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A new point mutation in the iron–sulfur subunit of succinate dehydrogenase confers resistance to boscalid in *Sclerotinia sclerotiorum*

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SUMMARY

Research has established that mutations in highly conserved amino acids of the succinate dehydrogenase (SDH) complex in various fungi confer SDH inhibitor (SDHI) resistance. For *Sclerotinia sclerotiorum* (Lib.) de Bary, a necrotrophic fungus with a broad host range and a worldwide distribution, boscalid resistance has been attributed to the mutation H132R in the highly conserved SdhD subunit protein of the SDH complex. In our previous study, however, only one point mutation, A11V in SdhB (GCA to GTA change in *SdhB*), was detected in *S. sclerotiorum* boscalid-resistant (BR) mutants. In the current study, replacement of the *SdhB* gene in a boscalid-sensitive (BS) *S. sclerotiorum* strain with the mutant *SdhB* gene conferred resistance. Compared with wild-type strains, BR and GSM (*SdhB* gene in the wild-type strain replaced by the mutant *SdhB* gene) mutants were more sensitive to osmotic stress, lacked the ability to produce sclerotia and exhibited lower expression of the *pac1* gene. Importantly, the point mutation was not located in the highly conserved sequence of the iron–sulfur subunit of SDH. These results suggest that resistance based on non-conserved vs. conserved protein domains differs in mechanism. In addition to increasing our understanding of boscalid resistance in *S. sclerotiorum*, the new information will be useful for the development of alternative antifungal drugs.

Keywords: boscalid, fungicide resistance, *Sclerotinia sclerotiorum*, SDHI fungicides.

INTRODUCTION

The plant-pathogenic fungus *Sclerotinia sclerotiorum* (Lib.) de Bary is widely distributed and can infect more than 400 species of cultivated plant (Wang *et al*., 2009). Sclerotinia stem rot caused by *S. sclerotiorum* seriously reduces the yield and quality of rapeseed. For many years, the control of Sclerotinia stem rot in China has been mainly dependent on the benzimidazole fungicide carbendazim and the dicarboximide fungicide dimethachlon (Kuang *et al*., 2011). Because of the extensive and repeated application of these fungicides, however, resistance has been found in populations of *S. sclerotiorum* in China (Kuang *et al*., 2011; Ma *et al*., 2009; Shi *et al*., 2000). The rapid development of fungicide resistance by *S. sclerotiorum* has also been documented in other countries (Jo *et al*., 2006; Mueller *et al*., 2002; Smith *et al*., 1995).

Boscalid, a small molecule, belongs to a novel fungicide class of succinate dehydrogenase inhibitors (SDHIs) (Stammler *et al*., 2007). It inhibits fungal respiration by binding to the ubiquinone-binding site (Q-site) of the mitochondrial succinate dehydrogenase (SDH) complex II in the electron transport chain, which is a functional part of the tricarboxylic acid cycle (Keon *et al*., 1991). The mitochondrial SDH complex consists of four subunits: flavoprotein (Fp) (SdhA), iron–sulfur protein (Ip) (SdhB) and two membrane-anchored proteins (SdhC and SdhD) (Hagerhall, 1997; Ito *et al*., 2004). Previous studies have demonstrated that the SDH binding site is highly conserved in bacteria and eukaryotes (Horsefield *et al*., 2006; Huang *et al*., 2006; Sun *et al*., 2005).

Because of their single site mode of action, SDH inhibitors (SDHIs) have selected for resistance in various fungi and bacteria. Numerous studies have indicated that mutations in highly conserved amino acid residues located in the SdhB, SdhC and SdhD subunits of SDH are responsible for SDHI resistance (Avenot and Michailides, 2010; Avenot *et al*., 2008; Matsson and Hederstedt, 2001; McGrath and Miazzi, 2008; Skinner *et al*., 1998). In *Ustilago maydis*, replacement of a highly conserved histidine residue [by either tyrosine or leucine (H257Y/L)] located at position 257 in the third cysteine-rich cluster of the mitochondrial iron–sulfur subunit SdhB is correlated with carboxin resistance (Broomfield and Hargreaves, 1992; Keon *et al*., 1991). In *Mycosphaerella graminicola*, carboxin resistance is conferred by the alteration of the equivalent codon at position 267 to either tyrosine or leucine (H267Y; H267L) (Skinner *et al*., 1998). Boscalid resistance has also been detected in *Alternaria alternata*, *Botrytis cinerea* and *Podosphaera xanthii* (Avenot and Michailides, 2007; Miazzi and McGrath, 2008; Stammler *et al*., 2007).

For *S. sclerotiorum*, Glättli *et al*. (2009) and Stammler *et al*. (2011) have reported that boscalid field resistance is conferred

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by the point mutation H132R in *SdhD*. In our previous study, five boscalid-resistant (BR) mutants, JK19R1, JK19R2, NT16R1, NT16R2 and CS51R, were obtained by fungicide induction. Interestingly, DNA sequence analysis showed that all BR mutants had only one point mutation A11V (GCA to GTA) in the iron–sulfur protein subunit (SdhB) (Wang *et al*., 2014). Therefore, the objectives of this study were to confirm whether the mutation confers resistance of *S. sclerotiorum* to boscalid, and to determine how the mutation affects the biological characteristics of *S. sclerotiorum*. We also analysed the phylogeny of the *SdhB* gene and determined whether the A11V mutation is located in a conserved or non-conserved protein domain.

