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A two-component histidine kinase *Shk1* controls stress response, sclerotial formation and fungicide resistance in *Sclerotinia sclerotiorum*

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SUMMARY

Fungal histidine kinases (HKs) are involved in osmotic and oxidative stress responses, hyphal development, fungicide sensitivity and virulence. Members of HK class III are known to signal through the high-osmolarity glycerol mitogen-activated protein kinase (HOG MAPK). In this study, we characterized the *Shk1* gene (SS1G_12694.3), which encodes a putative class III HK, from the plant pathogen *Sclerotinia sclerotiorum*. Disruption of *Shk1* resulted in resistance to phenylpyrrole and dicarboximide fungicides and increased sensitivity to hyperosmotic stress and H_2O_2-induced oxidative stress. The *Shk1* mutant showed a significant reduction in vegetative hyphal growth and was unable to produce sclerotia. Quantitative real-time polymerase chain reaction (qRT-PCR and glycerol determination assays showed that the expression of *SsHOG1* (the last kinase of the Hog pathway) and glycerol accumulation were regulated by the *Shk1* gene, but *PAK* (p21-activated kinase) was not. In addition, the *Shk1* mutant showed no change in virulence. All the defects were restored by genetic complementation of the *Shk1* deletion mutant with the wild-type *Shk1* gene. These findings indicate that *Shk1* is involved in vegetative differentiation, sclerotial formation, glycerol accumulation and adaption to hyperosmotic and oxidative stresses, and to fungicides, in *S. sclerotiorum*. Taken together, our results demonstrate, for the first time, the role of two-component HKs in *Sclerotinia*.

INTRODUCTION

Two-component histidine kinases (HKs) regulate responses to environmental stimuli in bacteria and eukaryotes, including yeasts, plants, slime moulds and filamentous fungi (Bahn, 2008; Nemecek *et al*., 2006; Tanaka and Izumitsu, 2010). Most eukaryotic and all fungal HKs are of the hybrid type, in that the HK domain and the response regulator (RR) domain are present in a single protein. In hybrid-type HKs, the phosphate group on the aspartate residue of the HK RR domain is transferred to a histidine residue of a histidine phosphotransfer (HPT) protein, and the RR protein is phosphorylated by the phosphorylated HPT protein (West and Stock, 2001). In eukaryotic cells, HKs are generally found at the head of intracellular signalling pathways that recruit more conventional downstream signalling modules, such as mitogen-activated protein kinase (MAPK) cascades (West and Stock, 2001). In the yeast *Saccharomyces cerevisiae*, one HK, Sln1, is involved in responses to high osmolarity (Posas *et al*., 1996). In filamentous fungi, HKs are classified into 11 groups based on the protein sequence (Catlett *et al*., 2003), and only members of HK class III have been associated with responses to high osmolarity; these HKs include Os-1 in *Neurospora crassa* (Ochiai *et al*., 2001), NikA in *Aspergillus nidulans* (Hagiwara *et al*., 2007), Bos1 in *Botrytis cinerea* (Cui *et al*., 2002; Viaud *et al*., 2006), Nik1 in *Alternaria brassicicola* and *Cochliobolus heterostrophus* (Avenot *et al*., 2005; Yoshimi *et al*., 2004) and Hik1 in *Magnaporthe oryzae* (Motoyama *et al*., 2005a). In addition, mutations in class III HKs can result in fungicide resistance and morphological defects (Hagiwara *et al*., 2007; Motoyama *et al*., 2005a; Ochiai *et al*., 2001; Viaud *et al*., 2006; Yoshimi *et al*., 2004). Class III HKs are also involved in the virulence of phytopathogenic fungi. Deletion of class III HKs strongly reduces the virulence of *Fusarium oxysporum*, *B. cinerea* and *A. brassicicola* (Cho *et al*., 2009; Liu *et al*., 2008; Rispail and Pietro, 2010; Viaud *et al*., 2006), but not of *M. oryzae* (Motoyama *et al*., 2005a). Similarly, point mutations in the class III HK *Mf-os1* decreased the virulence of *Monilinia fructicola* (Ma *et al*., 2006), but not of naturally occurring fungicide-resistant field isolates of *B. cinerea* or *A. brassicicola* (Avenot *et al*., 2005; Cui *et al*., 2002). These results indicate that the roles of two-component HKs vary significantly in different fungi.

Sclerotinia sclerotiorum is a necrotrophic, phytopathogenic, filamentous ascomycete with a broad host range and a worldwide distribution. Over 400 species of plant are susceptible to this pathogen, including many agronomic and horticultural crops (Boland and Hall, 1994; Duan *et al*., 2012; Purdy, 1979; Tu, 1997). Owing to inadequate levels of host resistance, the application of fungicides is the principal tool for the control of *Sclerotinia* diseases on most crops (Steadman, 1979). Although the dicarboximide fungicides iprodione and dimetachlone have been used for many years to control *Sclerotinia* diseases worldwide, and although the phenylpyrrole fludioxonil has strong fungicidal

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activity against *S. sclerotiorum*, the modes of action of these fungicides are not well known (Kuang *et al*., 2011; Ma *et al*., 2009; Shi *et al*., 2000; Zhang *et al*., 2002). Interestingly, dicarboximide fungicide-resistant strains of *B. cinerea*, *N. crassa* and *M. fructicola* show increased sensitivity to osmotic stress (Cui *et al*., 2002; Ma *et al*., 2006; Oshima *et al*., 2002). These studies suggest a relationship between osmoregulation and dicarboximide resistance in phytopathogenic fungi.

In *Fusarium graminearum*, *N. crassa* and *C. heterostrophus*, all deletion mutants of *Os1*, *Os2*, *Os4* and *Os5* genes, which are involved in osmoregulation, were highly resistant to dicarboximide fungicides (Fujimura *et al*., 2003; Ochiai *et al*., 2007; Yoshimi *et al*., 2004; Zhang *et al*., 2002). In *B. cinerea*, however, *BcOs1* deletion mutants were resistant to dicarboximide fungicides, and the *BcOs2*, *BcOs4*, *BcOs5* and *BcRrg1* deletion mutants remained sensitive to these fungicides (Liu *et al*., 2008; Segmüller *et al*., 2007; Viaud *et al*., 2006; Yan *et al*., 2010, 2011; Yang *et al*., 2012). These results suggest that the components in the osmotic signalling pathway have different roles in different phytopathogenic fungi. In the current research, we used a gene deletion and complementation strategy to characterize the class III HK *Shk1*, which is a key component of the osmotic signalling pathway in *S. sclerotiorum*.

RESULTS

Characterization of the *Shk1* gene

The *Shk1* gene was identified by a search of the *S. sclerotiorum* genome sequence deposited in the Broad Institute (http://www.broadinstitute.org) using the program BLAST. The *Shk1* nucleotide sequence was 4324 bp in length and was predicted to have five introns of 54, 64, 186, 54 and 36 bp, located at positions 712, 911, 2342, 3794 and 4277 of the sequence, respectively. The existence of these introns was verified by reverse transcription-polymerase chain reaction with the primer pair P1/P2 (Table 1), which amplified 3930- and 4324-bp fragments from cDNA and genomic DNA, respectively. Sequencing of the 3930-bp product obtained from cDNA verified the predicted position and size of the introns. The predicted 1310-amino-acid sequence of *Shk1* has 78% identity to *Os1* of *N. crassa* (NCU02815.5) and 98% identity to *BcOs1* (BC1G_00374) of *B. cinerea*. Analysis of the *Shk1* sequence with the InterProScan prediction server (http://www.ebi.ac.uk/Tools/InterProScan/) detected all the characteristic domains of class III HKs, including six HAMP repeats (IPR003660) (Aravind and Ponting, 1999), the HK a signal transducer domain (HK;

Table 1 Primers and probes of *Shk1* used in this study.

