Amino Acid Polymorphism in Succinate Dehydrogenase Subunit C Involved in Biological Fitness of *Botrytis cinerea*

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Accepted 9 January 2020.

Succinate dehydrogenase (SDH) is an important respiratory enzyme which participates in the tricarboxylic acid cycle and oxidative phosphorylation. A previous study of the baseline sensitivity of *Botrytis cinerea* against SDH inhibitors (SDHIs) showed that intrinsic sensitivity of the small population against the SDHIs exhibited significant differences. In the sequencing assay, we found five kinds of amino acid polymorphism in SDH subunit C (SdhC) of *B. cinerea* isolates which were never exposed to the SDHIs. To validate that amino acid polymorphism in the SdhC of *B. cinerea* confers intrinsic sensitivity against the SDHIs, the replacement mutants containing each kind of amino acid polymorphism of SdhC exhibited phenotype differences in intrinsic sensitivity to SDHIs, mycelial growth, sporulation, virulence, oxidative stress response, and carbon source utilization. These results indicated that SdhC of *B. cinerea* experienced positive selection during evolution and resulted in amino acid polymorphism which is involved in intrinsic sensitivity to SDHIs and biological fitness.

Keywords: amino acid polymorphism, biological fitness, *Botrytis cinerea*, fungal development, plant antifungal responses, population genetics, succinate dehydrogenase subunit C

Gray mold caused by *Botrytis cinerea* is one of the most important diseases worldwide. This pathogen can infect more than 200 host plants, including vegetables, fruit, and ornamental flowers, and was a classical “high-risk” pathogen (Elad et al. 2004; Veloukas and Karaoglaniadis 2012). Flowers of vegetables, fruit, and ornamental plants are susceptible to gray mold; in addition, because of the low priority of disease resistance at the growing stage, gray mold control mainly relies on application of fungicides. However, this strategy may result in fungicide resistance development and increasing concern about food safety.

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Funding: This work was supported by the National Science Foundation of China (grant number 31672065), the Special Fund for Agro-Scientific Research in the Public Interest (grant numbers 201303023 and 201303025), and Jiangsu Provincial Agricultural Plans CX (14)2054 and SXGC (2016) 154.

*The e-Xtra logo stands for “electronic extra” and indicates that two supplementary figures and one supplementary table are published online.

The author(s) declare no conflict of interest.

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Despite numerous botryticides available, chemical control of this disease has been hampered by the emergence of resistant populations in the field. This phenomenon is well documented by the application of benzimidazoles, dicarboximides, anilino-pyrimidines, and hydroxanilides (Leroux et al. 2002). Several novel-generation carboxamide fungicides have been recently applied to fields. Carboxamides, also known as succinate dehydrogenase inhibitors (SDHIs), inhibit the succinate dehydrogenase (SDH) in the fungal mitochondrial respiratory chain. SDH is a protein complex consisting of four SDH subunits (SdhA, SdhB, SdhC, and SdhD). SdhA (flavoprotein) and SdhB (iron-sulfur protein) are responsible for the SDH activity of the enzyme, whereas SdhC (cytochrome b) and SdhD (*CybS*) anchor the complex to the inner mitochondrial membrane (Hägerhäll 1997). However, the repetitive use of site-specific fungicides such as SDHIs leads to selection of resistant populations, which may cause a rapid decline in fungicide efficacy. Resistance of filamentous fungi against SDHIs is well documented. The case of resistance against first-generation carboxamides (e.g., carboxin and oxycarboxin) has been reported since 1977 (Abiko and Imai 1977; Leroux et al. 2010; Newcombe et al. 2000; White and Georgopoulos 1986), while the resistance to novel-generation carboxamides (e.g., boscalid) was first recorded in 2004 (Ito et al. 2004). Field and laboratory mutants resistant to the novel-generation SDHIs were identified in a limited number of plant pathogens, including *B. cinerea* from grape, apple, strawberry, greenhouse vegetables (Zhang et al. 2007), and kiwi fruit (Bardas et al. 2010); *Alternaria alternata* from pistachio (Avenot et al. 2009); *Corynespora cassiicola* from cucumber greenhouses (Miyamoto et al. 2009); *Didymella bryoniae* (a causal agent of gummy stem blight) from watermelon (Stevenson et al. 2008); *Mycosphaerella graminicola* from wheat (Skinner et al. 1998); and *Podosphaera xanthii* from cucurbits (Miazzi and McGrath 2008). To date, 27 mutations in the *sdh* gene have been found, which endow the above-described pathogens with resistance phenotypes to SDHIs (Dela and Helge 2013). In *B. cinerea*, only seven kinds of substitutions (e.g., P225F/L/R, N230I, and H272/L/R/Y) at gene *sdhB* were detected to be responsible for the resistance to SDHIs. The mutation P225F in *sdhB* leads to resistance of *B. cinerea* to all kinds of SDHIs, whereas other mutations confer either special sensitivity pattern (such as H272L and H272Y/R) (Lalève et al. 2014). Even mutations at the same codon can cause entirely different sensitivity patterns to SDHIs. For instance, the substitution H272Y in *sdhB* is responsible for the decreased sensitivity of *B. cinerea* to isopyrazam, boscalid, and bixafen but hypersensitivity to fluopyram (Dela and Helge 2013). The SdhD^{H132R} substitution was detected in another boscalid-resistant strain (Leroux et al.