RESULTS

Validation of the gene replacement mutants

To confirm whether the mutation in the *SdhB* gene confers resistance to boscalid, we generated targeted gene replacement mutants by transformation of the replacement cassette *Trpc-Neo-SdhB* (Fig. 1A). Gene replacement strains were subjected to polymerase chain reaction (PCR) amplification in order to detect the integration of the left and right portions of the replacement cassette. The primer pairs P9 + P10 and P11 + P12 amplified 1942-bp and 2587-bp fragments, respectively, from the gene replacement mutant, but did not amplify any fragments from the parental strain

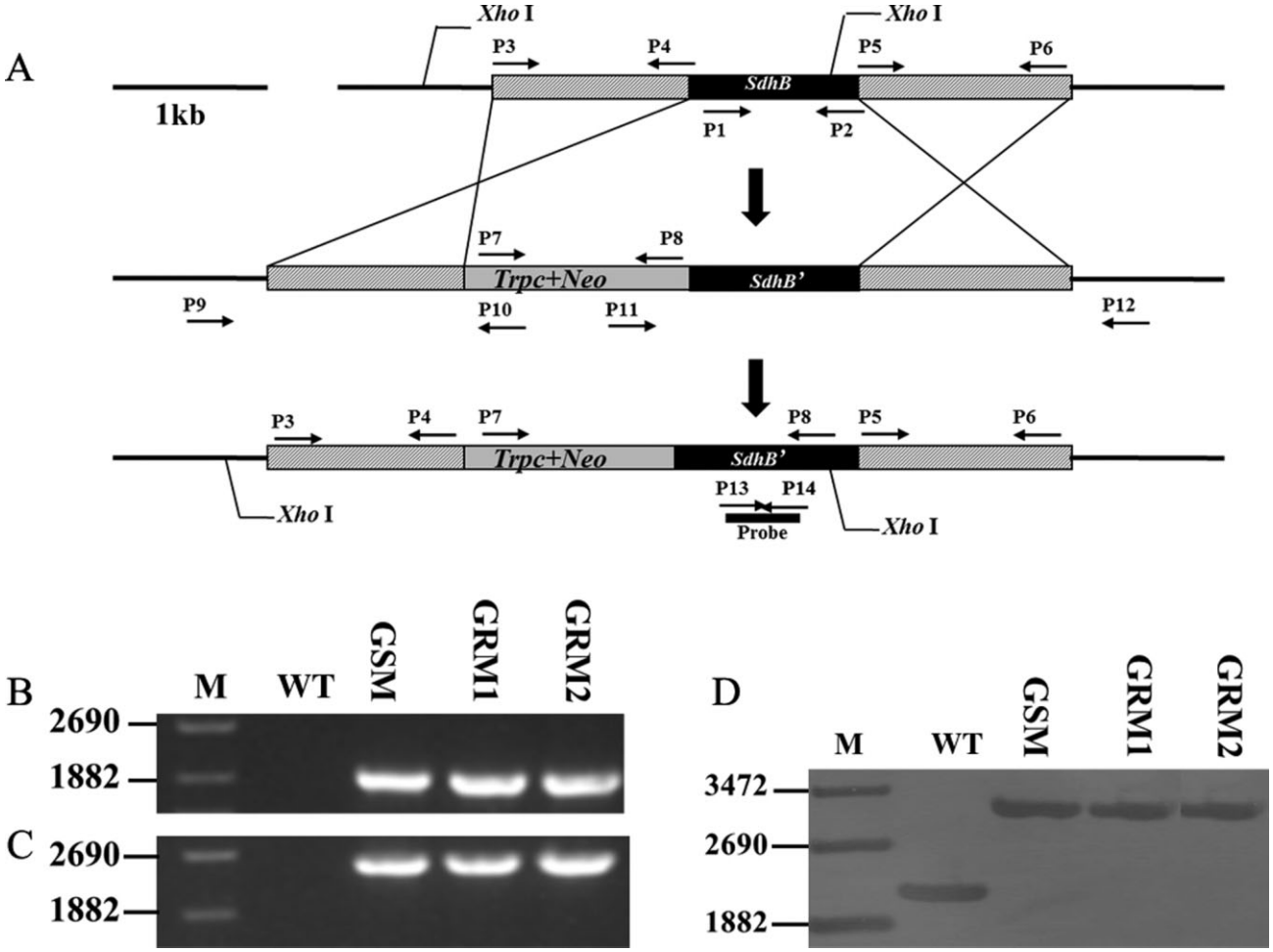

Fig. 1 Generation and identification of *Sclerotinia sclerotiorum SdhB* gene replacement mutants. (A) Schematic representation of the gene replacement strategy. The top part represents the genomic locus target of the replacement construct. Two grey fragments represent the left and right homologous arms, respectively, of the *SdhB* gene. The black fragment represents the target *SdhB* gene. The middle part represents the gene replacement cassette Trpc + Neo + *SdhB*' connecting the left and right homologous arms of *SdhB*, and the prime represents the selected *SdhB* mutation. The cassette Trpc + Neo + *SdhB* contains the neomycin resistance gene. Primer binding sites are indicated by arrows (see Table 1 for primer sequences). The bottom part of Fig. 1A represents the digested position with Xho1 in the genomic DNA. (B) Polymerase chain reaction (PCR) performed with primer pair P9/P10; a 1942-bp amplified fragment indicates integration of the replacement cassette Trpc + Neo + *SdhB* at the left junction. (C) PCR performed with primer pair P11/P12; a 2587-bp amplified fragment indicates integration of the replacement cassette Trpc + Neo + *SdhB* at the right junction. (D) Southern blot hybridization analysis of JK19 (WT, wild-type), GSM, GRM1 and GRM2 using a 400-bp fragment from the *SdhB* gene as probe; genomic DNA was digested with *Xho*1.