Primer code*	Sequence (5′→3′)†	Relevant characteristic
P1	ATGGGGGACACTACGATAGC	Amplify the full cDNA sequence of the *Shk1* gene
P2	CAGAACTGCTTAGTACAGGTT	
P3	AAAAGGTTGAACGGGCTTGTGTC	Amplify the left homologous arm of the *Shk1* gene of *S. sclerotiorum* (1450 bp)
P4	CCACCAGCCAGCCAACAGCTCCCATCGCAGTCGTGTGAGCTATCG	
P5	CAATACGCAAACCGCCTCTCCCCGTCCTTCACTTGTAACGGCAG	Amplify the right homologous arm of the *Shk1* gene of *S. sclerotiorum* (1541 bp)
P6	ATGACATCTTGCGGACTTGGGGC	
P7	GGGAGCTGTTGGCTGGCTGGTGG	Amplify the *hph* gene (1764 bp)
P8	GGGGAGAGGCGGTTTGCGTATTG	
P9	AGAACACTGACTGATGAAAGG	Amplify the knockout vector of the *Shk1* gene of *S. sclerotiorum* (4260 bp)
P10	CAGGGTGAAAGATAGGATACT	
P11	AACGGAGGGTATTCTCGGGG	Amplify a partial fragment of the *Shk1* gene of *S. sclerotiorum* (482 bp)
P12	TGTCTTTCAGCGAAGCGATT	
P13	CAAAGCATCAGCTCATCGAGAG	Amplify a partial fragment of the *hph* gene (503 bp)
P14	GAAAAGTTCGACAGCGTCTCC	
P15	GAGTAAAAACCGTTGATG	Confirm whether the *hph* genes homologously replaced the *Shk1* gene of *S. sclerotiorum* (2255 bp)
P16	GTCTGGACCGATGGCTGT	
P17	TCATTTGGATGCTTGGGTAG	Confirm whether the *hph* genes homologously replaced the *Shk1* gene of *S. sclerotiorum* (2333 bp)
P18	CCTTAAACCAGATTCCGTAC	
P19	CTGCAGAAccaccatgttggGTCGACAGAAGATGATATTG	Amplify the NEO cassette containing a trpC promoter (1181 bp)
P20	CCGctcgagTCAGAAGAACTCGTCAAGAAGGCG	
P21	TAAgcggccgcGCTGTACGAGTGATGCGA	Amplify the *Shk1* gene (include the control region of the *Shk1* gene) (6598 bp)
P22	TAActgcagATAGTAGCAAACACTGCCAG	
P23	TGAAGGGAAAGAGGAGAAGA	Amplify a probe for Southern blotting (560 bp)
P24	GATTGGGAGCAGGATAAGAA	
P25	TCAAGCCAAGCAACATCCTCGT	Amplify the *SsHOG1* gene for quantitative real-time PCR
P26	ATCCCGCACTCCAAACATCAAC	
P27	GAGTTTGTCGGTGCTCCCTTTG	Amplify the *PAK* gene for quantitative real-time PCR
P28	ATCATACGCTGCCATTCCTTCG	
P29	CCCCAGCGTTCTACGTCT	Amplify the reference gene actin for quantitative real-time PCR
P30	CATGTCAACACGAGCAATG	

*The number code refers to the primer binding sites shown in Fig. 1A.

†The respective restriction enzyme sites included in the primers are listed in lowercase letters in the sequence.

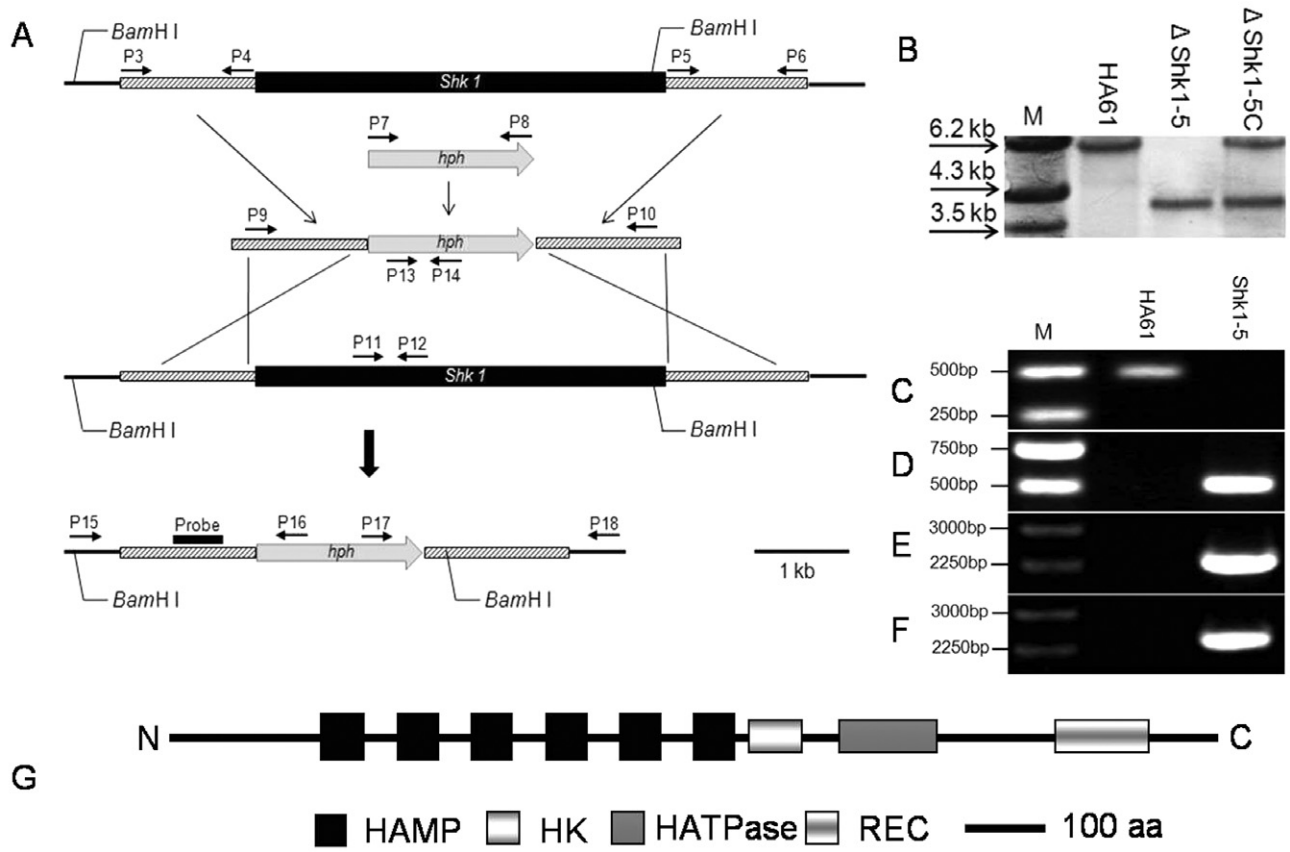

Fig. 1 Generation and identification of *Shk1* gene deletion mutants and the domain structure of Shk1 in *Sclerotinia sclerotiorum*. (A) Gene replacement strategy for *Shk1*. The hygromycin resistance cassette (*hph*) is denoted by the large grey arrow. Primer (P3–P18) binding sites are indicated by arrows (see Table 1 for the primer sequences). (B) Southern blot hybridization analysis of strains using the 5′-flanking region of *Shkl* as a probe. Genomic DNA of the wild-type progenitor HA61, *Shk1* deletion mutant ΔShk1-5 and complemented strain ΔShk1-5C were digested with *Bam*HI. (C) Primer pair P11/P12 was used to specifically amplify the partial Shk1 (482 bp). (D) Primer pair P13/P14 was used to validate the selectable marker *hph* (503 bp). (E, F) Primer pairs P15/P16 and P17/P18 were used to amplify the two homologous arms with a partial fragment of the connecting area (2255 bp in E and 2333 bp in F). (G) Schematic representation of the domain structure of the two-component histidine kinase gene (*Shk1*) in *S. sclerotiorum*. aa, amino acid.

