Expression of the Histidine Kinase Gene *Sshk* Correlates with Dimethachlone Resistance in *Sclerotinia sclerotiorum*

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ABSTRACT

Histidine kinases (HK) are implicated in virulence, vegetative mycelial growth, and osmotic and oxidative responses in pathogenic fungi. Our previous work showed that transcriptional levels of the group III HK gene *Sshk* are higher in field dimethachlone-resistant isolates of *Sclerotinia sclerotiorum* compared with sensitive isolates. However, it is not clear whether the overexpression of *Sshk* is the major mechanism for resistance to dimethachlone. In this study, we constructed *Sshk* silencing and overexpression vectors and assessed dimethachlone resistance levels, virulence, mycelial growth, and sensitivity to osmotic stress for the *Sshk*-silenced and -overexpression transformants. Overexpression of *Sshk* resulted in resistance to dimethachlone and increased sensitivity to various stresses and to the cell-wall-perturbing agents sodium dodecyl sulfate (SDS) and Congo red (CR). Compared with the parent isolate,

Sshk-silenced transformants had reduced resistance to dimethachlone, significantly higher ($P < 0.05$) mycelial growth and virulence, and lower sclerotium production, and were less sensitive to various exogenous stresses such as sodium chloride. Compared with the parent sensitive isolate HLJMG1, dimethachlone resistance ratios of the three overexpression transformants $\Delta C101$, $\Delta C21$, and $\Delta C10$ increased 168.1-, 189.5-, and 221.2-fold, respectively. The three overexpression transformants were more sensitive to CR and SDS than their parent isolate. These findings suggest that overexpression of *Sshk* is a major mechanism for dimethachlone resistance in some isolates of *S. sclerotiorum*, and that *Sshk* plays an important role in maintaining the integrity of the cell wall. Our findings reveal a novel molecular mechanism for dimethachlone resistance in plant-pathogenic fungi.

Sclerotinia sclerotiorum (Lib.) de Bary is a necrotrophic fungal plant pathogen with a worldwide distribution capable of infecting over 400 species of plants, leading to severe economic losses in the production of oil crops and vegetables (Bardin and Huang 2001; Bolton et al. 2006; Letham et al. 1976). Due to the lack of genetic resources that could be utilized in crop breeding programs, the control of *S. sclerotiorum* has been relying heavily on the application of fungicides (Bolton et al. 2006). The benzimidazole fungicide carbendazim had been widely used to control *S. sclerotiorum* in China prior to 2000, and high levels of carbendazim resistance were reported in the late 1990s in East China (Pan 1998). Since the early 2000s, the dicarboximide fungicide (DCF) dimethachlone has been widely used to control *S. sclerotiorum* in China (Kuang et al. 2011). Due to repeated applications of dimethachlone, medium to high levels of dimethachlone resistance have been reported subsequently in China (Ma et al. 2009; Zhou et al. 2014a,b). Previous studies indicate that the group III histidine kinase (HK) genes that function within a two-component signal transduction pathway are involved in DCF resistance in several filamentous fungi (Alex et al. 1996; Cui et al. 2002; Duan et al. 2013; Luo et al. 2012; Motoyama et al. 2005).

The two-component signal transduction system consists of an HK domain and a response regulator (RR) domain. Phosphorylation of histidine of HK and aspartic residues of RR leads to the activation of a signal transduction pathway, resulting in the recruitment of downstream mitogen-activated protein kinase cascades (West and Stock 2001). In pathogenic yeast and fungi, the two-component pathway mediates osmoregulation, mycelial development, and cell