Table 1 Oligonucleotide primers used in this study.

Primer	Sequence (5' to 3')	Use
P1	CCTTCTTGACGAGTTCTTCTGAATGGCATCTCTCCGCACAAG	Amplify the full DNA sequence of the *SdhB* gene (SS1G_04384.3)
P2	TGCTTTCCCATCATATCCC	
P3	AGGTCGCCAGTGCACTGCC	Amplification of the upstream *SdhB* fragment (1.0 kb)
P4	TTCAATATCATCTTCTGTCGACAGTTGTGGAGAAGGCGAAA	
P5	GGGATATGATGGGAAAGCAGAAGCCTTTAGCGAGATG	Amplification of the downstream *SdhB* fragment (1.0 kb)
P6	TCCATTCCCACAACAGAAGG	
P7	GTCGACAGAAGATGATATTG	Amplify the Neo cassette containing a Trpc promoter (1165 bp)
P8	TCAGAAGAACTCGTCAAGAAGGCG	
P9	CATCGAAGCGGCCATTCTG	Identification of *Trpc-Neo-SdhB** cassette integrated at left junction (1942 bp)
P10	CGATGCCTGCTTGCCGAATA	
P11	ACACGGCGGCATCAGAGCAG	Identification of *Trpc-Neo-SdhB** cassette integrated at right junction (2587 bp)
P12	TGGAGGGCAATTAGATAGTC	
P13	TACCATTTCACCATCTCGTCCTG	Amplify a probe for Southern blotting (400 bp)
P14	GGTCTCGTGCTTGGCGTCT	
P15	CCCCAGCGTTCTACGTCT	Amplify the *actin* gene (SS1G_08733) for quantitative real-time polymerase chain reaction
P16	CATGTCAACACGAGCAATG	
P17	TGAACATCGACGGAGTAAA	Amplify the *SdhB* gene for quantitative real-time polymerase chain reaction
P18	ATCTTGGTCTCGTGCTTGG	
P19	GCAGCATACCCTAATCTTCC	Amplify the *Pac1* gene (AY005467) for quantitative real-time polymerase chain reaction
P20	GTCCACCAGCACTTCTTTG	
P21	GGGAAGGGAAGACAACAGT	Amplify the *Sac1* gene (DQ526020) for quantitative real-time polymerase chain reaction
P22	TAGTGCAATCGGAATAGTGA	
P23	ATCCGATGATCATTGCCAG	Amplify the *Smk1* gene (AY351633) for quantitative real-time polymerase chain reaction
P24	ATCTAGCAAGACCAAAATCGCA	

(Fig. 1B,C). When probed with the partial *SdhB* gene region, the genomic DNA of mutants digested with *Xho*I had a single 3.1-kb hybridized DNA fragment instead of the 2.1-kb fragment found in the parental strain (Fig. 1D). These sizes are consistent with accurate replacement of the SdhB regions. Two mutants, GRM1 and GRM2, were obtained by replacing the *SdhB* gene in an *S. sclerotiorum* boscalid-sensitive (BS) strain with the mutant *SdhB* gene, and one mutant, GSM, was obtained by replacing the mutant *SdhB* gene with the *SdhB* gene from the BS *S. sclerotiorum* strain.

Fungicide sensitivity

The EC_{50} values for boscalid differed significantly ($P > 0.05$) among the strains (Table 2). As expected, the five BR mutants, GRM1 and GRM2 were resistant to boscalid, with RF (resistance factor: ratio of the EC_{50} value of the mutant to that of the wild-type) values between 86.5 and 127.6. The sensitivity to boscalid did not differ significantly among GSM and BS strains. We also assessed the susceptibility to other SDHI fungicides, carboxin and fluopyram. Pearson correlation analysis indicated that boscalid EC_{50} values were correlated with carboxin and fluopyram EC_{50} values.

Hyphal growth and sclerotial production

Hyphal growth was slower for the BR and GSM mutants than for the BS strains, but did not differ among GRM1, GRM2 and BS strains (Table 2). In our previous study, BR mutants lacked the ability to produce sclerotia. In the current study, however, the ability of GRM1 and GRM2 to produce sclerotia was similar to that of the BS strains (Fig. 2). Interestingly, GSM lacked the ability to produce sclerotia. These results indicate that the mutation A11V (GCA to GTA) in the *SdhB* gene does not affect hyphal growth or sclerotial development directly.

Table 2 Mycelial growth and sensitivity of *Sclerotinia sclerotiorum* strains or mutants exposed to succinate dehydrogenase inhibitor (SDHI) fungicides.