IPR003661), the HK-like ATPase domain (HATPase; IPR003594) and the RR domain (REC; IPR001789) (Fig. 1G).

Targeted knockout of the *S. sclerotiorum Shk1* gene and complementation

To explore the biological role of *Shk1* in *S. sclerotiorum*, a ΔShk1 null allele was generated by replacing the *Shk1* gene with the hygromycin resistance cassette, using a fusion polymerase chain reaction (PCR) method (Fig. 1A). Among 212 hygromycin-resistant transformants, three *Shk1* deletion mutants were identified by PCR with different combinations of gene-specific primers. Primer pair P11/P12 (Table 1, Fig. 1A), which amplifies a partial region in the *Shk1* gene, generated a 482-bp fragment in wild-type strain HA61, but not in the mutant ΔShk1-5 (Fig. 1C). Primer pair P13/P14 (Table 1, Fig. 1A) was used to amplify a 503-bp fragment of the *hph* gene, which was only detected in ΔShk1-5 (Fig. 1D). Then, primer pairs P15/P16 and P17/P18, which amplified the left and right flanking regions, generated 2255- and 2333-bp fragments from ΔShk1-5 (Fig. 1E, F), respectively. These amplification products indicated that the knockout fragment had replaced the *Shk1* gene locus after double-crossover homologous integration. As shown in Fig. 1B, when probed with the 560-bp 5′-flanking region of *Shk1*, the *Shk1* deletion mutant ΔShk1-5 had a 3.7-kb band, but lacked a 6.1-kb band, which was present in the wild-type progenitor. Southern blot analysis confirmed that the transformant ΔShk1-5 was the result of a single homologous replacement event at the *Shk1* locus, and the complemented strain ΔShk1-5C had integrated an intact copy of the *Shk1* gene into the genome of the mutant ΔShk1-5.

Involvement of *Shk1* in vegetative growth and sclerotial formation

To test whether *Shk1* was required for vegetative hyphal growth, colony growth was investigated on potato dextrose agar (PDA)

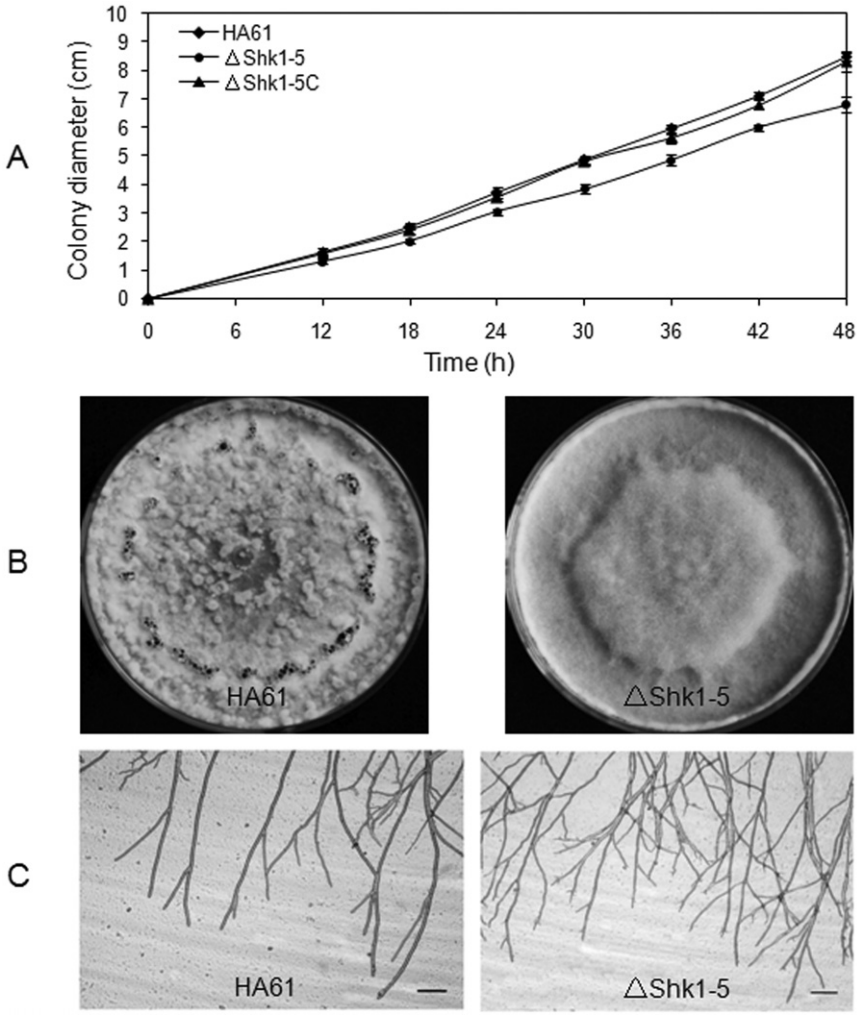

Fig. 2 Biological characteristics of the *Shk1* deletion mutant. (A) Mycelial growth rate of the wild-type strain HA61, the *Shk1* deletion mutant ΔShk1-5 and the complemented strain ΔShk1-5C on potato dextrose agar (PDA). Bars denote standard errors from three repeated experiments. (B) Colonies of the wild-type strain HA61 and the mutant ΔShk1-5 grown on PDA for 10 days. (C) Hyphal tip branching patterns of HA61 and ΔShk1-5 on PDA for 1 day. Bar, 100 μm.

plates. The growth rate of ΔSkh1-5 showed a significant reduction compared with that of the wild-type progenitor, but the growth rate did not differ between the complemented strain ΔShk1-5C and the wild-type progenitor (Fig. 2A). After incubation on PDA for 15 days, ΔSkh1-5 was unable to produce sclerotia and showed a distinct colony morphology (Fig. 2B). Microscopic examination of hyphae grown on PDA was observed. Compared with the wild-type progenitor, the *Shk1* deletion mutant had more hyphal branches and grew in close proximity to each other at the extension zone (Fig. 2C). These results indicate that *Skh1* plays a role in hyphal growth, branching and sclerotial formation in *S. sclerotiorum*.