growth (Calera et al. 2000; Li et al. 1998; Srikantha et al. 1998; Thomason et al. 1999; Wurgler-Murphy and Saito 1997). The two-component signal pathway in filamentous fungi is similar to but more complex than that of budding yeast because the latter has a sole HK gene, *SLN1*, and the deletion of this gene is lethal (Posas et al. 1996). Compared with yeast, filamentous fungi have a diverse number of HK genes (Ochiai et al. 2002; Tanaka and Izumitsu 2010). The putative HK genes are categorized into 11 groups (Catlett et al. 2003), of which group III HK lacks a transmembrane structure and is primarily involved in the response to high osmolarity (Duan et al. 2013; Izumitsu et al. 2010; Luo et al. 2012; Tanaka and Izumitsu 2010; Yoshimi et al. 2004) and in virulence (Cho et al. 2009; Liu et al. 2008; Luo et al. 2012; Rispaill and Pietro 2010; Viaud et al. 2006), conidial morphology (Viaud et al. 2006), and cell wall integrity (Zhang et al. 2010) of phytopathogenic fungi. Numerous studies have demonstrated that mutations of group III HK genes are responsible for DCF resistance in filamentous fungi, and these HK genes include *BcOS1* in *Botrytis cinerea*, *os-1* in *Neurospora crassa*, and *Dic1* in *Bipolaris maydis* (Cui et al. 2002; Ochiai et al. 2001; Oshima et al. 2002; Yoshimi et al. 2004). In *Pyricularia grisea*, *S. sclerotiorum*, and *Alternaria longipes*, deletion of the group III HK genes *HIK1*, *Shk1*, and *AlHK11*, respectively, leads to resistance to DCF and phenylpyrrole fungicides (Duan et al. 2013; Luo et al. 2012; Motoyama et al. 2005). In addition, lost expression of HK gene *SsOs1* may be involved in dimethachlone resistance in *S. sclerotiorum* (Firoz et al. 2016). These studies indicate that the group III HK genes play an important role in DCF resistance in various filamentous fungi.

Our previous studies showed that the expression levels of the group III HK gene *Sshk* are higher in field dimethachlone-resistant isolates than in sensitive ones of *S. sclerotiorum* (Li et al. 2017) but there is no direct evidence to show that increased transcription of the gene *Sshk* is the major mechanism for resistance to DCF. In the current research, therefore, we constructed *Sshk*-silencing and *Sshk* overexpression vectors to determine the correlation between *Sshk* expression and dimethachlone resistance in *S. sclerotiorum*.

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MATERIALS AND METHODS

Isolates and culture conditions. The dimethachlone-resistant *S. sclerotiorum* isolate HLJ4 and the sensitive isolate HLJMG1 were used in the present study, and their effective concentrations for 50% inhibition of mycelial growth (EC_{50} values) were 38.45 and 0.18 $\mu\text{g/ml}$, respectively (Li et al. 2017). Surface-sterilized sclerotia were placed on potato dextrose agar (PDA) dishes and incubated at 23°C in the dark for 2 days, mycelial plugs (5 mm in diameter) were cut from the fresh edge of 2-day-old colonies and transferred to fresh PDA dishes, and the resulting colonies were used for subsequent experiments. *Escherichia coli* strain TOP10 (Weidi Biotechnology, Shanghai, China) was used to propagate all plasmids, and *Agrobacterium tumefaciens* strain EHA105 (Weidi Biotechnology) was used for transformation of *S. sclerotiorum*. The *Sshk*-silenced transformants were cultured on PDA media amended with hygromycin B at 30 $\mu\text{g/ml}$ (Aladdin Biotech, Shanghai, China), and the *Sshk*-overexpression transformants were cultured on PDA media amended with geneticin G418 at 40 $\mu\text{g/ml}$ (Aladdin Biotech).

