Pesticide Biochemistry and Physiology 138 (2017) 15–21

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

Molecular and biochemical characterization of dimethachlone resistant isolates of *Sclerotinia sclerotiorum*

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ARTICLE INFO

Article history:
Received 11 October 2016
Received in revised form 30 January 2017
Accepted 5 February 2017
Available online 8 February 2017

Keywords:
Sclerotinia sclerotiorum
Dimethachlone
Fungicide resistance
Resistance mechanism

ABSTRACT

Sclerotinia sclerotiorum is a necrotrophic fungal plant pathogen with a broad host range. The dicarboximide fungicide dimethachlone has been used to control this pathogen for more than a decade and resistance to dimethachlone has recently been reported in China. Compared with sensitive isolates, the three dimethachlone resistant isolates with resistance ratios of 78.3, 85.5, and 94.8 exhibited significantly ($P < 0.05$) higher cell membrane permeability and peroxidase and polyphenol oxidase activities. Dimethachlone at 0.25 μg/mL significantly increased cell membrane permeability and enhanced activity of the two enzymes in both resistant and sensitive isolates. There were no significant differences in glycerol or oxalate content between the resistant and sensitive isolates. Dimethachlone treatment increased glycerol content in the resistant isolates and reduced in the sensitive isolates ($P < 0.01$). Sequencing of three genes involved in two-component signal pathway and of three genes in mitogen-activated protein (MAP) kinase cascade demonstrated that the dimethachlone resistant isolates HLJ4 and HLJ6 harbored point mutations of I232T and G1087D, respectively, in the deduce amino acid sequence of the histidine kinase (HK) gene *Sshk*. HLJ4 had a point mutation of P96L in the deduced amino acid sequence of the MAP kinase-kinase gene *SsPbs*. The expression levels of the *Sshk* gene were higher in HLJ4 and HLJ6 than in HLJ3 and the sensitive isolate HLJMG2, and transcription of the *Sshk* gene was up-regulated by dimethachlone for the three resistant isolates.

1. Introduction

Sclerotinia sclerotiorum (Lib.) de Bary, a cosmopolitan fungal plant pathogen with an extensive distribution worldwide, is capable of infecting over 400 species of plants, including oilseed rape, sunflower, soybean, carrot and onion [2,4,5,23]. *Sclerotinia sclerotiorum* causes 5 to 80% yield losses of oilseed rape annually in China [28,46]. Oilseed rape breeding programs for disease resistance have been hampered because of limited genetic resources, and the control of *S. sclerotiorum* mainly depends upon the application of fungicides [5]. The benzimidazole fungicide carbendazim has been widely used to control this pathogen for several decades, and high levels of resistance have been reported since 1997 in China [36,43]. Due to resistance to carbendazim, the dicarboximide fungicide (DCF) dimethachlone has been employed to control *S. sclerotiorum* in China since the early 2000s [22]. Several years of dimethachlone applications have resulted in reduced sensitivity in Jiangsu Province of China [28,43]. Recently, medium to high levels of dimethachlone resistance with frequencies of 3.8%, 2.7%, and 1.1% have been reported in Shaanxi, Heilongjiang, and Hunan Provinces, respectively [48–49].

Compared with sensitive isolates, plant pathogens resistant to DCFs usually exhibit fitness penalties such as reduced virulence, mycelial growth, and hyper-sensitivity to osmotic pressures [13,17,24,48]. Our previous study demonstrated that field dimethachlone resistant isolates of *S. sclerotiorum* were more sensitive to osmotic stress, less pathogenic to oilseed rape, and grew more slowly on potato dextrose agar (PDA) medium and sclerotia formation was delayed [48]. Laboratory and field mutants of *S. sclerotiorum* resistant to DCFs had lower fitness, were more sensitive to oxidative stress and released more electrolytes compared with the sensitive isolates [17,48]. Laboratory mutants of *S. sclerotiorum* with dual resistance to DCFs and the phenylpyrrole fungicide fludioxonil had significant increases in cell membrane permeability, glycerol and oxalate content, and activity of phenylalanine ammonia-lyase and peroxidase [16]. Previous studies indicated that DCFs resistance may be attributed to mutations or deletion of the two-component histidine kinase (HK) genes [15,27,31].