Shk1 is required for the sensitivity to fungicide, osmotic and oxidative stresses

Fungicide sensitivity tests showed that ΔShk1-5 was highly resistant to both dicarboximide and phenylpyrrole fungicides and grew well on PDA amended with 1 μg/mL fludioxonil, 5 μg/mL iprodione or 5 μg/mL dimetachlone, whereas the complemented strain ΔShk1-5C restored fungicide sensitivity to the wild-type level (Fig. 3). HA61, ΔShk1-5 and ΔShk1-5C exhibited similar sensitivity to the benzimidazole fungicide carbendazim (Fig. 3). Compared with the wild-type progenitor and the complemented strain, ΔShk1-5 exhibited significantly reduced mycelial growth on PDA supplemented with NaCl, KCl, glucose and D-sorbitol at the concentrations indicated in the figure legends (Fig. 4), and was more sensitive to salt stress than to D-sorbitol (Fig. 4). In addition, the mutant ΔShk1-5 showed increased sensitivity to oxidative stress generated by H_2O_2 on PDA plates. ΔShk1-5 was unable to grow on PDA amended with 50 mM H_2O_2, which inhibited only 30%–40% of mycelial growth of the wild-type progenitor and the complemented strain. These results indicate that *Shk1* is involved in the response of *S. sclerotiorum* to phenylpyrrole and dicarboximide fungicides, and hyperosmotic and oxidative stresses.

Shk1 regulates glycerol accumulation

Glycerol accumulation induced by fungicides and osmotic stress has been reported in *S. cerevisiae* and *N. crassa* via the activation

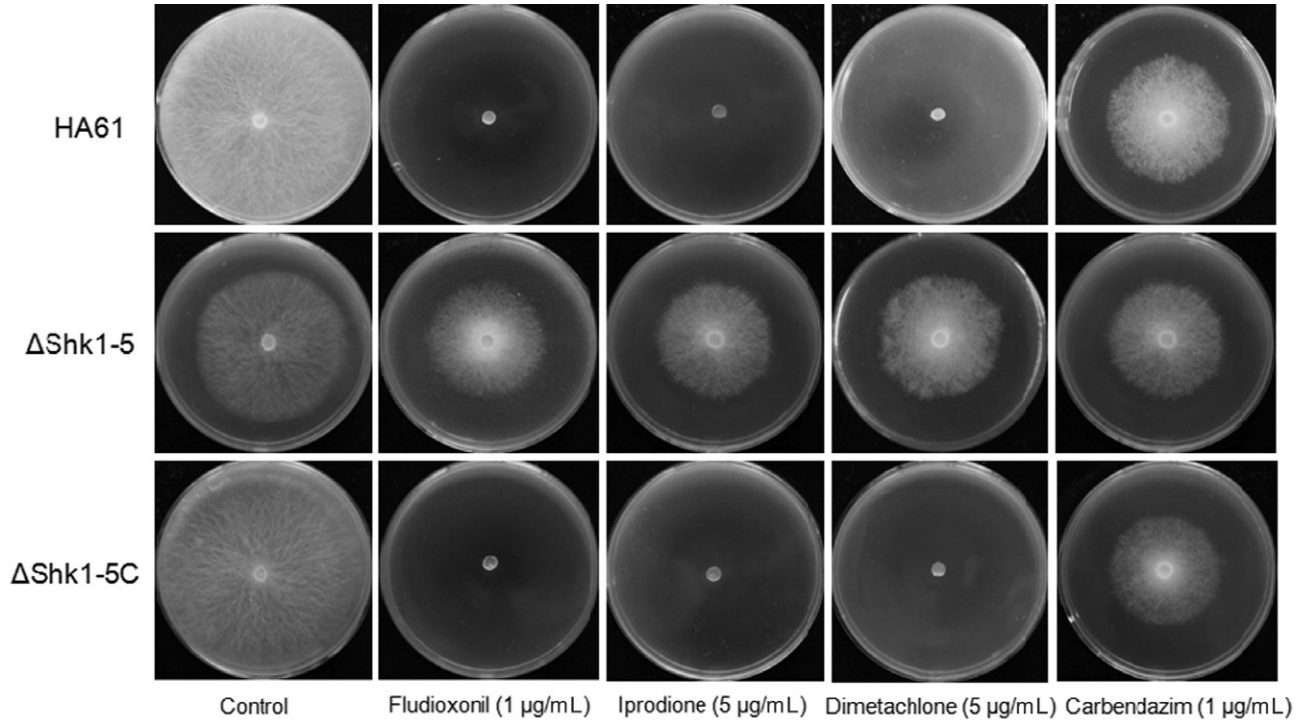

Fig. 3 Sensitivity of the wild-type strain HA61, the *Shk1* deletion mutant ΔShk1-5 and the complemented strain ΔShk1-5C of *Sclerotinia sclerotiorum* to different fungicides. Cultures were grown for 2 days on potato dextrose agar (PDA) amended with fungicide as indicated.

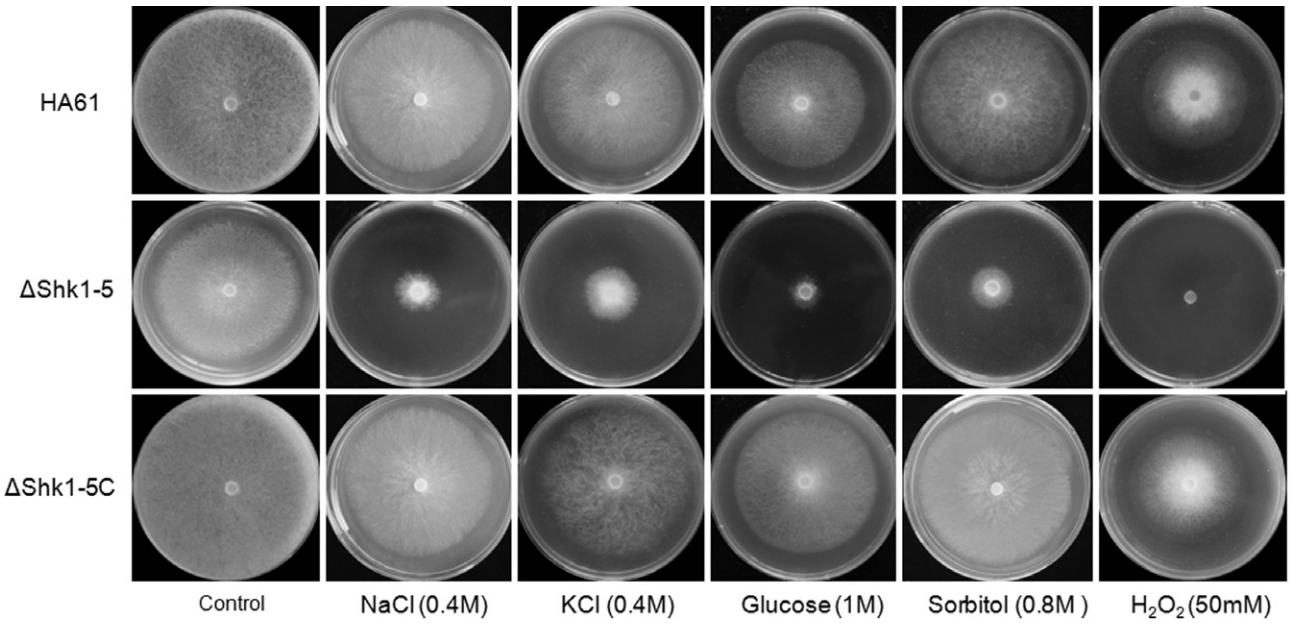

Fig. 4 Sensitivity of the wild-type strain HA61, the *Shk1* deletion mutant ΔShk1-5 and the complemented strain ΔShk1-5C to osmotic stress (generated by NaCl, KCl, glucose or sorbitol) and oxidative stress (generated by H_2O_2). Cultures were grown on potato dextrose agar (PDA).