Construction of *Sshk*-silencing and -overexpression vectors. *Sshk*-silencing and -overexpression vectors were constructed according to published protocols (Yu et al. 2012), with minor modifications. For the construction of the *Sshk*-silencing vector, a 306-bp fragment from *Sshk* was amplified with primers SSHKF and SSHKR (Table 1) from *S. sclerotiorum* cDNA and ligated to the T-Easy plasmid (Promega Biotech, Beijing, China) by T4 DNA ligase to produce the *Sshk*-4 plasmid. The plasmid *Sshk*-4 was digested with restriction endonucleases *Pst*I and *Bam*HI (Takara Biotech, Beijing, China) and the target fragment was subsequently ligated into the digested pCIT vector (Yu et al. 2012) to produce a pCIT1 vector. Subsequently, the plasmid *Sshk*-4 was digested with *Hind*III and *Cl*aI (Takara Biotech) and the target fragment was ligated into the digested pCIT1 to produce a pCIT2 vector. The vector pCIT2 containing the two *Sshk* gene fragments in the opposite orientation was digested with *Xho*I and *Sac*I (Takara Biotech) and ligated into the pCH plasmid to generate an *Sshk*-SM silencing vector.

For the construction of the *Sshk*-overexpression vector, a 3.9-Kb full-length *Sshk* gene was amplified with primers OEHKF and OEHKR (Table 1) from *S. sclerotiorum* cDNA and then ligated into the digested pCETNS-4 vector (Yang et al. 2018) at the *Sac*I and *Sma*I (Takara Biotech) sites to generate the overexpression vector *Sshk*-OE. All constructs were assessed by sequencing analysis and introduced into *A. tumefaciens* EHA105 (Weidi Biotechnology) by the electroporation-mediated method (Yu et al. 2012).

Transcription levels of the *Sshk*-silenced and -overexpression transformants. Mycelial plugs (5 mm in diameter) cut from the edge of 2-day-old colonies were transferred to PDA dishes overlaid with cellophane (Bo'ao Biotechnology), and mycelia of each isolate were harvested after 48 h of incubation at 23°C. Genomic DNA was extracted according to the manufacturer's instructions

(HP Fungal DNA kit; OMEGA Biotek, Shanghai, China), and T-DNA insertions in different transformants were examined by polymerase chain reaction (PCR) using the primers Hyg-F: CAGCGTCTCCGACCTGAT and Hyg-R: TCTGCGGGCGATT TGT (Jiang 2007). To evaluate transcriptional levels of *Sshk* in different transformants, total RNA from each isolate was extracted using TRIZOL Reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized and quantitative reverse-transcription (qRT)-PCR was conducted as previously described (Li et al. 2017). Transcriptional levels of *Sshk* were evaluated by qRT-PCR using the primers RT-F/R (Table 1) and normalized to the β -tubulin gene using the primers β tub-F/R (Table 1), and the relative levels of gene transcription were calculated with the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001). Three replicates for each isolate were analyzed and the experiment was independently repeated three times.

Determination of dimethachlone sensitivity. Dimethachlone sensitivity of the *Sshk*-silenced transformants and the parent isolate HLJ4 was measured on PDA amended with dimethachlone at 0, 10, 20, 30, 40, 50, and 60 $\mu\text{g/ml}$, whereas dimethachlone at 0, 5, 10, 20, 40, 60, and 80 $\mu\text{g/ml}$ was used for the *Sshk*-overexpression transformants. Mycelial plugs (5 mm in diameter) cut from the edge of 2-day-old colonies were transferred onto PDA dishes amended with dimethachlone as described above, and PDA dishes free of dimethachlone were used as the controls. After incubation at 23°C for 2 days, the diameter of each mycelial colony was measured twice at right angles. EC_{50} values were calculated according to Zhou et al. (2014a). The experiment was performed in triplicate and repeated independently four times.

Determination of sclerotial production and mycelial growth on PDA. Mycelial plugs (5 mm in diameter) cut from the edge of 2-day-old colonies were transferred to PDA dishes. Six replicate dishes of each isolate were incubated at 23°C for 30 days. During the first 60 h of incubation, colony diameters were measured once every 12 h. The sclerotial production on PDA was recorded after 30 days of incubation. The experiment was repeated independently twice.

