato, 20 g glucose, 20 g agar and 1 L water) and MM (10 mM K₂HPO₄, 10 mM KH₂PO₄, 4 mM (NH₄)₂SO₄, 2.5 mM NaCl, 2 mM MgSO₄, 0.45 mM CaCl₂, 9 µM FeSO₄, 10 mM glucose and 1 L water, pH 6.9) for mycelial growth. In addition, each strain was grown on sterilized cucumber fragments and PDA for conidiation assays.

Mycelial growth tests under various conditions were performed on PDA plates with supplements at the concentrations indicated in the legends of the figures. Each plate was inoculated with a 5-mm-diameter mycelial plug taken from the edge of a 3-day-old colony grown on PDA. Each experiment was repeated three times independently.

Sequence analysis of *BcSKN7*

BcSKN7 (XP_001554269.1) was originally identified during homology search of the *B. cinerea* genome sequence (http://www.broadinstitute.org/annotation/genome/botrytis_cinerea/) using the BLASTP algorithm with Skn7 of *S. cerevisiae* (Morgan *et al.*, 1997) as a query. To verify the existence and size of the introns, RNA was extracted from mycelia of the wild-type 38B1 with a TaKaRa RNAiso Reagent (TaKaRa Biotech. Co., Dalian, China) and used for reverse transcription with a RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas Life Sciences, Burlington, ON, Canada) according to the manufacturer's instructions. RT-PCR was performed with the primer pair YES2-BcSkn7-F and YES2-BcSkn7-R (Table S1). The resulting PCR product was purified, cloned and sequenced.

Complementation of the yeast *SKN7* mutant

The yeast strain BY4741 (wild-type) and *SKN7* deletion mutant BY4741Δ*SKN7* were ordered from the EUROpean *Saccharomyces Cerevisiae* Archive for Functional analysis (EUROSCARF). The full-length *BcSKN7* cDNA was amplified using the primer pair YES2-Skn7-F and YES2-Skn7-R. The PCR product was digested with *Bam*HI and *Xho*I, cloned into the pYES2 vector (Invitrogen Co., CA, USA) and transferred into the yeast mutant BY4741Δ*SKN7* according to the lithium acetate/single-stranded carrier DNA/polyethylene glycol (LiAc/SS-DNA/PEG) transformation procedure (Schiestl and Gietz, 1989). Yeast transformants were selected on synthetic medium lacking uracil. In addition, the wild-type strain BY4741 and BY4741Δ*SKN7* mutant transformed with empty pYES2 vector were used as controls. For the complementation assay, the yeast transformants were grown on YPRG medium (1% yeast extract, 2% peptone, 1% galactose, 1% raffinose, 2% agar) supplied with H₂O₂, triadimefon and tebuconazole at the concentrations indicated in the figure legends. The experiments were repeated three times.

Construction of *BcSKN7* deletion and complemented mutants

A *BcSKN7* deletion vector pCA-BcSkn7-UD was constructed by inserting two flanking sequences of *BcSKN7* into two sides of *HPH* (hygromycin resistance) in the pBS-HPH1 vector (Liu *et al.*, 2007). An 896-bp upstream flanking fragment of *BcSKN7*, amplified from 38B1 genomic DNA using the primer pair BcSkn7-up-F and BcSkn7-up-R, was inserted into the *Xho*I-*Sal*I site of the pBS-HPH1 vector to generate the plasmid pBS-BcSkn7-up. Subsequently, a 905-bp downstream flanking fragment of *BcSKN7*, amplified from 38B1 genomic DNA using the primer pair BcSkn7-down-F and BcSkn7-down-R, was inserted into the *Bam*HI-*Sac*I site of the pBS-BcSkn7-up vector to generate the plasmid pBS-BcSkn7-UD. The linear DNA fragment for transformation was amplified from pBS-BcSkn7-UD using the primer pair BcSkn7-up-F and BcSkn7-down-R, and transferred into the wild-type 38B1. Protoplast formation and transformation of *B. cinerea* were performed using a previously published protocol (Gronover *et al.*, 2001).

The BcSkn7 deletion mutant (ΔBcSkn7-1) was complemented with the full-length *BcSKN7* to confirm that phenotypic changes of the mutant were caused by the gene deletion. As ΔBcSkn7-1 could not survive on 20 mM H₂O₂, we used 20 mM H₂O₂ as a selective marker. The complete *BcSKN7* gene, including the 392-bp upstream and 394-bp terminator regions, was amplified from genomic DNA of the wild-type strain with the primer pair BcSkn7-com-F and BcSkn7-com-R, and the resulting PCR product was purified and used in the protoplast transformation, except that 20 mM H₂O₂ was used as a selective marker.

Nucleic acid manipulations

Fungal genomic DNA was isolated as described by McDonald and Martinez (1990). Plasmid DNA was isolated using a Plasmid Miniprep Purification Kit (BioDev Co., Beijing, China). Southern blot hybridization analysis of *BcSKN7* in the transformants of *B. cinerea* was performed using a 905-bp downstream fragment of *BcSKN7* as a probe. The probe was

labelled with digoxigenin (DIG) using the High Prime DNA Labelling and Detection Starter Kit II, according to the protocol of the manufacturer (Roche Diagnostics; Mannheim, Germany). DNA extracted from *B. cinerea* was digested with *Xho*I and used for Southern hybridization analysis.