2010). Four conserved key residues of *B. cinerea*—SdhB (W229 and H272), SdhC (S84), and SdhD (Y144)—were involved in the binding of ubiquinone and carboxamides through hydrogen bonds. In addition, the *sdhB* residues (P225, W229, and I274) are involved in the hydrophobic interactions (Horsefield et al. 2006; Ruprecht et al. 2009; Yankovskaya et al. 2003).

Previous studies showed that intrinsic sensitivity of *B. cinerea* to SDHIs varies markedly. For instance, baseline sensitivity of *B. cinerea* to boscalid was evaluated in terms of colony growth on potato dextrose agar (PDA) (effective concentration that provides 50% growth reduction [EC₅₀] = 0.09 to 3.69 mg/liter, minimum inhibitory concentration [MIC] = 10 to 30 mg/liter) (Zhang et al. 2007) and conidial germination on acetate water agar (EC₅₀ = 0.03 to 0.1 mg/liter, MIC = 1 to 3 mg/liter) (Fernández-Ortuño et al. 2012), while baseline sensitivity of *B. cinerea* to fluopyram ranges from 0.05 to 5.98 mg/liter for mycelial inhibition on PDA (Zhang et al. 2014). Therefore, the mechanism underlying different intrinsic sensitivities of *B. cinerea* to SDHIs is elusive. In the current study, we found that amino acid polymorphism in SdhC of *B. cinerea* in the field was responsible for intrinsic sensitivity to the SDHIs, and SdhC was positively selected during evolution. To confirm the hypothesis, we constructed five *sdhC* in-situ-complemented mutants. The data supported the idea that amino acid polymorphism of the SdhC conferred not only intrinsic sensitivity of *B. cinerea* to SDHIs but also virulence, oxidative stress response, carbon sources utilization, and other fitness.

RESULTS

Positive selection on the SdhC of *B. cinerea*.

The 52 *B. cinerea* isolates were isolated from Jiangsu Province, Shanxi Province, Liaoning Province, Chongqing City, Hubei Province, and Xinjiang Province of China (Table 1). All of the isolates were never exposed to SDHIs, and the four subunits (SdhA, SdhB, SdhC, and SdhD) of SDH of these isolates were analyzed by sequencing. Sequence alignment showed that no nucleotide substitution occurred in *sdhA*, *sdhB*, and *sdhD* from all of the tested isolates but abundant single-nucleotide polymorphism was detected only in *sdhC*. Among the 52 isolates, 29 nucleotide sites were variable in SdhC.

All of the strains were categorized into five groups (G-B05.10 and G1 to G4) according to amino acid polymorphism of the SdhC (Table 2). Amino acid sequence of the SdhC from the sequencing strain B05.10 was used as a reference and designated G-B05.10, which was the most common, with 63.5%. The second group, G1, possessing four substitutions (G85A, I93V, M158V, and V168I in the SdhC) ranked next, with 28.9%, and G2, with one substitution (G37S), ranked third (3.9%). G3, with one substitution (G37S), and G4, with three substitutions (I79V, G85A, and L151I), had the same frequency (1.9%).