Virulence assays on detached leaves. The virulence assay was performed according to Li et al. (2015). For determination of virulence to cucumber (cultivar Xinyou 1) and oilseed rape (Zhongshuang 10), leaves of similar size and position were detached from approximately 35-day-old plants and transferred to 15-cm Petri dishes lined with wet filter paper. The adaxial surfaces of the leaves were inoculated with mycelial plugs (5 mm in diameter) cut from the fresh edge of 2-day-old colonies of each isolate and transformant. The diameter of each disease lesion was measured twice at right angles after 2 days of incubation (23°C, 85% relative humidity, photoperiod of 16 h of light and 8 h of darkness) for the detached oilseed rape leaves and after 3 days of incubation for the detached cucumber leaves. Six replicates were used for each isolate in each of two independent experiments.

TABLE 1. Polymerase chain reaction primers used in this study

Primer	Sequence (5'–3') ^a	Relevant characteristics ^b
SSHKF	CCCAagcttctgcagATGCGTGAGATGTTGGG	Amplify the silenced fragment of the <i>Sshk</i> gene (306 bp)
SSHKR	CCCatcgatggatccCGAGTCCACAACGATCACATCATAA	
PCIT-F	ACTAGAAGGCACTCTTTGCTGC	Confirm whether the <i>Sshk</i> silenced fragment replaced the PCIT vector (630 bp)
PCIT-R	GACGACGAGGTCAGCAACT	
PCIT-F1	TTCGTGGTCAAGGTCCATC	
PCIT-R1	AGCAGACAGGAACGAGGAC	
OEHKF	CgagctcATGGGGGACACTACGATAGCTC	
OEHKR	CCcccgggTtagTACAGGTTCTTGCAAGTGG	Amplify the overexpression fragment of the <i>Sshk</i> gene (3,920 bp)
β tub-F	TTGGATTGCTCCTTTGACCAG	Amplify the β -tubulin gene for quantitative real-time PCR (104 bp)
β tub-R	AGCGGCCATCATGTTCTTAGG	
Rt-F	AAGGGTAGGGAAGTAAGGCAATC	Amplify the <i>Sshk</i> gene for quantitative real-time PCR (153 bp)
Rt-R	GTTTATAGGGCCAGTGGTAGTGTATG	

^a Lowercase letters represent the restriction enzyme sites in the primer sequence.

^b PCR = polymerase chain reaction.

Sensitivity to osmotic and oxidative stresses. For determination of sensitivity to sodium chloride (NaCl), glucose, sucrose, glycerol, and hydrogen peroxide (H₂O₂), mycelial plugs (5 mm in diameter) cut from the fresh edge of 2-day-old colonies of the parent isolate HLJ4 and the *Sshk*-silenced transformants were transferred onto PDA amended with NaCl at 5, 10, 20, 30, 40, 50, 60, 70, and 80 g/liter; glucose at 10, 20, 40, 60, 80, 100, 150, 200, and 300 g/liter; sucrose at 10, 50, 100, 150, 200, 250, 300, and 350 g/liter; glycerol at 5, 10, 20, 40, 80, 100, and 200 g/liter; or H₂O₂ at 5, 10, 20, 40, 80, 100, 200, 300, and 400 mM. Mycelial plugs (5 mm in diameter) cut from the fresh edge of 2-day-old colonies of the parent isolate HLJMG1 and the *Sshk*-overexpression transformants were transferred onto PDA amended with NaCl at 1.25, 2.5, 5, 10, 20, 30, 40, 50, 60, 70, 80, and 120 g/liter; glucose at 10, 20, 40, 60, 80, 100, 160, 320, and 500 g/liter; sucrose at 5, 10, 20, 40, 80, 100, 150, 200, and 400 g/liter; glycerol at 5, 10, 20, 40, 80, 100, 200, and 400 g/liter; or H₂O₂ at 5, 10, 20, 40, 80, 100, 200, and 400 mM. PDA dishes without amendments were used as the controls. After incubation at 23°C for 2 days, the diameter of each mycelial colony was measured twice at right angles, and percent inhibition of mycelial growth relative to the controls was calculated. The experiment was performed in triplicate and repeated independently twice.