	EC_{50} (μg/mL)			
Strain or mutant	Boscalid	Carboxin	Fluopyram	Colony diameter (cm)
JK19	0.134f*	0.883f	0.113f	8.09ab
NT16	0.126f	0.819f	0.124f	7.98b
CS51	0.158f	0.932f	0.098f	8.15a
JK19R1	12.7d	3.52e	3.65c	7.32dc
JK19R2	13.5cd	4.34bc	3.42cd	7.07d
NT16R1	11.2e	3.96d	2.99e	7.23cd
NT16R2	10.9e	4.00d	3.17de	7.25cd
CS51R	14.3c	4.17cd	3.28d	7.18cd
GSM	0.153f	0.915f	0.155f	7.34c
GRM1	15.6b	4.52b	4.07b	8.12a
GRM2	17.1a	5.01a	4.75a	8.05ab

*Values in a column followed by the same letter are not significantly different ($P > 0.05$) according to Fisher's least-significant difference.

Sensitivity to temperature, osmotic stress and oxidative stress

The BR strain and GSM mutants were more sensitive than the BS strain JK19 to osmotic stress. The sensitivity to osmotic stress, however, did not differ significantly among GRM1, GRM2 and BS strains (Fig. 3). Previous studies have demonstrated that paraquat

Table 3 Analysis of the conserved domains of the *SdhB* and *SdhD* genes in *Sclerotinia sclerotiorum*.

List of domain hits*					
Subunit	Name	Accession	Description†	Interval	*E*-value
SdhB	Fer2-3	pfam 13085	2Fe–2S iron–sulfur cluster binding domain	71–177	2.52e-39
	Fer4-17	pfam 13534	4Fe–4S dicluster domain	214–287	8.16e-07
	PLN00129	PLN00129	Succinate dehydrogenase [ubiquinone] iron–sulfur subunit	44–300	5.52e-160
SdhD	SQR-TypeC-Cybs	cd03496	SQR catalyses the oxidation of succinate to fumarate coupled to the reduction of quinone to quinol	88–191	1.78e-49

*Amino acid sequences of the *SdhB* and *SdhD* genes in *S. sclerotiorum* were submitted and analysed on the website http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi.

†More detailed information of the description can be found on the above website.

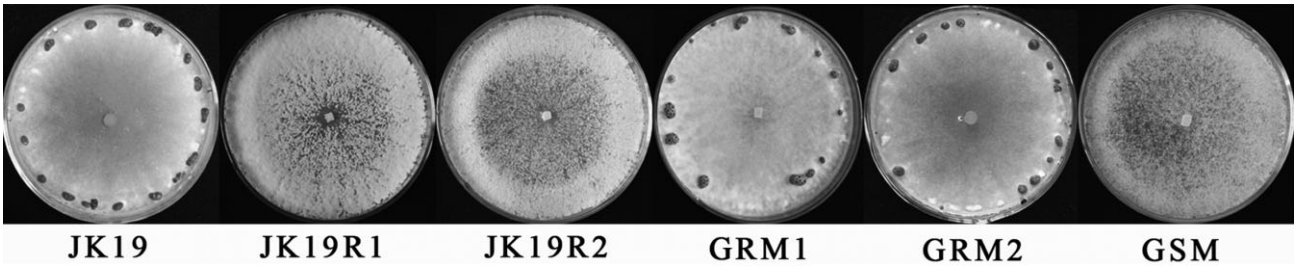

Fig. 2 Sclerotial production by *Sclerotinia sclerotiorum* strains and mutants.

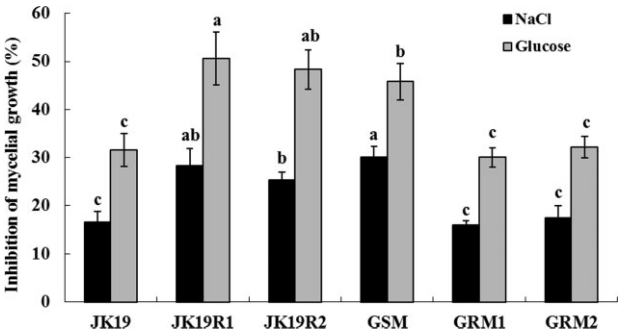

Fig. 3 Sensitivity of the wild-type strain JK19, boscalid-resistant (BR) mutants (JK19R1 and JK19R2) and gene replacement mutants (GSM, GRM1 and GRM2) of *Sclerotinia sclerotiorum* to osmotic stress (generated by 0.4 M NaCl or 1 M glucose). Values on the bars followed by the same letter are not significantly different ($P > 0.05$) according to Fisher's least significant difference.

can induce oxidative stress in organisms by generating superoxide ions in the cytoplasm or mitochondria (Betarbet *et al*., 2002; Yanase *et al*., 2002). In the current study, the strains did not differ in their sensitivity to oxidative stress induced by paraquat (data not shown). The strains also did not differ in their sensitivity to temperature (data not shown).

Phylogenetic analysis and determination of the position of the mutation

The *SdhB* gene from *S. sclerotiorum* was compared with the *SdhB* genes from other selected plant pathogens based on amino acid sequence. A phylogenetic tree was generated by alignment and cluster analysis. This analysis grouped *SdhB* of *S. sclerotiorum* in a branch together with *SdhB* from *B. cinerea* (AAW52509.1) (Fig. 4). The identities between the *S. sclerotiorum SdhB* amino acid sequence and that of other plant pathogens in the National Center for Biotechnology Information (NCBI) GenBank database were different, as shown in Fig. 4.