of the high-osmolarity glycerol (HOG) pathway (Lew, 2010; San-Jose *et al*., 1996; Wojda *et al*., 2003). We therefore analysed glycerol accumulation in the mycelia of ΔShk1-5 after 1, 2 and 4 h of treatment with 0.1 μg/mL fludioxonil, 1 μg/mL dimetachlone and 0.4 M NaCl. As shown in Fig. 5, in the absence of fungicides and osmotic stress, very little glycerol was detected in the wild-type progenitor or the ΔShk1-5 mutant. When treated with fungicides and NaCl, glycerol concentration in the wild-type progenitor

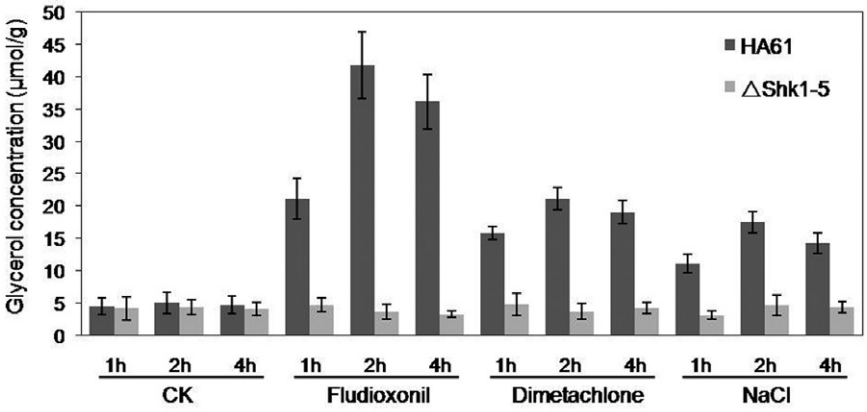

Fig. 5 Intracellular glycerol concentration (in micromoles per gram of dried mycelia) of the wild-type strain HA61 and the *Shk1* deletion mutant ΔShk1-5. The mycelia of each strain were treated with 0.1 μg/mL fludioxonil, 1 μg/mL dimetachlone or 0.4 M NaCl for 1, 2 and 4 h after growth in YEPD (10 mg/mL peptone, 3 mg/mL yeast extract, 20 mg/mL glucose) for 2 days. The cultures without treatment were used as the controls (CK). Bars denote standard errors from three repeated experiments.

increased gradually with time, but that in the ΔShk1-5 mutant was not obvious. This suggests that fungicides and osmotic stress failed to induce glycerol accumulation in the ΔShk1-5 mutant (Fig. 5). In the complemented strain ΔShk1-5C, glycerol accumulation with or without fungicides and NaCl was similar to that of the wild-type (data not shown). These results show that the accumulation of glycerol in response to fungicides and osmotic stress is under the control of *Shk1* in *S. sclerotiorum*.

Regulation of *SsHOG1* and p21-activated kinase (*PAK*) expression

The SS1G_07590 gene is orthologous to the MAPK gene of the HOG pathway in the budding yeast (Saito and Tatebayashi, 2004), and is named as *SsHOG1* in this study. *SsHOG1* is the last kinase of the Hog pathway in *S. sclerotiorum*; we were therefore interested in analysing the effects of *Shk1* deletion on the expression of *SsHOG1*. Quantitative real-time PCR (qRT-PCR) assays showed that the expression of *SsHOG1* was up-regulated by various treatments in the wild-type strain and ΔShk1-5 (Fig. 6). The expression levels of *SsHOG1* in ΔShk1-5 were significantly lower than those in the wild-type strain under various stress conditions (Fig. 6). In addition to the osmoregulating MAPK *HOG1*, *PAK* is also involved in osmotic stress responses (Leberer *et al*., 1992; Liu *et al*., 1993; O'Rourke and Herskowitz, 1998; Raitt *et al*., 2000; Ramer and Davis, 1993; Roberts and Fink, 1994). Therefore, the expression of *PAK* in ΔShk1-5 was determined. The results indicated that the expression of *PAK* did not differ between the wild-type and ΔShk1-5, even in various stress conditions (Fig. 6). This indicates that *PAK* may act in independent and additive pathways, but not in the Hog MAPK pathway.

Shk1 is not required for pathogenicity

The pathogenicity of the *Shk1* deletion mutant was evaluated on several plant leaves. On wounded leaves of rapeseed, strawberry, tomato and cucumber plants, HA61, ΔShk1-5 and ΔShk1-5C caused serious disease lesions of similar size 2 days after inoculation (Fig. 7A–E). Studies of the infection process of *S. sclerotiorum* on bean hypocotyls and pea pods have documented the formation of infection cushion structures which may participate in the penetration and pathogenicity process (Huang and Kokko, 1992; Lumsden and Dow, 1973). To determine whether the *Shk1* deletion mutant confers a change in the production of infection cushions, we placed agar plugs colonized with the wild-type or the *Shk1* deletion mutant on a transparent hydrophobic surface (empty Petri dishes) (Harel *et al*., 2006). The wild-type and *Shk1* deletion mutant both developed typical infection cushions after 24 h (Fig. 7F). These results indicate that, unlike certain other class III HKs (Rispail and Pietro, 2010), *Shk1* is not required for pathogenicity on plant leaves.

DISCUSSION

Fungal HKs have essential functions in osmotic and oxidative stress responses, cell cycle control and virulence (Motoyama *et al*., 2005b). In particular, class III HKs mediate the cellular responses to osmotic and oxidative stresses (Motoyama *et al*., 2005a; Viaud *et al*., 2006), conidiation and asexual morphology (Viaud *et al*., 2006), the mould-to-yeast transition of dimorphic fungi (Nemecek *et al*., 2006), fungicide resistance (Motoyama *et al*., 2005a; Ochiai *et al*., 2001; Viaud *et al*., 2006; Yoshimi *et al*., 2004) and pathogenicity (Liu *et al*., 2008; Nemecek *et al*., 2006; Viaud *et al*., 2006; Yamada-Okabe *et al*., 1999). However, the function of class III HKs in these physiological processes differs greatly according to the type of mutation and fungal species. In this study, we characterized *Shk1*, a class III HK from the plant pathogen *S. sclerotiorum*, and demonstrated that *Shk1* controls the resistance of *S. sclerotiorum* to phenylpyrrole and dicarboximide fungicides (Fig. 3). This result is consistent with that reported in *N. crassa*, *M. grisea*, *C. heterostrophus*, *B. cinerea*, *F. oxysporum*, and other fungi (Liu *et al*., 2008; Motoyama *et al*., 2005a; Ochiai *et al*., 2001; Viaud *et al*., 2006; Yoshimi *et al*., 2004).