Expression analysis of *BcSKN7* under various stresses

Expression levels of the *BcSKN7* gene in the wild-type 38B1 were measured by a real-time PCR method. Briefly, the strain was grown in potato dextrose broth at 25 °C for 2 days in a shaker. After treatment with 0.5 M NaCl, 20 mM H₂O₂ or 2.5 µg/mL triadimefon for 2 h, mycelia of each strain were harvested and ground in liquid nitrogen. RNA extraction and reverse transcription were performed using the protocol described previously (Yan *et al.*, 2010). The real-time PCR amplifications were conducted in a DNA Engine Opticons 4 System (MJ Research, Inc., Waltham, MA, USA) using TaKaRa SYBR Premix Ex Taq (TaKaRa Biotech. Co.). There were two replicates for each sample. For each sample, PCR amplifications with the primer pair β -tubulin-F and β -tubulin-R for the quantification of expression of the β -tubulin gene were performed as a reference. The experiment was repeated three times independently. Gene expression levels were calculated using the 2^{- $\Delta\Delta C_t$} method (Livak and Schmittgen, 2001).

Western blot analysis

Each strain was grown in potato dextrose broth at 25 °C for 2 days in a rotary shaker. After treatment with 0.5 M NaCl or 20 mM H₂O₂ for 2 h, mycelia of each strain were harvested and ground in liquid nitrogen. The extraction of protein and Western blot were performed as described previously (Liu *et al.*, 2011; Yang *et al.*, 2012). For the detection of BcSak1, an anti-Hog1 antibody (C-terminal anti-Hog1) from Santa Cruz Biotechnology (Santa Cruz, CA, USA) was used. Phosphorylation of BcSak1 in *B. cinerea* was examined using an antibody against dually phosphorylated p38 (Thr180/Tyr182) (Cell Signaling Technology, Beverly, MA, USA) (Liu *et al.*, 2008, 2011; Segmüller *et al.*, 2007). The experiment was repeated four times. The intensities of the Western blotting bands were quantified with the program Quantity One (Bio-Rad, Hercules, CA, USA).

Pathogenicity assays

Leaves of 3-week-old tomato and cucumber were inoculated with 5-mm-diameter plugs of 4-day-old cultures. The pathogenicity test was performed on both wounded and unwounded leaves. Inoculated tissues were incubated at 25 °C with 16 h of daylight for up to 60 h. The experiment was repeated four times. In addition, the inoculated leaves were examined for the presence of H₂O₂ and O₂⁻ using the DAB and NBT staining methods, as described previously (Doke, 1983; Thordal-Christensen *et al.*, 1997).

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SUPPORTING INFORMATION

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

Fig. S1 Amino acid alignments of *Botrytis cinerea* Skn7 (BcSkn7, XP_001554269.1) with those of *Alternaria alternata* Skn7 (AaSkn7, AFK08432.1), *Aspergillus parasiticus* Skn7 (ApSkn7, AAZ14865.1), *Candida albicans* Skn7 (CaSkn7, XP_716869.1), *Clavispota lusitanae* Skn7 (ClSkn7, XP_002616177.1), *Neurospora crassa* Skn7 (NcSkn7, XP_959541.2) and *Saccharomyces*

cerevisiae Skn7 (ScSkn7, EEU07729.1). Boxshade program was used to highlight identical (black shading) and similar (grey shading) amino acids. The conserved N-terminal DNA-binding domain and the C-terminal receiver domain are underlined.

Fig. S2 Generation and identification of the *BcSKN7* deletion mutant of *Botrytis cinerea*. (A) Gene deletion strategy of *BcSKN7*. The hygromycin-resistant cassette (*HPH*) is denoted by the large grey box. Primer (codes 1–8) binding sites are indicated by arrows (see Table S1 for the primer sequences). (B) Identification of *BcSKN7* deletion and complemented mutants by polymerase chain reaction (PCR) analyses with the primer pair BcSkn7-out-F and BcSkn7-out-R. (C) Southern blot hybridization analysis for the identification of the *BcSKN7* deletion mutant and complemented strain using the downstream fragment of *BcSKN7* as a probe. Genomic DNA preparations from the wild-type progenitor 38B1, *BcSKN7* deletion mutant Δ BcSkn7-1 and complemented strain Δ BcSkn7-1-C were digested with *XhoI*.

Fig. S3 Pathogenicity of the wild-type strain 38B1, the deletion mutant Δ BcSkn7-1 and the complemented strain Δ BcSkn7-1-C on wounded tomato and cucumber leaves. Agar plugs without fungal mycelia were used as negative controls (CK). The images were taken 60 h after inoculation.

Fig. S4 Accumulation of O_2^- and H_2O_2 in tomato leaf inoculated with mycelial plugs of 38B1, Δ BcSkn7-1 and Δ BcSkn7-1-C after 10 h of inoculation. The agar plug without fungal mycelia was used as a control (CK). The accumulations of H_2O_2 and O_2^- at inoculation sites (indicated by the light brown spots) were stained with 3,3'-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT), respectively.

Table S1 Oligonucleotide primers used in this study.