The molecular mechanisms of DCFs resistance involve two signal transduction pathways: the two-component HK system and mitogen-activated protein (MAP) kinase cascades [3]. Compared with yeasts, filamentous fungi have a larger number of HKs. The putative HKs are categorized into 11 groups [7], of which group III HK plays a primary role in

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http://dx.doi.org/10.1016/j.pestbp.2017.02.001
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response to high osmolarity [38]. Group III HKs are known to regulate the high-osmolarity glycerol (HOG) MAP kinase [15]. In fungi, HKs are of the hybrid type, since the HK domain and the response regulator (RR) domain are present in a single protein. When the cells response to environment stimuli, the histidine residue in the HK domain is auto-phosphorylated, and this phosphate is transmitted to an aspartic acid residue in the RR protein, thus activating the MAP kinase cascade [8,44]. A point mutation (V238A) in the *SsOs1* gene involved in the HOG signaling pathway was found in one dimethachlone resistant isolate of *S. sclerotiorum* [17]. Mutations of the deduced amino acid sequence located in the second, third, fifth or sixth 90-amino acid repeat of the coiled-coil region in the HK genes were detected in iprodione resistant isolates of *S. sclerotiorum* [24]. Iprodione and fludioxonil resistance in *Neurospora crassa* might be related to mutations in the fifth and sixth tandem repeats of HKs [34]. Amino acid changes in the coiled-coil region of the putative HKs might cause DCFs resistance in *Botrytis cinerea* [11,35].

Previous studies indicate that fungal two-component HK systems and MAP kinase cascades play important roles in glycerol accumulation, the response to osmotic stress, pathogenicity and resistance to DCFs [10,17,24,37,39,40,48]. With respect to *S. sclerotiorum*, most studies on DCFs resistance focused on laboratory mutants [14–17], and the molecular mechanisms for DCFs resistance in field resistant isolates of *S. sclerotiorum* are still unclear. In the present study, physiochemical characteristics including glycerol and oxalate contents, cell membrane permeability, peroxidase (POD) and polyphenol oxidase (PPO) activities were measured, and genes involved in two-component signal transduction system (*Sshk, SsYpd, Sssk1*) and a MAP kinase cascade (*Sssk2, SsPbs, SsHog*) were cloned and sequenced in three arbitrarily selected sensitive isolates and three dimethachlone resistant isolates. The objectives of this study were to compare the physiochemical characteristics between dimethachlone resistant and sensitive isolates and to determine the molecular mechanism for dimethachlone resistance.

2. Materials and methods

2.1. Isolates of S. sclerotiorum

Three dimethachlone resistant isolates (HLJ3, HLJ4 and HLJ6) and three sensitive isolates (HLJMG2, HLJMG3, and HLJMG5) were collected from soybean fields of Heilongjiang Province in 2011 [48]. All six *S. sclerotiorum* isolates were named after their collection region, with HLJ representing Heilongjiang Province and MG representing sensitive to dimethachlone. The mature sclerotia were stored at 4 °C in a refrigerator. The resistance ratios of three dimethachlone resistant isolates HLJ3, HLJ4 and HLJ6 were 85.5, 94.8 and 78.3, respectively, compared with the baseline sensitivity of dimethachlone [47].

Sclerotia were surface sterilized in 75% ethanol for 3 min, in 1% sodium hypochlorite solution for 3 min, rinsed in sterile water for 30 s, and dried on sterilized filter paper for 30 min [48]. The sterilized sclerotia were placed on potato dextrose agar (PDA) plates and incubated at 23 °C in the dark for 2 days in a growth chamber. The mycelia were subcultured at 23 °C for subsequent experiments. For each of the six *S. sclerotiorum* isolates, mycelial plugs (5 mm in diameter) from edges of 2-day-old colonies were transferred to cellophane-overlaid PDA plate. For dimethachlone treatment, the final concentration of the fungicide in PDA was 0.25 μg/mL. After incubating at 23 °C for 3 days, mycelia of each isolate were harvested for the following experiments.

2.2. Fungicide

Technical grade dimethachlone (96.2% active ingredient, Wenzhou Pesticide Co. Ltd. Zhejiang Province, China) was dissolved in methanol to produce a 1000 μg/mL stock solution, and the stock solution was stored at 4 °C.

2.3. Determination of glycerol content in mycelia

Glycerol content in mycelia was measured by the cupric glycerinate colorimetric method [14,45]. Briefly, glycerol and copper ions react in an alkaline solution and generate a dark reddish blue complex (cupric glycerinate). This complex has a maximum absorption peak at 630 nm. The standard curve for glycerol content was prepared as follows: 1 mL $CuSO_4$ solution at 0.05 g/L and 3.5 mL NaOH solution at 0.05 g/mL were mixed and added to 50 mL tubes containing 10 mL glycerol. The final concentrations of glycerol were 0, 0.0025, 0.003, 0.004, 0.005, 0.006, 0.008, and 0.01 g/mL. The tubes were centrifuged at 100 rpm for 12 min, and the supernatant was filtered through double filter paper. Absorption at 630 nm of cupric glycerinate was measured with a spectrophotometer.