Higher sensitivity to osmotic stress, including stress generated by salts and polyols, and to H_2O_2 has been reported in most mutants lacking class III HKs (Alex *et al*., 1996; Hagiwara *et al*.,

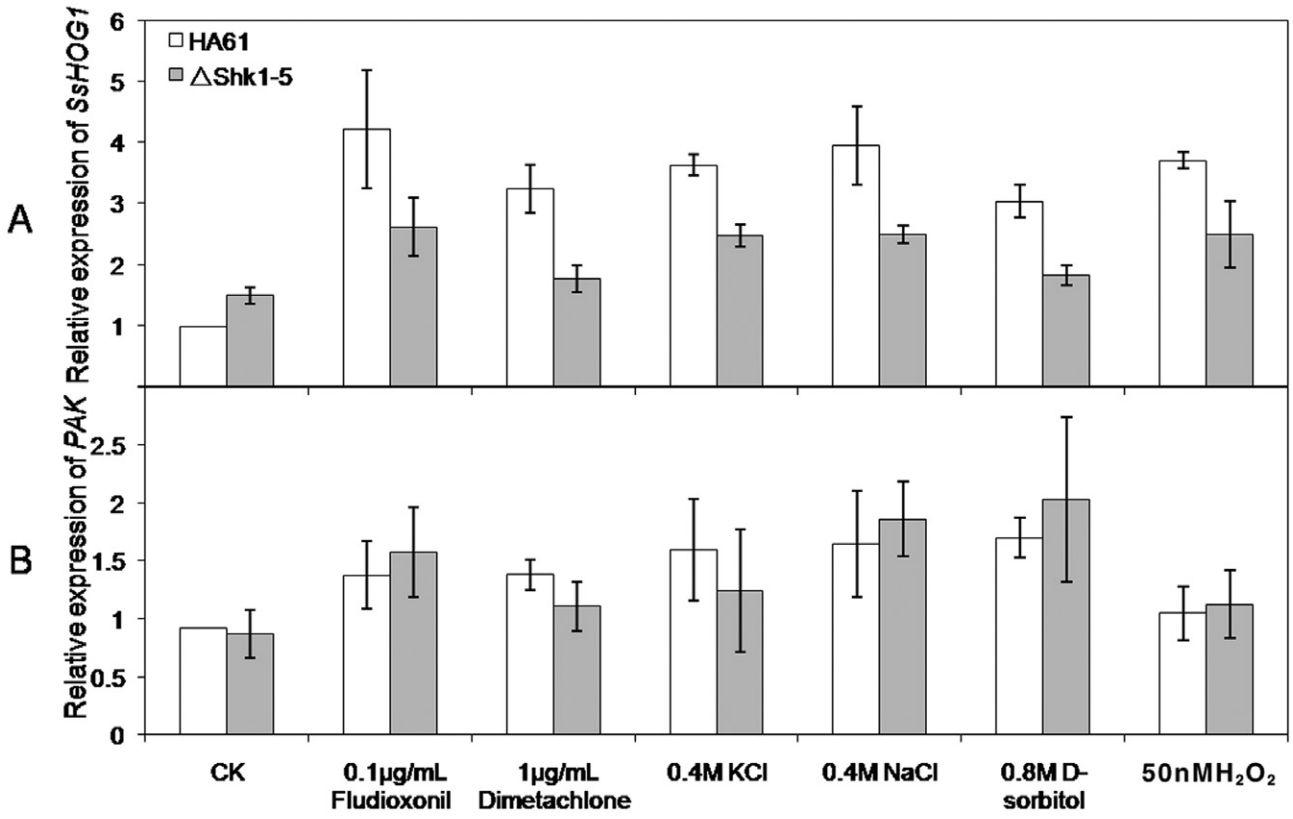

Fig. 6 Relative expression levels of the *SsHOG1* (A) and *PAK* (B) genes in the wild-type strain HA61 and the *Shk1* deletion mutant ΔShk1-5. RNA samples were extracted from the mycelia of each strain untreated or treated with the fungicide fludioxonil (0.1 μg/mL), dimetachlone (1 μg/mL), 0.4 M NaCl, 0.4 M KCl, 0.8 M D-sorbitol or 50 mM H_2O_2 for 3 h after being grown in YEPD (10 mg/mL peptone, 3 mg/mL yeast extract, 20 mg/mL glucose) for 2 days. The cultures without treatment were used as the controls (CK). The relative expression levels of *SsHOG1* and *PAK* in the wild-type strain or ΔShk1-5 under different stress conditions were calculated as the amount of *SsHOG1* and *PAK* mRNA divided by the amount in the wild-type strain without treatment. Vertical bars in each column denote the standard errors of three repeated experiments.

2007; Motoyama *et al*., 2005a; Ochiai *et al*., 2001; Viaud *et al*., 2006; Yoshimi *et al*., 2004). In addition, the sensitivity of these mutants to osmotic stress differs depending on the osmoticum. For instance, mutants of *M. oryzae* and *F. oxysporum* show a strong growth reduction on polyols, but not on salt (Motoyama *et al*., 2005a; Rispail and Pietro, 2010), whereas mutants of *N. crassa*, *C. heterostrophus* and *B. cinerea* show a higher sensitivity to salt (NaCl or KCl) than to polyols (glycerol, sorbitol) (Alex *et al*., 1996; Viaud *et al*., 2006; Yoshimi *et al*., 2004). *Sclerotinia sclerotiorum* showed a similar trend, i.e. ΔShk1 mutants were more sensitive to salt stress than to sorbitol.

In the filamentous fungus *F. graminearum*, the HOG MAPK pathway is involved in the regulation of hyphal growth and branching (Zheng *et al*., 2012). Therefore, we determined the effects of *Shk1* deletion on vegetative hyphal growth in *S. sclerotiorum*. In addition to the reduced growth rate, the *Shk1* deletion mutant had more hyphal branches and grew in close proximity to each other at the extension zone. Surprisingly, ΔSkh1-5 was unable to produce sclerotia and showed a distinct colony morphology on PDA plates (Fig. 2B). Glycerol production and accumulation are well known to be regulated by the HOG pathway in *S. cerevisiae*. Our results indicated that glycerol production was also regulated by *Shk1* in *S. sclerotiorum*, and glycerol accumulation was also induced by fungicides and NaCl treatment in the wild-type but not in the *Shk1* mutant (Fig. 5). In summary, these data showed that the HOG pathway activated by *Shk1* regulates hyphal growth and branching in *S. sclerotiorum*. It also plays a critical role in sclerotial formation, glycerol production and accumulation. In eukaryotic cells, HKs are found to act at the head of the HOG pathway (West and Stock, 2001). Therefore, it is important to characterize further the functions of the HOG pathway in hyphal growth, sclerotial formation and glycerol accumulation.

Ste20 activates distinct MAPK cascades that control filamentous growth, mating and osmotic stress responses (Leberer *et al*., 1992; O'Rourke and Herskowitz, 1998). The *PAK* gene (SS1G_02450) is orthologous to Ste20 and is involved in osmoadaptation in *S. sclerotiorum* (Duan Y.B., Ge C.Y. and Zhou M.G., unpublished data). *SsHOG1* (SS1G_07590) is orthologous to the *Fghog1*, *BcOs2* and Os2 genes of *F. graminearum*, *B. cinerea* and *N. crassa*, respectively, and the last kinase of the Hog pathway in