Cell wall and cell membrane integrity assays. Chitin is a crucial component of the cell wall of pathogenic fungi. Congo red (CR) interferes with the integrity of the cell wall by binding to chitin (Ram and Klis 2006). Sodium dodecyl sulfate (SDS) is an ionic surfactant that can dissolve membrane proteins and damage the cell membrane structure (Bulawa 1993). In this study, mycelial plugs (5 mm in diameter) cut from the fresh edge of 2-day-old colonies were transferred onto PDA amended with CR at 0.5, 1, 2, 4, 6, and 8 mg/ml or SDS at 0.0025, 0.005, 0.0075, 0.01, 0.015, 0.02, 0.03, and 0.04% (wt/vol). PDA media without CR or SDS was used as the

control. After incubation for 48 h at 23°C, the diameter of each mycelial colony was measured twice at right angles, and percent inhibition of mycelial growth relative to the control was calculated. The experiment was performed in triplicate and repeated independently twice.

Transmission electron microscopy observations on hyphal cell wall morphology. Transmission electron microscopy (TEM) observations were according to Yu et al. (2012). Mycelia of the parent isolate HLJ4 and the *Sshk*-silenced transformants grown at 23°C for 48 h on cellophane-overlaid PDA were harvested by gentle scraping with a scoop. The samples were fixed at 4°C for 6 h in 4% (vol/vol) glutaraldehyde in 100 mM phosphate buffer (pH = 7.2), thoroughly rinsed with phosphate buffer, postfixed with osmium tetroxide at 4°C for 2 h, and dehydrated in a graded acetone series from 30 to 100%. The samples were then embedded in Epon-821 resin (Hexion, Columbus, OH) and polymerized at 60°C for 24 h. Ultrathin sections (50 nm) were cut from the samples on a Leica Ultracut UCT ultramicrotome with a diamond knife and collected onto 200-mesh copper grids. After the contrasting procedure with uranyl acetate and lead citrate, the grids were examined with an electron microscope H-7650 (Hitachi, Tokyo) at 80 kV. The experiment was performed in triplicate and repeated independently twice.

Data analysis. A paired *t* test was used to analyze data of lesion diameter and sclerotial production, and Fisher's least significant difference test following one-way analysis of variance in SPSS (ver. 17; SPSS Inc., Chicago) was used to evaluate significant differences in expression levels of the *Sshk* gene and EC₅₀ values of dimethachlone. A general linear model was used to evaluate significant differences in slopes of the fitted mycelial growth lines between the parent isolates and the transformants.

RESULTS

Transcriptional levels of *Sshk* in the *Sshk*-silenced and -overexpression transformants. Compared with the parent isolate HLJ4, the transcription levels of *Sshk* in the three silenced transformants Δ HLJ4-108, Δ HLJ4-65, and Δ HLJ4-63 declined by 99.9, 94.1, and 95.1%, respectively (Fig. 1A). Compared with the parent isolate HLJMG1, the *Sshk* transcription levels in the three *Sshk*-overexpression transformants Δ C101, Δ C21, and Δ C10 increased (*P* < 0.01) 7.7-, 10.2-, and 14.1-fold, respectively (Fig. 1B).

Fig. 1. Relative mRNA levels of *Sshk* in **A**, the parent isolate HLJ4 of *Sclerotinia sclerotiorum* and its *Sshk*-silenced transformants and **B**, HLJMG1 and its *Sshk*-overexpression transformants. Bars denote the standard error of the mean of nine observations (three replicates in each of three independent experiments). Different lowercase letters above the bars indicate statistically significant differences ($\alpha = 0.05$).

Fig. 2. Dimethachlone sensitivity in **A**, the parent isolate HLJ4 of *Sclerotinia sclerotiorum* and its *Sshk*-silenced transformants and **B**, HLJMG1 and its *Sshk*-overexpression transformants. EC₅₀ values = effective concentrations for 50% inhibition of mycelial growth. Bars denote the standard error of the mean of 12 observations (three replicates in each of four independent experiments). Different lowercase letters above the bars indicate statistically significant differences ($\alpha = 0.05$).