Mycelia grown on PDA amended with dimethachlone at 0.25 μg/mL were harvested as described above in section 2.1. Mycelia grown on PDA free of dimethachlone were used as the control. Half a gram of mycelia of each isolate was lyophilized and ground in the presence of quartz sand. The mycelial powder was suspended in 20 mL of sterile-distilled water. The suspensions were transferred to 50 mL centrifuge tubes and incubated in water bath at 80 °C for 15 min. After centrifugation at 8500 rpm for 10 min, absorption of the supernatants was measured at 630 nm with sterile-distilled water as the control. The concentrations of glycerol were calculated using the standard curve. The experiment was performed in triplicate and repeated independently twice.

2.4. Determination of oxalic acid content

The method for measuring oxalic acid content was according to Di et al. [12]. Iron (III) and oxalic acid form a complex which will change color under acidic conditions. Hence, the content of oxalate was calculated from absorbance of the complex at 510 nm. The standard curve for oxalate was prepared as follows: 2 mL of $FeCl_3$ solution (0.5 mg/mL), 20 mL of HCl-KCl buffer solution (KCl 50 mM, pH = 2), and 1.2 mL of sulfosalicylic acid solution (5 mg/mL) were mixed and pipetted to 50 mL flasks. Different volumes (0, 0.1, 0.2, 0.4 or 0.8 mL) of sodium oxalate solution (2 mg/mL) were added, and double-distilled water was added to bring the final volume up to 25 mL. The flasks were vortex-stirred for 10 s and incubated at 25 °C for 30 min. Then, absorbance was measured at 510 nm with a spectrophotometer.

To determine oxalic acid content, three mycelial plugs were placed into 250-mL flasks containing 100 mL potato dextrose broth (PDB). For fungicide treatment, the final concentration of dimethachlone in PDB was 0.25 μg/mL. After incubating at 23 °C for 3 days on an orbital shaker at 175 rpm and centrifuging at 1500 rpm for 10 min, absorption of supernatants at 510 nm was determined, with the double-distilled water as a blank. The experiment was performed in triplicate and repeated independently twice.

2.5. Determination of cell membrane permeability

Relative electrical conductivity is used to quantify cell membrane permeability [14]. Mycelia were prepared as described in section 2.3. Mycelia (0.3 g) from each isolate were placed into a 50 mL centrifuge tube containing 30 mL of double-distilled water. Electrical conductivity was measured at room temperature with an electrical conductivity meter (BANTE900, BANTE instruments, Shanghai, China) at 0, 5, 10, 20, 40, 60, 80, 120 and 160 min. After 160 min, mycelia were boiled for 10 min and cooled to room temperature, and the final conductivity was measured. The relative conductivity of mycelia was calculated as follows: Relative conductivity (%) = Conductivity / Final conductivity × 100. The experiment was performed in triplicate and independently repeated twice.

2.6. Determination of peroxidase and polyphenol oxidase activity

Approximately 0.35 g of mycelia was prepared as described in section 2.3 and ground by a homogenizer in the presence of 0.1 g of polyvinyl-polypyrrolidone and 0.8 mL of 0.05 M sodium phosphate buffer (pH = 5.5 for POD and 6.8 for PPO). The suspension was centrifuged at 12000 rpm, 4 °C for 20 min.

POD activity was assayed using guaiacol as the substrate according to Duan et al. [14]. The total reaction volume was 1.5 mL, containing 0.04 mL of supernatant and 0.475 mL of 0.2% guaiacol substrate dissolved in 50 mM sodium phosphate buffer. The increase in absorbance at 470 nm was measured spectrophotometrically at room temperature after 0.5 mL of 0.3% H_2O_2 was added. The unit of enzyme activity was defined as the change in absorbance per min.

PPO activity was assessed according to Wang et al. [41] with minor modifications. Sixty microliters of the supernatant were mixed with 0.69 mL of 0.05 M sodium phosphate buffer (pH 6.8) and 0.75 mL of 0.02 M catechol and incubated at 30 °C for 2 min. Absorbance at 398 nm was measured. The experiment was performed in triplicate and independently repeated twice.