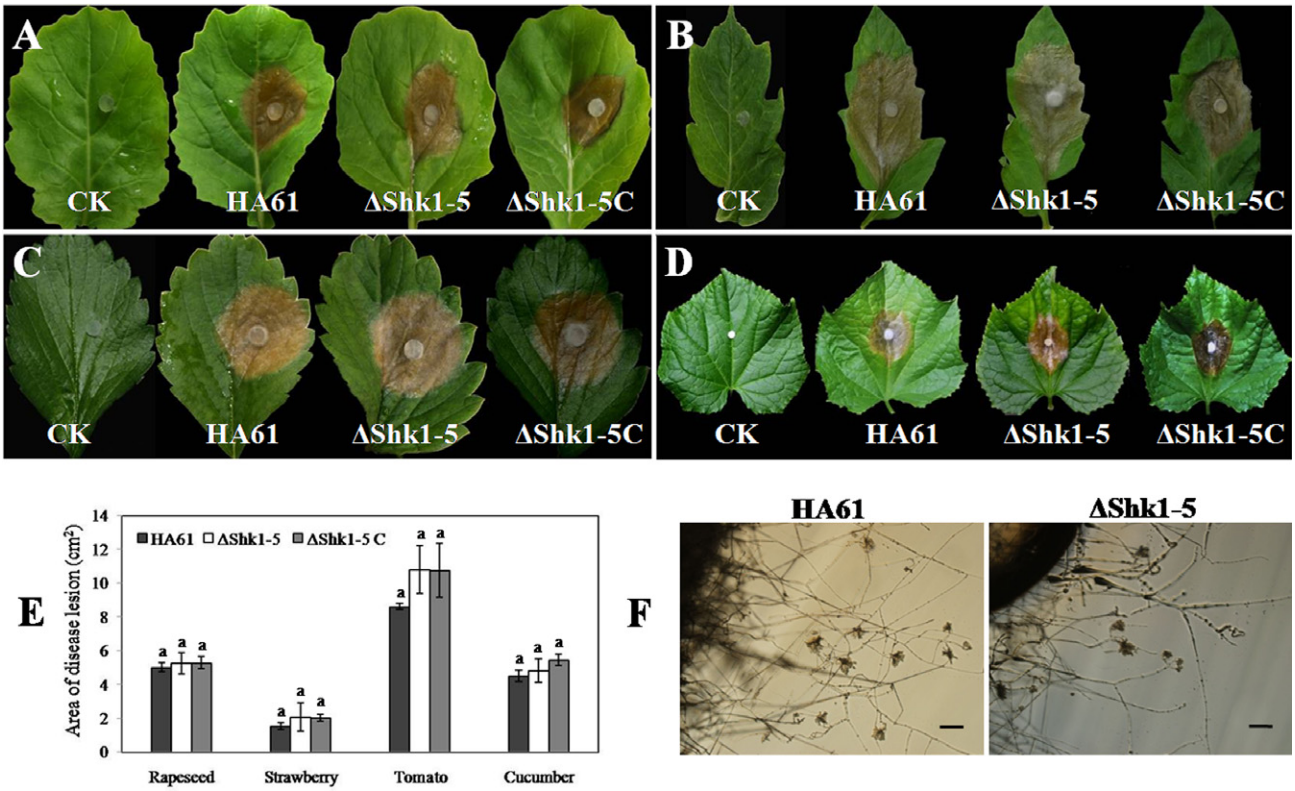

Fig. 7 Pathogenicity assays on detached leaves of four host plants and infection cushion formation. Photographs of leaves of rapeseed (A), tomato (B), strawberry (C) and cucumber (D) were taken after culture for 2 days. (E) Area of disease lesions. Bars denote the standard errors of four repeated experiments. The lowercase letters above the bars indicate that the lesion size for each host was unaffected by the strain ($P > 0.05$). (F) Infection cushion formation assay. Potato dextrose agar plugs colonized with the wild-type (left) or ΔShk1-5 (right), 1 day after transfer to a hydrophobic surface. Bar, 100 μm.

S. sclerotiorum. In the current study, we found that the expression of *SsHOG1* was regulated by the *Shk1* gene, but *PAK* was not involved in the HOG pathway in *S. sclerotiorum*. To further explore relationships between the HOG pathway and the pathways controlled by *PAK*, side-by-side comparisons of various phenotypes among the mutants involved in the pathways could provide clearer information.

The role of class III HKs in virulence has been studied in plant pathogens, and phenotypes of mutants range from highly virulent to completely avirulent, depending on the type of mutation, genetic background and fungal species. For instance, class III HKs are required for full virulence in *F. oxysporum*, *B. cinerea* and *A. brassicicola*, but not in *M. oryzae* (Cho *et al*., 2009; Liu *et al*., 2008; Motoyama *et al*., 2005a; Rispail and Pietro, 2010; Viaud *et al*., 2006). It was therefore necessary to determine the role of class III HKs in the virulence of *S. sclerotiorum*. The results obtained with four species of host plant indicated that *Shk1* is not required for virulence in *S. sclerotiorum*. We conclude that the function of HKs in pathogenicity differs among phytopathogenic fungi.

That fludioxonil-resistant isolates of several filamentous fungi are also resistant to dicarboximide fungicides indicates that the mechanism of resistance is similar for both groups of fungicide (Avenot *et al*., 2005). Mutations in class III HKs increase dicarboximide resistance, indicating that class III HKs could be a primary target of the dicarboximides. This inference, however, must be confirmed with direct binding studies. In addition to involving the *os-1* gene that encodes the class III HK, resistance to dicarboximides in *N. crassa* also involves *os-2*, *os-4* and *os-5* (Fujimura *et al*., 2000; Yang *et al*., 2012). Therefore, it will be interesting to isolate and characterize such genes from *S. sclerotiorum* in order to fully understand the resistance of *S. sclerotiorum* to dicarboximide fungicides. The findings from this study and previous reports have shown that the roles of osmotic signalling systems differ among fungi. An understanding of these differences should aid in the development of new fungicides and other methods of fungal control.

EXPERIMENTAL PROCEDURES

Strains and culture conditions

Strain HA61 of *S. sclerotiorum* was used for transformation in this study. It was isolated from rapeseed in Jiangsu Province, China, and is sensitive to fludioxonil and dicarboximide (Duan *et al*., 2013). The hyphal structure of

each strain growing on PDA was examined using an Olympus microscope (Olympus Optical Co., Ltd, Tokyo, Japan).

Autoclaved PDA was used to culture the wild-type progenitor HA61 and its derived mutants in routine assays for *in vitro* sensitivity to fludioxonil, dimetachlone, iprodione, carbendazim, NaCl, KCl, H_2O_2, glucose and D-sorbitol at the concentrations indicated in the figure legends (Chen *et al*., 2012). Each plate was inoculated with a plug (diameter, 5 mm) of mycelium from the leading edge of a 2-day-old colony. After the plates had been incubated at 25 °C for 2 days, the mean colony diameters (minus the diameter of the inoculation plug) were measured. The percentage of radial growth inhibition (RGI) was calculated using the formula RGI (%) = $[(C - N)/(C - 5)] \times 100$, where *C* is the colony diameter of the control and *N* is the colony diameter of the treatment. The experiment was performed three times.

Identification of the kinase gene *Shk1* in *S. sclerotiorum*

The *Shk1* (SS1G_12694.3) gene was originally identified through a homology search of the *S. sclerotiorum* genome sequence (available at http://www.broadinstitute.org/annotation/genome/sclerotinia_sclerotiorum/MultiHome.html) using BLAST with the *Os1* gene from *N. crassa* (Alex *et al*., 1996; Schumacher *et al*., 1997) and the *BcOs1* gene from *B. cinerea* (Liu *et al*., 2008; Viaud *et al*., 2006) as queries. The autocalled gene *Shk1* has five predicted introns. To verify the existence and size of the introns, RNA was extracted from the mycelia of the wild-type progenitor HA61 with the RNAsimple Total RNA Kit (Tiangen Biotech. Co., Beijing, China) and was used for reverse transcription with the PrimeScript RT Reagent Kit (TaKaRa, Dalian, China). Reverse transcription PCR amplification of the cDNA was conducted with the primers P1/P2 (Table 1). The resultant PCR product was purified, cloned and sequenced.