To determine whether the mutation is located in the highly conserved amino acid sequences, we analysed the conserved domain of the *SdhB* and *SdhD* gene in *S. sclerotiorum*. As shown in Table 3, the conserved amino acids of *SdhB* in *S. sclerotiorum* ranged from position 44 to 300, and the conserved amino acids of *SdhD* in *S. sclerotiorum* ranged from position 88 to 191 (Table 3). This indicates that the new point mutation A11V is not located in the highly conserved domain of the SdhB subunit, whereas the mutation H132R is located in the highly conserved domain of the SdhD subunit.

Gene expression level analysis

In *S. sclerotiorum*, the *pac1* gene (AY005467) is required for sclerotial development; transcription of the *smk1* gene (AY351633) and mitogen-activated protein kinase (MAPK) enzyme activity are dramatically induced during sclerotiogenesis and are required for sclerotial development; and the adenylate cyclase *sac1* gene (DQ526020) affects multiple developmental pathways and pathogenicity (Chen *et al*., 2004; Erental *et al*., 2007; Jurick and Rollins, 2007; Rollins, 2003). Quantitative reverse transcriptase-polymerase chain reactiuon (qRT-PCR) was used to determine whether the expression of *pac1*, *smk1* and *sac1* differs among the BR and BS strains and mutants. qRT-PCR was also used

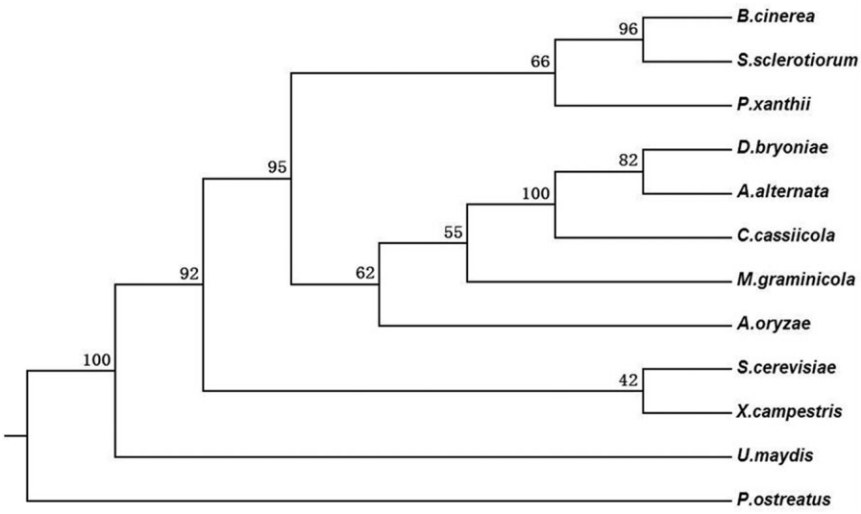

Fig. 4 Phylogenetic relationship of the *SdhB* gene among *Sclerotinia sclerotiorum* and other plant pathogens based on amino acid sequence. The identities between the *S. sclerotiorum SdhB* amino acid sequence and that of other plant pathogens are as follows: *Botrytis cinerea*, 94% identity; *Podosphaera xanthii* (AB547417), 90% identity; *Didymella bryoniae* (HQ156462.1), 80% identity; *Mycosphaerella graminicola* (Mg, AAB97419.1), 77% identity; *Pleurotus ostreatus* (AB007363.1), 75% identity; *Alternaria alternata* (B2BZ64), 74% identity; *Saccharomyces cerevisiae* (Sc, B3LTD3), 73% identity; *Corynespora cassiicola* (AB548738), 73% identity; *Aspergillus oryzae* (Ao, Q2TWM0), 71% identity; *Xanthomonas campestris SdhB* (WP_017113179.1), 69% identity; *Ustilago maydis SdhB* (GenBank: CAA44612.1), 65% identity.

to assess the effect of the point mutation on the expression of the *SdhB* gene in these strains and mutants. The expression of *pac1* was lower in JK19R1, JK19R2 and GSM than in JK19, GRM1 or GRM2 (Fig. 5); the expression of *smk1* and *sac1* did not differ among these strains and mutants (data not shown). The expression of the *SdhB* gene was higher in the other five mutants than in JK19 (Fig. 5).

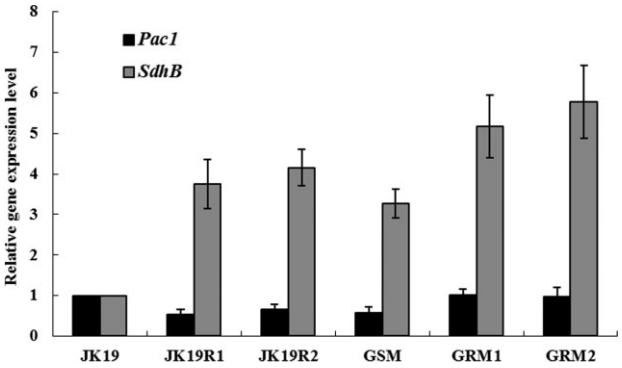

Fig. 5 Relative expression levels of the *pac1* and *SdhB* genes in JK19, JK19R1, JK19R2, GSM, GRM1 and GRM2 of *Sclerotinia sclerotiorum*. Values are means and standard errors.