2.7. Cloning and sequencing of genes involved in two-component HK system and MAP kinase cascades

Genomic DNA was extracted from mycelia by the CTAB (Hexadecyl trimethyl ammonium Bromide) method [33]. According to gene functions reported in *S. sclerotiorum* [1], all primers were designed on the basis of the partial sequences of a group III HK gene (*Sshk*), a response regulator gene (*Sssk1*), a His-phosphotransfer gene (*SsYpd*), a MAPK gene (*SsHog*), a MAPKK gene (*SsPbs*), and a MAPKKK gene (*Sssk2*) by Primer Premier 5.0 (ver 5.0, Premier Biosoft, Palo Alto, CA), which were listed in Table 1. Amplification reaction mixtures contained 50 ng of template DNA, 1-5™ 2 × High-Fidelity Master Mix and 400 μM of each primer (Tsing KE Biological Technology, Beijing, China). All amplification reactions were performed using the following protocol: an initial preheating for 2 min at 98 °C, followed by 35 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 15 s, and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min in a DNA Engine System (Bio-Rad, C1000 thermal cycler). Amplified polymerase chain reaction (PCR) products were analyzed in 1% agarose gel electrophoresis and purified with the E.Z.N.A.® Gel Extraction Kit (OMEGA), then cloned into the pGEM-T Easy vector (Promega), and sequenced by Tsing KE Co., Ltd. To avoid sequence mismatch during gene cloning and sequencing, the experiment was performed independently three times. The program DNAman (ver 6.0, Lynnon Corporation, San Ramon, CA) and the InterProScan (http://www.ebi.ac.uk/interpro/search/sequence-search) prediction server were used to compare DNA sequences, deduce and analyze amino acid sequences of the amplified genes from dimethachlone resistant and sensitive isolates.

Table 1
Polymerase chain reaction primers used in this study.

Primer	Sequence (5′–3′)	Target gene
F1	ATGGGGGACACTACGATAGCTC	*Sshk*
R1	TTAGTACAGGTTCCTTGCAAGTGG	
SsHog-F	ATGGCTGAATTCGTTAGAG	*SsHog*
SsHog-R	GACCATCCACCAGATTTTGGGCGT	
SsPbs-F	ATGACAGATAATCCTCAATTAGATTCG	*SsPbs*
SsPbs-R	CATCAAACCCTGCTTTTTCCTCTCA	
SsSk2-F	ATGGAGCGTCCACATATTG	*SsSk2*
SsSk2-R	ACTGAGAGCTTCCACTATCGCTTG	
SsSk1-F	GACGATAGGCGATCTCAAAACACGA	*SsSk1*
SsSk1-R	ATCCCACAACCACCTCATTATCG	
SsYpd-F	ATGTCCTCTTCCACTTCTACCTC	*SsYpd*
SsYpd-R	GTGGCATAAAACTTCTTCAAG	
βtub-F	TTGGATTTGCTCCTTTGACCAG	β-tubulin
βtub-R	AGCGGCCATCATGTTCTTAGG	
Rt-F	AAGGGTAGGGAAGTAAGGCAATC	*Sshk*
Rt-R	GTTTATAGGGCCAGTGGTAGTGTATG	

2.8. Quantitative RT-PCR

Total RNA was extracted by the TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized with the Prime Script ™ RT reagent Kit (TaKaRa). All quantitative RT-PCR experiments were performed in a MyIQ2 real-time PCR detection system (Bio—Rad). The reaction mixture contained 5 μL of SoFast™ EvaGreen® Supermix (Bio-Rad, Hercules, CA, USA), 1 μL of the reverse transcription product, 0.5 μL of (10 mM) each of the forward and reverse primers (Table 1), and 3.0 μL of nuclease-free water. The thermal cycling conditions consisted of 95 °C for 30 s, 40 cycles of at 95 °C for 5 s, at 60 °C for 10 s. The cycles were followed by a melting curve analysis: 10s at a temperature increasing from 55 °C to 95 °C in 0.5 °C increments. The transcription levels of *Sshk* was normalized to the β-tubulin gene. The relative levels of gene transcription were calculated with the $2^{-\Delta\Delta CT}$ method [25]. Three biological replicates for each isolate were used to calculate the mean and standard error.

2.9. Data analysis

We used ANOVAs with independent *t*-tests to analysis data of glycerol content, oxalic acid content, cell membrane permeability and POD, PPO activity in the statistical software SPSS (Statistical Product and Service Solutions, ver. 17.0, SPSS Inc., Chicago, IL). Graphs were constructed with Microsoft Excel (ver. 2010, Microsoft Corporation, Redmond, WA).

3. Results

3.1. Glycerol content

There were no significant differences ($P = 0.065$) in glycerol content between the dimethachlone resistant and sensitive isolates (Fig. 1). Nevertheless, dimethachlone treatment significantly ($P < 0.01$) increased glycerol content by 30.3%, 86.1%, and 27.3% for resistant isolates HLJ3, HLJ4, and HLJ6, respectively; whereas dimethachlone significantly ($P < 0.01$) reduced glycerol content by 13.9%, 19.1%, and 15.5% for sensitive isolates HLJMG2, HLJMG3, and HLJMG5, respectively.