Construction of the Δ*Shk1* null allele and fungal transformation

The knockout construct Shk1up-hph-dn of *S. sclerotiorum Shk1* was generated by fusion PCR (Yu *et al*., 2004). First, 1.4-kb upstream and 1.5-kb downstream flanking fragments of *Shk1* were amplified from the genomic DNA of *S. sclerotiorum* strain HA61 with the primer pairs P3/P4 and P5/P6 (Table 1, Fig. 1A), respectively. The 5′ regions of P4 and P5 contained the complementary sequences of primers P7 and P8, respectively, which were used to amplify a 1.8-kb cassette containing the hygromycin B resistance gene under the control of the *Aspergillus nidulans* trpC promoter (Mullins *et al*., 2001). Then, the two *Shk1* flanking fragments were mixed with the hygromycin B resistance cassette in a molar proportion of 1:1:3 and fused using the external P9 and P10 primers (Table 1, Fig. 1A). The construct generated was used to transform protoplasts of the wild-type strain HA61.

Polyethylene glycol (PEG)-mediated protoplast transformation was performed as described previously, but with some modifications (Maier *et al*., 2005). To generate protoplasts, plugs from the edge of a 2-day-old colony on PDA were placed in 250-mL flasks containing 100 mL of liquid YEPD (10 mg/mL peptone, 3 mg/mL yeast extract, 20 mg/mL glucose), and the flasks were placed on a rotary shaker (175 rpm, 25 °C) for 36 h. Mycelia were collected on a sterile filter and washed twice with distilled water to remove medium. Then, 0.1 g of mycelia was resuspended in 20 mL of protoplast solution (10 mg/mL of lysing enzymes from *Tichoderma harzianum*; Sigma, St. Louis, MO, USA) and digested for 2.5 h at 30 °C and 85 rpm. Protoplasts were transformed and plated on regeneration medium (0.5 g/L yeast extract, 0.5 g/L casein hydrolysate, 0.7 M sucrose and 16 g/L agar powder). After 12 h, regeneration plates were overlaid with 10 mL of selective medium (0.5 g/L yeast extract, 0.5 g/L casein hydrolysate, 1 M sucrose and 12 g/L agarose) containing 100 μg/mL hygromycin B; the plates were incubated at 25 °C. After 4–10 days, hygromycin-resistant colonies appeared, and individual transformants were transferred onto PDA plates amended with hygromycin B at 100 μg/mL. The plates were incubated at 25 °C for 1 day, and single hyphal tips from each transformant were picked with a sterile needle, transferred to PDA containing hygromycin B (100 μg/mL) and used in subsequent experiments. Transformants showing the homologous insertion of the construct were detected by PCR amplification of genomic DNA with four pairs of primers, P11/P12, P13/P14, P15/P16 and P17/P18 (Table 1, Fig. 1A), and confirmed by Southern analysis of genomic DNA digested with *Bam*HI and hybridized with a labelled probe obtained by PCR amplification with primers P23/P24 (Table 1, Fig. 1A).

To confirm that the phenotype of the *Shk1* deletion mutant resulted from deletion of the gene, a *Shk1* deletion mutant was complemented with the full-length *Shk1* gene. The *Shk1* complement plasmid pNEO-Shk1-Com was constructed using the backbone of pCAMBIA 1300 (CAMBIA, Canberra, Australia). First, a *Bst*XI-*Xho*I NEO cassette containing a trpC promoter (resistance to neomycin) was amplified from plasmid PII99-Pro(DOHH)GFP with primers P19/P20 (Table 1) and cloned into the *Bst*XI-*Xho*I site of pCAMBIA 1300 to create plasmid pNEO. Then, the full-length *Shk1* gene, including the 1891-bp upstream and 383-bp terminator regions, was amplified from genomic DNA of the wild-type strain with primers P21/P22 (Table 1), and subsequently cloned into the *Sac*I-*Sma*I site of pNEO to generate the complement plasmid pNEO-Shk1-Com. Before plasmid pNEO-Shk1-Com was transformed into strain ΔShk1-5, *Shk1* in this plasmid was sequenced to ensure sequence correctness. Transformation of ΔShk1-5 with the full-length *Shk1* gene was conducted as described above, except that neomycin (100 μg/mL) was used as a selection agent.

Determination of intracellular glycerol accumulation

Glycerol accumulation in the mycelia of each strain was measured as described previously with some modifications (Henkel and Stoltz, 1982). Briefly, each strain was grown in YEPD for 2 days at 25 °C in a shaker. After treatment with 0.1 μg/mL fludioxonil, 1 μg/mL dimetachlone or 0.4 M NaCl for 1, 2 and 4 h, the mycelia of each strain were harvested and ground in liquid nitrogen. Then, the mycelial powders (0.1 g) were transferred to a 2-mL microcentrifuge tube containing 1 mL glycerol extraction buffer (Applygen Technologies Inc., Beijing, China). After vortexing for 5 min, the tubes were centrifuged at 5000 ***g*** for 20 min; 5 μL of supernatant in each tube was mixed with 195 μL of detection buffer (Applygen Technologies Inc.). After the mixture had been incubated at 37 °C for 20 min, the glycerol concentration was determined at a wavelength of 550 nm using a SpectraMax M5 microplate reader (Molecular Devices Inc., Sunnyvale, CA, USA). The experiment was repeated twice.

Determination of the expression of *SsHOG1* and *PAK* in the *Shk1* deletion mutant

To extract total RNA, the mycelial plugs of the wild-type strain HA61 or the *Shk1* deletion mutant ΔShk1-5 were transferred to YEPD and cultured for

2 days at 25 °C in the dark. Before the total RNA was extracted, the culture was treated for 3 h with fludioxonil, dimetachlone, NaCl, KCl, D-sorbitol or H_2O_2 at the concentrations indicated in the legend of Fig. 5. Extraction of total RNA and reverse transcription were performed as described above. The expression of *SsHOG1* and *PAK* was determined by qRT-PCR. The qRT-PCR amplifications were performed in a 7500 ABI PRISM Sequence Detector System (Applied Biosystems, Foster City, CA, USA) using SYBR Green I fluorescent dye detection. Amplification was conducted in a 20-μL volume containing 10 μL of iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 2 μL of the reverse transcription product and 1 μL each of the forward and reverse primers (500 nM each) (Table 1). There were three replicates for each sample. For each sample, PCR amplifications with primers P29/P30 (Table 1) for the quantification of the expression of the actin gene were performed as a reference. The experiment was repeated twice. The expression of *SsHOG1* and *PAK* in the wild-type progenitor or ΔShk1-5 under fungicide, osmotic or oxidative stress conditions, relative to that in the wild-type strain without treatment, was calculated using the $2^{-\Delta\Delta Ct}$ method (Aravind and Ponting, 1999).

Plant infection assays

To determine whether the pathogenicity of the *Shk1* deletion mutant varied on different host plants, healthy, fully expanded leaves of rapeseed, strawberry, tomato and cucumber plants were inoculated with 5-mm-diameter plugs of 2-day-old cultures growing on PDA; for controls, leaves were inoculated with plugs that lacked fungi. Before inoculation, the leaves were wounded with a sterile needle tip to facilitate the penetration of the fungus into plant tissue. Inoculated plant leaves were incubated in a growth chamber (25 °C; relative humidity, 85%; 16 h daylight). The diameter of the lesion on each leaf was recorded 2 days after inoculation. There were 10 replicate leaves for each strain, and the experiment was repeated three times.

Infection cushion formation assay

Five-millimetre-diameter plugs of 2-day-old cultures from the wild-type and the *Shk1* deletion mutant growing on PDA were placed on the surface of empty Petri dishes (nine per dish). The Petri dishes were maintained at 100% relative humidity and 25 °C for 24 h. The formation of infection cushions was monitored by light microscopy using an Olympus microscope (Olympus).

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