Fig. 3. Mycelial growth of **A**, the parent isolate HLJ4 of *Sclerotinia sclerotiorum* and its *Sshk*-silenced transformants and **B**, HLJMG1 and its *Sshk*-overexpression transformants; and **C**, sclerotial formation of the *Sshk*-silenced transformants on potato dextrose agar. Photos were taken after incubation for 30 days. Bars denote the standard error of the mean of 12 observations (six replicates in each of two independent experiments).

Dimethachlone resistance in *Sshk*-silenced and -over-expression transformants. Compared with the parent isolate HLJ4, dimethachlone resistance ratios of the three *Sshk*-silenced transformants Δ HLJ4-108, Δ HLJ4-65, and Δ HLJ4-63 declined by 62.9, 42.5, and 49.8%, respectively, and EC_{50} values were 14.3, 22.1, and 19.3 μ g/ml, respectively (Fig. 2A). Compared with the parent isolate HLJMG1, dimethachlone resistance ratios of the three overexpression transformants Δ C101, Δ C21, and Δ C10 increased ($P < 0.01$) 168.1-, 189.5-, and 221.2-fold, respectively, and EC_{50} values were 29.4, 33.2, and 38.7 μ g/ml, respectively (Fig. 2B). These findings indicate that the transcriptional upregulation of the *Sshk* gene is a major mechanism for dimethachlone resistance in some isolates of *S. sclerotiorum*.

Effects of *Sshk* expression on mycelial growth and sclerotial production. Compared with the parent isolate HLJ4, colony diameter of the *Sshk*-silenced transformants Δ HLJ4-108, Δ HLJ4-65, and Δ HLJ4-63 on PDA media increased (Fig. 3A), and there were significant differences in slopes of the fitted mycelial growth lines between the parent isolate and the *Sshk*-silenced transformants (the three *Sshk*-silenced transformants were used as three biological replicates). The silenced transformant Δ HLJ4-65 was incapable of producing sclerotia (Fig. 3C), and the number of sclerotia produced by the transformants Δ HLJ4-108 and Δ HLJ4-63 declined. Compared with the parent isolate HLJMG1, colony diameter of the three overexpression transformants Δ C101, Δ C21, and Δ C10 decreased (Fig. 3B), and there were significant differences in slopes between the parent isolate and the overexpression transformants (the three overexpression transformants were used as three biological replicates), indicating that expression of the *Sshk* gene affects mycelial growth and sclerotial production of *S. sclerotiorum*.

Effects of *Sshk* expression on virulence. Compared with the parent isolate HLJ4, lesion diameters for the silenced transformants Δ HLJ4-108, Δ HLJ4-65, and Δ HLJ4-63 increased significantly ($P < 0.05$) (Fig. 4). Lesion diameters for the overexpression transformants Δ C101, Δ C21, and Δ C10 decreased significantly ($P < 0.05$) compared with the parent isolate HLJMG1. These findings indicate that overexpression of *Sshk* has a negative effect on the virulence of *S. sclerotiorum*.

Effects of *Sshk* expression on sensitivity to osmotic and oxidative stresses. Percent inhibitions of mycelial growth on PDA media supplemented with glucose (Fig. 5A), sucrose (Fig. 5B), NaCl (Fig. 5C), and glycerol (Fig. 5D) were lower for the *Sshk*-

silenced transformants compared with the parent isolate HLJ4, and higher for the overexpression transformants compared with the parent isolate HLJMG1. Percent inhibitions of mycelial growth on PDA amended with H_2O_2 at 200 mM for the parent isolate HLJ4 and *Sshk*-silenced transformants were 100 and 71.8% (average), respectively. Percent inhibitions on PDA amended with 200 mM H_2O_2 were 70.0 and 100% for the parent isolate HLJMG1 and *Sshk*-overexpression transformants, respectively (Fig. 5E). These results indicate that transcription levels of the *Sshk* gene influence osmoadaptation and tolerance to oxidative stress in *S. sclerotiorum*.