3.2. Cell membrane permeability and oxalic acid content

There were no significant differences ($P = 0.853$) in oxalic acid content between the dimethachlone resistant and sensitive isolates; and

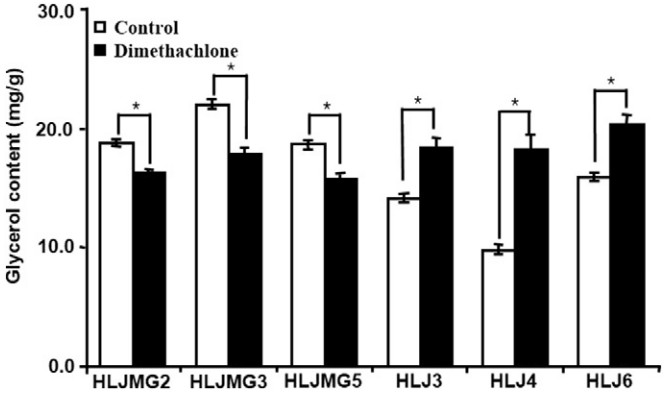

Fig. 1. Glycerol content of dimethachlone resistant and sensitive isolates of *Sclerotinia sclerotiorum*. "*" indicates statistically significant differences (α = 0.05). Values are means (±SE) of two independent repeats. Error bars denote the standard error of the mean according to *t*-test.

dimethachlone did not have a significant effect on oxalic acid levels either in dimethachlone resistant or sensitive isolates (data not shown).

Compared with the three dimethachlone sensitive isolates, the three resistant isolates had significantly higher ($P < 0.05$) cell membrane permeability. For all isolates, dimethachlone at 0.25 μg/mL significantly ($P < 0.05$) increased relative electrical conductivity (Fig. 2), indicating that dimethachlone treatment is conductive to hyphal cell electrolyte leakage.

3.3. Peroxidase and polyphenol oxidase activity

The dimethachlone resistant isolates had significantly ($P < 0.05$) higher POD and PPO activities than the sensitive isolates grown on PDA plates without dimethachlone (Fig. 3). Dimethachlone treatment significantly ($P < 0.05$) increased POD and PPO activities for all isolates tested.

3.4. Newly identified point mutations in the Sshk and SsPbs genes

Sequence analysis showed that isolate HLJ4 had a point mutation in the group III HK gene *Sshk*: ATT was replaced by ACT, leading to an I232T mutation in the deduced amino sequence in the first 90-amino-acid repeat (Fig. 4). Isolate HLJ6 had a point mutation of GGT to GAT at codon position 1087 in the *Sshk* gene. The mutation was located between the kinase core domain and the RR domain, rather than in the 90-amino-acid repeat region. The MAPKK gene *SsPbs* also contained a point mutation of CCA to CTA at codon position 96 in isolate HLJ4. Several synonymous mutations were detected in the *SsYpd* (T324C), *Sssk*1 (A759G), and *Sssk*2 genes (C48T, A3447T and T1736G), which had no effect on the amino acid sequence. In the *SsHog* gene, there were no mutations in the nucleotide sequence for all the three dimethachlone resistant isolates.