SDHI intrinsic sensitivity assay.

The three SDHIs (i.e., fluopyram, boscalid, and carboxin) were used to determine the intrinsic sensitivity of the five SdhC groups by mycelial growth inhibition. The results indicated that the five groups differed dramatically in sensitivity to the three fungicides. G-B05.10 had significantly higher EC₅₀ values against fluopyram and boscalid than the other groups, whereas groups G1 and G3 were more sensitive to carboxin than groups G-B05.10, G2, and G4. Group G4 exhibited the highest sensitivity against fluopyram (Fig. 1).

Generation of the *sdhC* in-situ-complemented mutants.

To confirm the hypothesis that amino acid polymorphism of the *sdhC* was involved in the sensitivity of *B. cinerea* against

SDHIs and to exclude the influence of different genetic backgrounds of the five groups, the *sdhC* in-situ-replacement mutants were constructed via gene replacement strategy. The *sdhC* of the five groups which containing the intrinsic site-directed mutation was separately transformed into the wild-type strain BT8-12 (Supplementary Fig. S1). The gene replacement transformants were verified by PCR and sequencing. The primer pairs P9/P10 and P11/P12 can amplify 1,506- and 2,373-bp fragments, respectively, in the replacement mutant but not in wide-type strain BT8-12 (Supplementary Figs. S1A and S2A and B). These results indicated that the replacement construct had replaced the target gene via homologous recombination. In the Southern blotting assay, the 576 bp at the 3' flanking region of the *sdhC* was probed. The replacement

Table 1. Origin of the *Botrytis cinerea* strains tested in this study

Strains	Origin ^a	Host	Location
YC-3	Yancheng, Jiangsu	Celery	E120°15' N33°35'
YC-4	Yancheng, Jiangsu	Celery	E120°15' N33°35'
YC-5	Yancheng, Jiangsu	Celery	E120°15' N33°35'
YC-6	Yancheng, Jiangsu	Celery	E120°15' N33°35'
YC-7	Yancheng, Jiangsu	Celery	E120°15' N33°35'
YC-11	Yancheng, Jiangsu	Celery	E120°15' N33°35'
YC-12	Yancheng, Jiangsu	Celery	E120°15' N33°35'
YC-13	Yancheng, Jiangsu	Celery	E120°15' N33°35'
BT1-9	Jvrong, Jiangsu	Strawberry	E119°17' N31°95'
BT1-27	Jvrong, Jiangsu	Strawberry	E119°17' N31°95'
BT1-34	Jvrong, Jiangsu	Strawberry	E119°17' N31°95'
BT2-9	Jvrong, Jiangsu	Strawberry	E119°17' N31°95'
BT2-12	Jvrong, Jiangsu	Strawberry	E119°17' N31°95'
BT3-12	Jvrong, Jiangsu	Strawberry	E119°17' N31°95'
BT3-13	Jvrong, Jiangsu	Strawberry	E119°17' N31°95'
BT3-29	Jvrong, Jiangsu	Strawberry	E119°17' N31°95'
BT5-3	Jvrong, Jiangsu	Strawberry	E119°17' N31°95'
BT8-10	Jvrong, Jiangsu	Strawberry	E119°17' N31°95'
BT9-1	Jvrong, Jiangsu	Strawberry	E119°17' N31°95'
BT9-8	Jvrong, Jiangsu	Strawberry	E119°17' N31°95'
BT13-15	Jvrong, Jiangsu	Strawberry	E119°17' N31°95'
BT14-1	Jvrong, Jiangsu	Strawberry	E119°17' N31°95'
B4-3	Nanjing, Jiangsu	Strawberry	E118°50' N32°02'
B4-4	Nanjing, Jiangsu	Strawberry	E118°50' N32°02'
B7	Nanjing, Jiangsu	Strawberry	E118°50' N32°02'
B8	Nanjing, Jiangsu	Strawberry	E118°50' N32°02'
NJ3-6	Nanjing, Jiangsu	Strawberry	E118°50' N32°02'
NJ4-1	Nanjing, Jiangsu	Strawberry	E118°50' N32°02'
NJ4-12	Nanjing, Jiangsu	Strawberry	E118°50' N32°02'
NJ5-1	Nanjing, Jiangsu	Strawberry	E118°50' N32°02'
NJ5-10	Nanjing, Jiangsu	Strawberry	E118°50' N32°02'
NJ5-15	Nanjing, Jiangsu	Strawberry	E118°50' N32°02'
TM01	Yangling, Shanxi	Tomato	E108°07' N34°28'
TM02	Yangling, Shanxi	Tomato	E108°07' N34°28'
TM03	Yangling, Shanxi	Tomato	E108°07' N34°28'
TM04	Yangling, Shanxi	Tomato	E108°07' N34°28'
TM05	Yangling, Shanxi	Tomato	E108°07' N34°28'
HLD-08	Shenyang, Liaoning	Tomato	E123°24' N41°50'
HLD-09	Shenyang, Liaoning	Tomato	E123°24' N41°50'
LD-02	Shenyang, Liaoning	Tomato	E123°24' N41°50'
LY-3	Shenyang, Liaoning	Tomato	E123°24' N41°50'
LY-5	Shenyang, Liaoning	Tomato	E123°24' N41°50'
BCHMAX	Chongqing, China	Cabbage	E106°32' N29°32'
HDLHM01	Chongqing, China	Phalaenopsis	E106°32' N29°32'
YCHMOY	Chongqing, China	Oilseed rape	E106°32' N29°32'
Garlic-02	Enshi, Hubei	Garlic	E109°29' N30°16'
Garlic-38	Enshi, Hubei	Garlic	E109°29' N30°16'
Onion76	Enshi, Hubei	Onion	E109°29' N30°16'
B5	Shihezi, Xinjiang	Tomato	E85°94' N44°27'
H4	Shihezi, Xinjiang	Tomato	E85°94' N44°27'
H5	Shihezi, Xinjiang	Tomato	E85°94' N44°27'
H7	Shihezi, Xinjiang	Tomato	E85°94' N44°27'