Effects of *Sshk* expression on the integrity of hyphal cell wall and cell membrane. Compared with the parent isolate HLJ4, percent inhibitions of mycelial growth on PDA amended with CR were significantly lower according to a paired *t* test ($P < 0.05$) for the three silenced transformants (Fig. 6A), indicating that the *Sshk*-silenced transformants were less sensitive to CR than the parent isolate. Compared with the parent isolate HLJMG1, percent inhibitions of mycelial growth on PDA supplemented with CR

Fig. 4. Lesion development on detached leaves of A1 and B1, cucumber and A2 and B2, rapeseed 3 and 2 days, respectively, after inoculation with *Sclerotinia sclerotiorum*, and the diameters of disease lesions caused by the C, *Sshk*-silenced transformants and D, *Sshk*-overexpression transformants. Bars denote the standard error of the mean of 12 observations (six replicates in each of two independent experiments).

Fig. 5. Inhibition of mycelial growth of the parent isolates HLJ4 and HLJMG1 of *Sclerotinia sclerotiorum* and their *Sshk*-silenced or *Sshk*-overexpression transformants on potato dextrose agar amended with A, glucose; B, sucrose; C, NaCl; D, glycerol; and E, H_2O_2 . Bars denote the standard error of the mean of six observations (three replicates in each of two independent experiments).

increased significantly according to a paired *t* test ($P < 0.05$) for the three overexpression transformants (Fig. 6A). Percent inhibitions of mycelial growth on PDA supplemented with SDS for both the *Sshk*-silenced and -overexpression transformants increased significantly according to a paired *t* test ($P < 0.05$) compared with their respective parent isolates (Fig. 6B). TEM observations indicated that the cell wall and plasma membrane of most cells shrank for the *Sshk*-silenced transformants, the organelles of some cells were destroyed and the contents leaked in the transformants Δ HJLJ4-108 and Δ HJLJ4-65, and the organelles of almost every cell collapsed in Δ HJLJ4-63, leading to cell lysis (Fig. 7). These results suggest that adequate expression of the gene *Sshk* is necessary for maintaining the integrity of the cell wall and cell membrane.

DISCUSSION

Mutations or deletions of group III HK genes were previously shown to mediate resistance to DCF, phenylpyrrole, and aromatic hydrocarbon fungicides (Cui et al. 2002; Duan et al. 2013; Luo et al. 2012; Motoyama et al. 2005; Ochiai et al. 2001; Oshima et al. 2002; Yoshimi et al. 2004). Our previous studies demonstrated that the field dimethachlone-resistant isolates have no cross resistance with the phenylpyrrole fungicide fludioxonil (Zhou et al. 2014a), and expression levels of the group III HK gene *Sshk* are higher in the field isolates than in the sensitive isolates of *S. sclerotiorum* (Li et al. 2017). In this study, therefore, we constructed group III *Sshk*-silencing and -overexpression vectors and demonstrated that silencing of *Sshk* led to a significant decrease in dimethachlone resistance and overexpression of *Sshk* resulted in a dramatic increase in dimethachlone resistance. The transcription levels of *Sshk* in three silenced transformants were downregulated by 99.9, 94.1, and 95.1%, respectively, and dimethachlone resistance ratios declined by 62.9, 42.5, and 49.8%, respectively. The silenced transformants still retained medium levels of dimethachlone resistance. Compared with the dimethachlone-sensitive parent isolate HLJMG1, the *Sshk* transcription levels of three overexpression transformants increased 7.7-, 10.2-, and 14.1-fold, respectively, and the dimethachlone resistance ratio increased 168.1-, 189.5-, and 221.2-fold, respectively. According to a recent study, lost expression of a group III HK gene *SsOs1* may be involved in dimethachlone resistance in *S. sclerotiorum* (Firoz et al. 2016). These results indicate that different mechanisms exist for target resistance to DCF, and transcriptional upregulation of *Sshk* is a

novel mechanism for dimethachlone resistance in some isolates of *S. sclerotiorum*. This is one case of overexpression of the target protein as a resistance mechanism and corroborates that the action targets of the DCF are the group III HK in the two-component signal pathway.