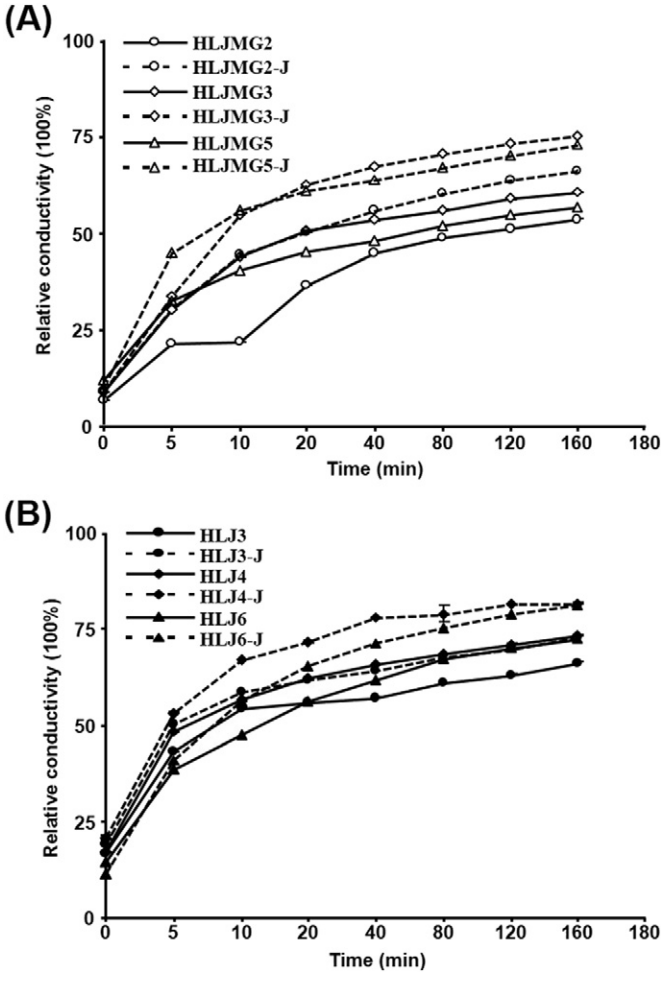

Fig. 2. Relative electrical conductivity of mycelia of *Sclerotinia sclerotiorum* isolates. (A) Relative conductivity values of distilled water containing mycelia of dimethachlone sensitive isolates with and without dimethachlone treatment. (B) Relative conductivity values of dimethachlone resistant isolates. The same labels denote the same isolates, values are means (±SE) of two independent repeats. Error bars denote the standard error of the mean according to *t*-test.

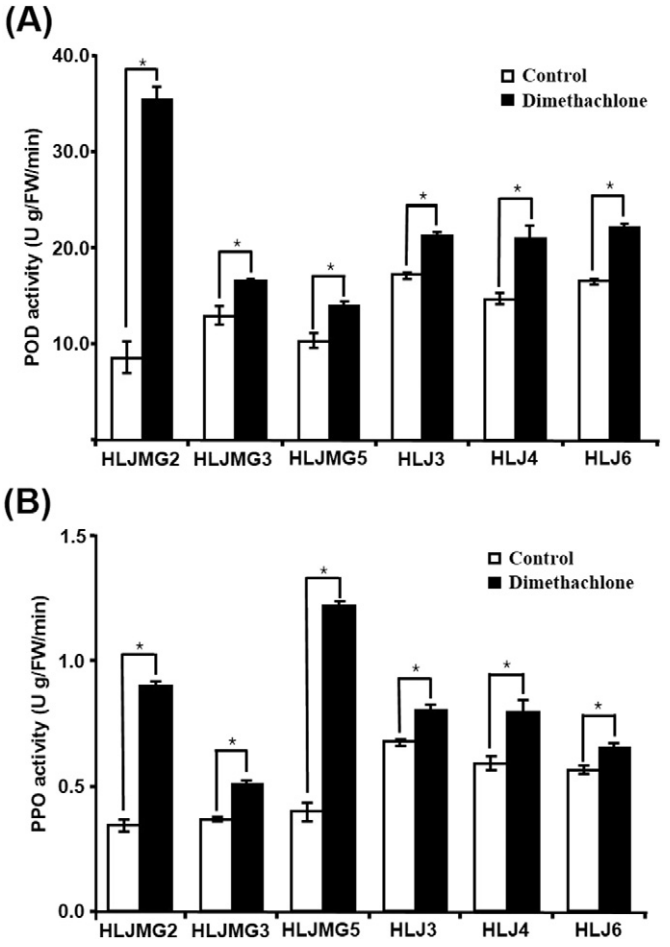

Fig. 3. Peroxidase (POD) (A) and polyphenol oxidase (PPO) (B) activities of dimethachlone resistant and sensitive isolates of *Sclerotinia sclerotiorum*. "*" indicates statistically significant differences ($\alpha = 0.05$). Values are means (±SE) of two independent repeats. Error bars denote the standard error of the mean according to *t*-test.

3.5. Transcription levels of the Sshk gene

Transcription levels of the *Sshk* gene were significantly higher ($P < 0.05$) in the isolates HLJ4 and HLJ6 than in the dimethachlone sensitive isolate and the resistant isolate HLJ3 (Fig. 5). For all the three dimethachlone resistant isolates, transcription levels of *Sshk* were significantly ($P < 0.05$) up-regulated by dimethachlone compared with the non-treated control. These results indicate that the point mutations and induced transcriptions of this gene in isolates HLJ4 and HLJ6 may be related to dimethachlone resistance.