DISCUSSION

Boscalid resistance has been reported for *A. alternata* on pistachio, *B. cinerea* on grapevine and strawberry, *P. xanthii* on cucurbits, and for other fungi on various crops in several countries. Mutation in *SdhB* is the most frequent cause of boscalid resistance in *B. cinerea* (Avenot and Michailides, 2007; Avenot *et al*., 2008; McGrath, 2008; McGrath and Miazzi, 2008; Miazzi and McGrath, 2008; Stammler, 2008). In *S. sclerotiorum*, however, boscalid resistance in one field strain resulted from the mutation H132R in the *SdhD* gene. In our previous study, a new point mutation A11V (GCA to GTA) in the SdhB subunit protein was detected in *S. sclerotiorum* BR mutants (Wang *et al*., 2014). Because the *SdhB* gene in *S. sclerotiorum* has over 90% identity with the *SdhB* gene in *B. cinerea*, this new point mutation in *SdhB* was not unexpected. To confirm whether the mutation in *SdhB* confers resistance to boscalid, we replaced the *SdhB* gene in the sensitive strain with that from the BR mutant in the current study. The gene replacement mutants exhibited significant resistance to boscalid, as well as to carboxin and fluopyram, which was consistent with previous findings of positive cross-resistance among boscalid, carboxin and fluopyram (Wang *et al*., 2014). Similarly, replacement of the mutant *SdhB* gene with the *SdhB* gene from an *S. sclerotiorum* BS strain conferred sensitivity. These results indicate that the point mutation A11V in SdhB (as a result of a GCA to GTA change in the *SdhB* gene) confers resistance not only to boscalid, but also to carboxin and fluopyram, which could be explained by the similar chemical structures of these fungicides. In this research, we also attempted to knock out the *SdhB* gene from the BR mutant. Unfortunately, no gene knockout mutant was obtained (data not shown). We therefore infer that the *SdhB* gene is essential for *S. sclerotiorum* survival and that the A11V mutation leads to a functional SdhB protein.

Numerous studies have shown that the ubiquinone-binding site in the mitochondrial matrix is highly conserved between fungi and

bacteria, and is formed by residues from subunits B, C and D (Horsefield *et al*., 2006; Sun *et al*., 2005; Yankovskaya *et al*., 2003). Previous studies have also demonstrated that mutations in highly conserved amino acids located in the SdhB, SdhC or SdhD subunit of the SDH complex are responsible for SDHI resistance (Avenot and Michailides, 2010; Avenot *et al*., 2008; Matsson and Hederstedt, 2001; McGrath and Miazzi, 2008; Skinner *et al*., 1998). Unexpectedly, the new point mutation, A11V in SdhB, is not located in the highly conserved sequence of the iron–sulfur subunit of the SDH complex. A docking model, which has been used to predict the binding pockets formed by the SdhB, SdhC and SdhD subunits in other studies, has also demonstrated that the new mutation is not located in these binding pockets (Fraaije *et al*., 2012; Scalliet *et al*., 2012; Shima *et al*., 2009). In addition, the expression levels of the *SdhB* gene were higher in BR and GRM mutants than in the parental strain, which may be consistent with three possible hypotheses. First, over-expression of the *SdhB* gene may prevent boscalid from reaching the binding site. Second, the amino acid substitution by this mutation may alter the conformation of the binding site of the SDH complex, which, in turn, may reduce the binding affinity to boscalid (Shima *et al*., 2009). Third, the A11V mutation may reduce the efficiency of the SdhB protein instead of inactivating it, and therefore the cells may be trying to overcompensate for this inferior protein by making more of it. These results suggest that different mechanisms operate for non-conserved and conserved protein domains to gain resistance.

Sclerotia are hard, asexual, resting structures that facilitate the survival and spread of *S. sclerotiorum*. Sclerotia of *S. sclerotiorum* can germinate carpogenically to form apothecia from which ascospores are liberated, or myceliogenically to produce hyphae (Abawi and Grogan, 1979; Steadman, 1979). We have reported previously that the BR mutants induced in our laboratory lacked the ability to produce sclerotia, and thus we suspected that resistance was associated with this inability. The gene replacement mutants GRM1 and GRM2 obtained in this research, however, exhibited no loss in the ability to produce sclerotia, even though they were quite resistant to boscalid. Interestingly, GSM still lacked the ability to produce sclerotia, even though it was quite sensitive to boscalid. As noted earlier, the *pac1* gene is required for sclerotial development in *S. sclerotiorum*, and qRT-PCR has shown that *pac1* expression is lower in BR strains and the GSM mutant than in the wild-type strain JK19 and GRM mutants. These differences in *pac1* expression may account for our observations. Further work concerning the molecular relationship between sclerotium production and the mutation A11V in SdhB is underway in our laboratory.