Biological characteristics, including mycelial growth, sclerotium formation, and osmotic response, have been reported in HK-deletion mutants of phytopathogenic fungi (Duan et al. 2013; Firoz et al. 2016; Luo et al. 2012; Rispaill and Pietro 2010; Zhang et al. 2010). Group III HK play different roles in virulence of different fungal species with different genetic backgrounds. For example, the genes *Shk1* and *HIK1* are not required for virulence of *S. sclerotiorum* and *P. oryzae* (Duan et al. 2013; Motoyama et al. 2005) but are required in *Fusarium oxysporum* and *Botrytis cinerea* (Rispaill and Pietro 2010; Viaud et al. 2006). In this study, we demonstrated that overexpression of *Sshk* resulted in resistance to dimethachlone, increased sensitivity to hyperosmotic and oxidative stresses, and reduced virulence. For *S. sclerotiorum*, sclerotia play a major role in long-term survival (Willettts and Wong 1980), and mycelial growth is a primary indicator of aggressiveness (Garg et al. 2010). Our results showed that the *Sshk*-silenced transformants had increased mycelial growth but reduced sclerotium-production capability and shriveled sclerotia. These results indicate that transcription of *Sshk* is involved in sclerotium development and vegetative differentiation in *S. sclerotiorum*.

The fungal cell wall regulates responses of fungal cells to environmental stimuli (Jeon et al. 2008), and chitin is an essential component of the fungal cell wall. The anionic dye CR can combine with chitin, resulting in changes in hyphal morphology such as hyphal tips lysis, cell separation, or activation of responses of the cell wall to stresses (Boorsma et al. 2004; Damveld et al. 2005a,b; García et al. 2004; Lagorce et al. 2003; Pancaldi et al. 1984; Roncero and Duran 1985; Vannini et al. 1983). In *P. oryzae*, the Δ *Mos1* mutant has increased resistance to the cell wall stressor Calcofluor white (Zhang et al. 2010). In the present study, the *Sshk*-silenced transformants became less sensitive to CR, indicating an increase in chitin contents in the cell wall of *S. sclerotiorum*. Interestingly, we also found that the cell walls of most cells were

Fig. 6. Inhibition of mycelial growth of the parent isolates HLJ4 and HLJMG1 of *Sclerotinia sclerotiorum* and their *Sshk*-silenced or *Sshk*-overexpression transformants on potato dextrose agar amended with **A**, Congo red (CR) and **B**, sodium dodecyl sulfate (SDS). Bars denote the standard error of the mean of six observations (three replicates in each of two independent experiments).

Fig. 7. Microscopic structures of the parent isolate HLJ4 of *Sclerotinia sclerotiorum* and *Sshk*-silenced transformants observed by transmission electron microscopy. CW = cell wall, CM = cell membrane, PM = plasma membrane, V = vacuole, C = cytoplasm, M = mitochondrion, and N = nucleus.

damaged and the organelles of some cells collapsed in the *Sshk*-silenced transformants.

In conclusion, the present study demonstrated that transcriptional upregulation of the group III HK gene *Sshk* was a vital mechanism for dimethachlone resistance in some isolates of *S. sclerotiorum*. The transcription levels of *Sshk* had a great impact on fitness-related parameters of *S. sclerotiorum*. Overexpression of *Sshk* reduced mycelial growth and virulence to host plants while it increased sensitivity of *S. sclerotiorum* to exogenous stresses. The fitness costs associated with dimethachlone resistance may account for the slow development of dimethachlone resistance in the field. These findings will advance our understanding of the mechanisms of resistance to the dicarboximide fungicides in *S. sclerotiorum* and should be taken into account in designing resistance management strategies.

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