4. Discussion

The present study revealed no significant differences in glycerol content level between the field dimethachlone resistant and sensitive isolates of *S. sclerotiorum*, and glycerol content levels were increased in resistant isolates but reduced in sensitive ones by dimethachlone treatment. In *B. cinerea* and *S. sclerotiorum,* no significant differences in

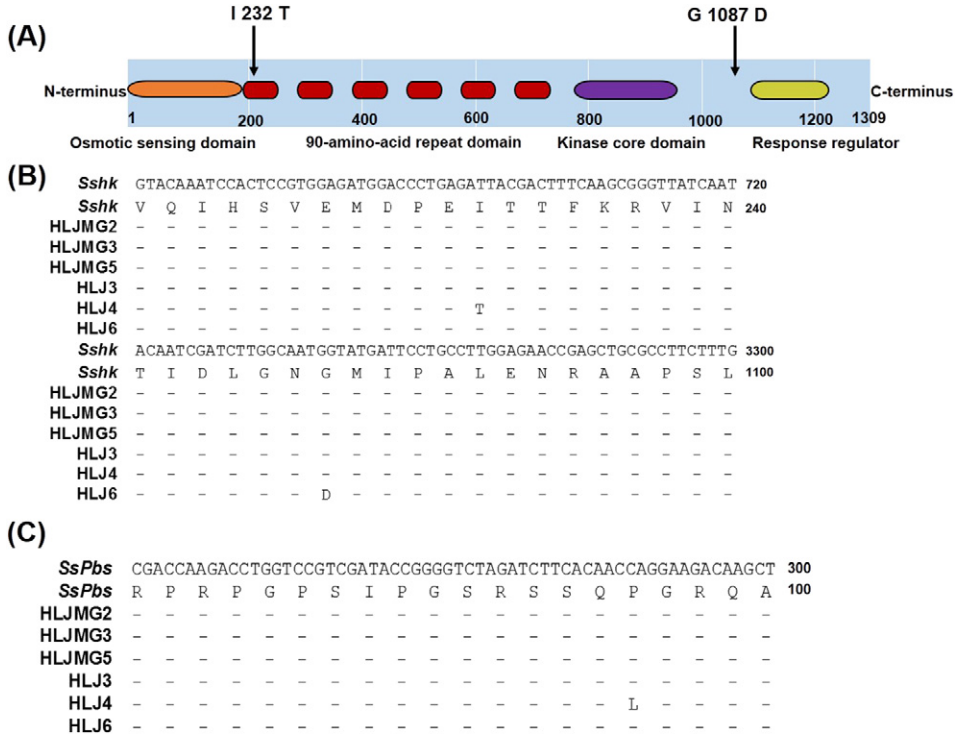

Fig. 4. Cloning the *Sshk* (SS1G_12694.3) gene. (A) Schematic representation of the structure of the *Sshk* gene (InterProScan prediction server). (B) By analyzing the *Sshk* gene with BLAST and the DNAman program, a mutation in isolate HLJ4 and HLJ6 were uncovered in the *Sshk* gene. (C) The gene *SsPbs* had a point mutation at codon position 96 in isolate HLJ4.

glycerol content were found between laboratory-induced procymidone resistant mutants and their wild type parents [6,42], and fludioxonil treatment induced glycerol accumulation in *S. sclerotiorum* [14]. These results indicate that the increased glycerol content by dimethachlone in dimethachlone resistant isolates might be due to the fact that the dimethachlone resistant isolates were more sensitive to osmotic stress and dimethachlone interfered with the HK signaling pathway [11,13,15,31,48]. Previous studies have shown that DCFs resistant isolates of *S. sclerotiorum* were more sensitive to hyperosmosis, this may be related to the fact that resistant isolates released more electrolytes than sensitive isolates [9,16–17]. The result of the current study was in accord with previous studies in that relative conductivity was increased by dimethachlone treatment, and the three resistant isolates had significantly higher cell membrane permeability compared with that of the three dimethachlone sensitive isolates. Electrolyte leakage caused by dimethachlone may be related to the mode of action of this fungicide, i.e., interfering with osmolarity regulation.

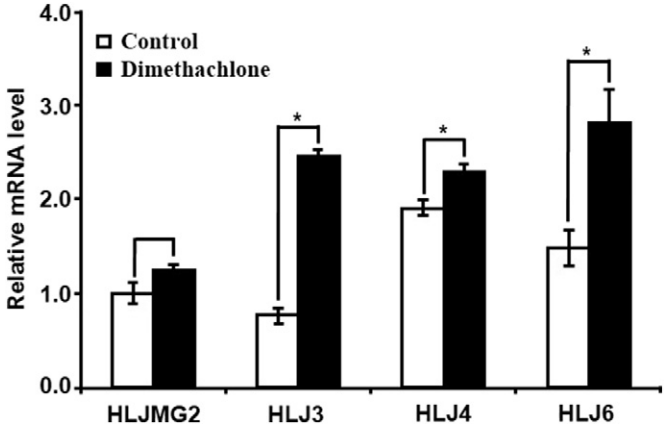

Fig. 5. Relative mRNA levels of *Sshk* in dimethachlone resistant and sensitive isolates of *Sclerotinia sclerotiorum*. "*" indicates statistically significant differences ($\alpha = 0.05$). Values are means ($\pm$SE) of two independent repeats. Error bars denote the standard error of the mean according to *t*-test.