In previous studies, benomyl resistance has been positively correlated with cold sensitivity in *Fusarium moniliforme* and *Monilinia fructicola* (Ma, 2003; Yan and Dickman, 1996). In addition, BR mutants of *A. alternata* or *Saccharomyces cerevisiae* were more sensitive to oxidative stress than BS *A. alternata* or *Sa. cerevisiae* strains (Avenot *et al*., 2009; Szeto *et al*., 2007). In the present study, in contrast, BS and BR strains of *S. sclerotiorum* did not differ in their sensitivity to temperature or oxidative stress. Compared with BS strains and the GRM mutants, however, BR and GSM mutants grew more slowly, could not produce sclerotia and were more sensitive to osmotic stress. Previous research has also shown that BR mutants exhibited lower virulence than their parental strains (Wang *et al*., 2014). At least three possible explanations could account for our observations. First, the new point mutation located in the non-conserved domain of SdhB may reduce *S. sclerotiorum* fitness. Second, the non-conserved domain may regulate indirectly a variety of signal transduction pathways in *S. sclerotiorum* and may have a major impact on biodiversity in biological evolution. Third, altered phenotypes may also be a result of multiple mutations induced in the fungicide-treated mutants described in our previous study, and further research is still needed. We therefore infer that the conserved domains explain the commonalities among species, whereas the non-conserved domains explain the differences among species, and that there is a balance between conserved and non-conserved domains (Dickman and Figueiredo, 2011).

In conclusion, our results indicate that the new point mutation A11V in the SdhB subunit protein confers resistance to boscalid in *S. sclerotiorum*. Importantly, the point mutation is not located in the highly conserved sequence of the SdhB subunit of the SDH complex. These results suggest that mechanisms differ for resistance arising from non-conserved vs. conserved protein domains. This information increases our understanding of boscalid resistance in plant-pathogenic fungi and should be useful for the development of new fungicides.

EXPERIMENTAL PROCEDURES

Fungal strains and media

The strains of *S. sclerotiorum* used in this study were collected from oilseed rape fields in Jiangsu Province, China during 2010 and 2013. Five BR mutants (JK19R1, JK19R2, NT16R1, NT16R2 and CS51R) were induced from their parental strains (JK19, NT16 and CS51) in our previous study (Wang *et al*., 2014).

Regeneration medium (RM) was prepared with 0.5 g of yeast extract, 0.5 g of casein hydrolysate, 0.7 M sucrose and 16 g of agar per litre of distilled water. Selective regeneration medium (SRM) was prepared with 0.5 g of yeast extract, 0.5 g of casein hydrolysate, 1 M sucrose and 10 g of agarose per litre of distilled water. Potato dextrose agar (PDA) was prepared with 200 g of potato, 20 g of agar and 20 g of dextrose per litre of distilled water (Duan *et al*., 2013).

Construction of the *SdhB* gene replacement vector

The gene replacement vector for the targeted gene was generated as described previously (Laleve *et al*., 2014; Zheng *et al*., 2014). First, the

primer pair P1 + P2 was used to amplify the entire *SdhB* gene (Gene ID: SS1G_04384) from the genomic DNA of the *S. sclerotiorum* BR strain JK19R1. Similarly, primer pairs P3 + P4 and P5 + P6 were used to amplify the sequence 1.0 kb upstream (1.0-up) and 1.0 kb downstream (1.0-down) of the *SdhB* gene, respectively (all primers are listed in Table 1). In addition, primer pair P7 + P8 was used to amplify a 1.2-kb Neo cassette containing a Trpc promoter (resistance to neomycin) according to a previous study (Duan *et al*., 2013). The first two fragments (1.2-kb Trpc + Neo, *SdhB*) were connected by double-joint PCR, and then the three fragments (1.0-up, Trpc + Neo + *SdhB* and 1.0-down) were mixed in a 1:3:1 molar ratio and employed as a template for the fusion round, which was performed using La Taq Polymerase (TaKaRa, Dalian, China) without primers (Yu *et al*., 2004). A 1-μL volume of product from the second PCR round by the three fragments was used as DNA template to amplify a 4.1-kb DNA fragment employing the primers P3 + P6. The PCR products were purified with the AxyPreTP DNA Gel Extraction Kit (Axygen, USA), and the entire gene replacement vector was confirmed by sequencing. In addition, we also replaced the mutant *SdhB* gene with the *SdhB* gene from the BS *S. sclerotiorum* strain as described above.

Fungal transformation and confirmation of the gene replacement mutants

Transformation was carried out as described previously (Duan *et al*., 2013). Mycelial plugs taken from the edge of a 2-day-old colony of the BS strain JK19 were placed in 250-mL flasks (five plugs per flask) containing 100 mL of liquid PDB (PDA without agar). After the flasks had been shaken at 175 rpm and 25 °C for 36 h, the mycelia were collected and washed twice with distilled water. Then, 0.1 g of fresh mycelia was treated with 20 mL of protoplast solution (10 mg/mL of lysing enzyme, Sigma, USA) and digested at 85 rpm and 30 °C for 2 h. The enzyme solution was then filtered to eliminate mycelial residues. The protoplasts in the filtrate were washed twice with STC (0.8 M sorbitol, 0.05 M Tris, pH 8.0, 50 mM $CaCl_2$) and resuspended in SPTC (STC with 40% w/v PEG 6000) buffer (STC : SPTC = 4:1). Protoplasts were transformed as described previously (Zheng *et al*., 2014). For transformation, 10^7 protoplasts in 200 μL of SPTC buffer and 30 μg gene replacement vector DNA in 10 μL of heparin sodium were mixed and incubated on ice for 30 min; 1 mL SPTC was mixed with the suspension and incubated at room temperature for 20 min. Protoplasts were mixed into 200 mL RM at 43 °C, poured into 9-cm-diameter Petri plates (20 mL per plate) and incubated at 25 °C. After 12 h, RM plates were overlaid with 10 mL of SRM containing 100 μg/mL neomycin. Four days post-transformation, the transformants were transferred to fresh PDA containing 100 μg/mL neomycin.