Some studies indicated that oxalate content of the resistant mutants of *S. sclerotiorum* was higher than their wild-type isolates and oxalate content significantly decreased after fungicide treatment [14,16], and the field dimethachlone resistant isolates were less pathogenic to oilseed rape [48]. In order to determine whether the pathogenicity is related to the dimethachlone resistance, the oxalic acid content were observed. In the present experiment, the oxalic acid content were did not differ significantly between dimethachlone resistant and sensitive isolates. Dimethachlone did not show any significant effects on oxalic acid production. But not all, study showed oxalic acid was not the only pathogenic factor [16], and oxalic acid may play a role in some of resistance to dimethachlone in *S. sclerotiorum*.

In fungi, POD and PPO have been reviewed as the defense enzymes [16,32], and PPO is involved in pathogenesis and melanin formation [19,32]. In the present study, POD and PPO activities for dimethachlone resistant isolates were significantly higher compared to sensitive isolates, and dimethachlone treatment increased their activities in both resistant and sensitive isolates. Similarly, it was stated that POD activity in laboratory-induced mutants of *S. sclerotiorum* was greater than wild-type isolates, and fungicide treatment increased POD activity [14,16]. The activity of POD in tebuconazole resistant mutants of *Fusarium oxysporum* f. sp. *fragariae* was higher compared with wild-type isolates [20]. These results indicate that the dicarboximide fungicide is conducive to enhancing the activities of POD and PPO. And this is the reason because these enzymes are capable of defending against *S. sclerotiorum* infection, might be resulted in dimethachlone resistance.

Previous studies indicated that DCFs might target the HK signal pathway, and point mutations located within the 90-amino-acid repeats conferring resistance to DCFs have been reported in several phytopathogenic fungi, including *S. sclerotiorum*, *B. cinerea*, *A. alternata*, and *M. fructicola* [13,16,29–30]. In the current study, we found a new point mutation of I232T located in the first 90-amino-acid repeat of HK in isolate HLJ4, and a G1087D mutation located between the kinase core domain and the RR domain in isolate HLJ6. To our knowledge, this is the first

report of a point mutation located between the kinase core domain and the RR domain in HK of *S. sclerotiorum*. A null mutation of CGA to TGA at amino acid position 1040 located in the kinase core domain of the two-component histidine kinase gene (*Bos1*) associated with DCFs resistance has been reported in *B. cinerea* [31]. It is interesting that synonymous mutations were found in *SsYpd*, *Sssk*1, and *Sssk*2 genes in dimethachlone resistant isolates. At present, it is not clear whether or not these synonymous mutations are associated with dimethachlone resistance. Synonymous mutations may alter the phenotype via affecting transcription, splicing, mRNA transport or translation. It has been reported that a synonymous polymorphism in the multidrug resistance 1 (MDR1) gene of mammal cancer cells changes substrate specificity [21].

The transcription levels of the *Sshk* gene in isolates HLJ4 and HLJ6 were remarkably higher than in the sensitive isolate HLJMG2 and resistant isolate HLJ3. Isolate HLJ3 had no mutations in the *Sshk* gene. According to recent studies, molecular mechanisms other than point mutations in HK genes should exist for resistance to DCFs in *S. sclerotiorum*, *A. longipes* and *B. cinerea* [17,18,26]. A previous study stated that the lost expression or the V238A point mutation of *Ssos1* gene in the field resistant isolate may confer dimethachlone resistance in *S. sclerotiorum* [17]. Our results indicated that a linkage between *Sshk* gene expression, the point mutations and resistance to dimethachlone might exist in *S. sclerotiorum*.

In conclusion, over-expression or the point mutations (I232T and G1087D) in the *Sshk* gene and the activities of POD and PPO may confer resistance to dimethachlone in *S. sclerotiorum*. The present studies provided valuable insight into the resistance mechanisms of *S. sclerotiorum* to dimethachlone. According to the previous studies, our results may present good evidence to control dimethachlone resistance in *S. sclerotiorum* by site-specific mutagenesis in the *Sshk* gene or decreasing the transcriptional levels of the *Sshk* gene and the activities of POD, PPO, which is important for integrated pest management (IPM) program [13,16,20,29–31]. The molecular mechanisms for dimethachlone resistance merit further study in the future since the mechanisms for dimethachlone resistance were complicated, and might exist more than one factor contributing to dimethachlone resistance.

Competing interests

The authors have declared that no competing interests exist.

Acknowledgements

This study was supported by National Natural Science Foundation of China (31371964).

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