The total DNA of the transformants was extracted by the cetyltrimethylammonium bromide (CTAB) method (Nicholson and Parry, 1996). Primer pairs P9 + P10 and P11 + P12 (Table 1) were used to amplify the fragment of the connecting area to confirm that the gene replacement vector had been inserted into the site in which *SdhB* is normally located in the genome, and the PCR products amplified by primer pairs P9 + P12 from transformants genome were sequenced. The transformants were further confirmed by Southern analysis of genomic DNA digested with *Xho*1 and hybridized with a labelled probe obtained by PCR amplification with the primers P13/P14 (Table 1, Fig. 1A). Three gene replacement mutants, GSM, GRM1 and GRM2, were obtained (see Results).

Fungicide susceptibility test

Sensitivity of the strains listed in Table 2 to the SDHIs boscalid, carboxin, and fluopyram was assessed as previously described (Leroux *et al*., 1999, 2010; Wang *et al*., 2009). Fungicide concentrations used for the BR or the gene replacement mutants were 0, 1.56, 3.13, 6.25, 12.5, 25, and 50 μg/mL. Fungicide concentrations used for the BS strains were 0, 0.0625, 0.125, 0.25, 0.5, 1, and 2 μg/mL. After the cultures were incubated at 25 °C for 2 days, the colony diameters were measured, and the EC_{50} (50% of mycelia growth inhibition) values were calculated by regressing the percentage of growth inhibition against the log of fungicide concentration (Kuang *et al*., 2011).

Hyphal growth and sclerotial production

Mycelial plugs (5 mm in diameter) taken from the margin of a 2-day-old colony of the strains used for the sensitivity test described in the previous section were placed on PDA plates (one plug per plate). Colony diameters were measured perpendicularly after incubation at 25 °C for 2 days. Sclerotial production was assessed as follows: the colonies of the wild-type strain JK19, BR mutants (JK19R1 and JK19R2) and gene replacement mutants (GSM, GRM1 and GRM2) were photographed 1 month post-inoculation. Each strain or mutant was represented by three plates, and the experiment was performed three times.

Sensitivity to temperature, osmotic and oxidative stresses

The strains used to test sclerotial production were also used to test the sensitivity to temperature, osmotic and oxidative stresses. Mycelial plugs (5 mm in diameter) taken from the edge of a 2-day-old colony were transferred to PDA plates (one plug per 9-cm-diameter plate) and incubated at 5, 15 and 35 °C. Plates incubated at 25 °C were used as controls. Colony diameters were measured after 2 days of incubation.

Similar mycelial plugs were also transferred to plates containing PDA amended with 0.4 M NaCl, 1 M glucose or paraquat (1, 10 or 50 μg/mL); NaCl and glucose were used to generate osmotic stress and paraquat was used to generate oxidative stress (Duan *et al*., 2013; Wang *et al*., 2014). Plates without NaCl, glucose or paraquat served as controls. The plates were incubated at 25 °C in a growth chamber (12-h photoperiod) for 2 days before colony diameters were measured. The percentage of inhibition of mycelial radial growth (PIMG) was calculated using the formula, PIMG = $[(C - N)/(C - 5)] \times 100$, where C is the colony diameter of the untreated control and N is that of the NaCl, glucose or paraquat treatment. Each combination of strain and treatment was represented by three plates, and the experiment was performed three times.

Phylogenetic analysis and determination of the position of the mutation

Phylogenetic analysis was carried out as described previously (Chen *et al*., 2009). Amino acid sequences of the *SdhB* gene from *S. sclerotiorum* and other plant pathogens were obtained from the public database GenBank. For phylogenetic analysis, the deduced protein sequences were aligned

with CLUSTALW using the Blosum matrix and standard default parameters. The phylogenetic tree was established using MEGA software with the neighbour-joining method, excluding positions with gaps.

The positions of the mutations A11V in the *SdhB* gene and H132R in the *SdhD* gene, which conferred resistance to boscalid, were determined. Amino acid sequences of the *SdhB* and *SdhD* genes in *S. sclerotiorum* were submitted and analysed on the website http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi. The conserved domains of the *SdhB* and *SdhD* genes are listed in Table 3.

qRT-PCR

Total RNA was extracted with the RNeasy kit (Tiangen Biotech. Co., Beijing, China) from 2-day-old mycelia grown in potato sucrose broth. First-strand cDNA was synthesized with the PrimeScript® RT reagent kit (TaKaRa) (Zheng *et al*., 2014). qRT-PCRs were performed with an ABI 7500 real-time detection system (Applied Biosystems, Foster City, CA, USA). The primers used for qRT-PCR are listed in Table 1. All data were normalized to *actin* gene expression, and relative changes in gene expression levels were analysed by ABI 7500 SDS software (Applied Biosystems, Foster City, CA, USA), which automatically sets the baseline. Data from three biological replicates were used to calculate the means and standard deviations.

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