^a Isolates were provided by Nanjing Agricultural University (Jiangsu), Northwest Agricultural and Forestry University (Shanxi), Shenyang Agricultural University (Liaoning), Southwest University (Chongqing), and Huazhong Agricultural University (Hubei).

mutant had a 4.9-kb band while a 3.8-kb band was detected in wild-type strain BT8-12 (Supplementary Fig. S1B). These results showed that the replacement mutant was the result of a single homologous replacement event at the *sdhC* locus. The five groups of replacement mutants were named R-GB05.10, R-G1, R-G2, R-G3, and R-G4. The replacement mutant R-GB05.10 containing the *sdhC* from B05.10 was assigned as the reference strain. The *sdhC* replacement mutants were used in further analyses.

Effect of the SdhC amino acid polymorphism on intrinsic sensitivity against the SDHIs.

The sensitivity of the *sdhC* in-situ replacement mutants to SDHIs (fluopyram and boscalid) was determined by mycelial growth inhibition in vitro. The results showed that R-G1, R-G2, R-G3, and R-G4 exhibited significantly higher sensitivity to both fluopyram and boscalid than R-G-B05.10 (Fig. 2A).

Effect of the SdhC amino acid polymorphism on mycelial growth, sporulation, and virulence.

To further assess fitness of the five groups of replacement mutants, mycelia growth rate, sporulation, and virulence were analyzed in vitro. Compared with the reference strain R-G-B05.10, the mycelia growth rate of R-G1 and R-G2 decreased while that of R-G3 increased (Fig. 2B). The sporulation of R-G4 was significantly reduced in comparison with that of R-G1 (Fig. 3A). The virulence of the five groups of mutants varied with the hosts plant tissue (leaves of strawberry and tomato and fruit of apple and onion). Compared with the reference group strain R-G-B05.10, the virulence of R-G2 and R-G4 on strawberry leaves, R-G2 and R-G3 on tomato leaves, and R-G4 on onion fruit were increased, whereas the virulence of R-G1 on apple fruit was decreased (Fig. 3B). This suggested that the favorite hosts for the five groups of isolates varied during selection in the field.

Effect of the SdhC amino acid polymorphism on mycelial respiration in response to fluopyram.

SDH plays a pivotal role in both the tricarboxylic acid (TCA) cycle and electron transport chain in fungi, and serves as a target of the SDHIs (Matuz-Mares et al. 2018). After exposition to fluopyram at 5 µg/ml for 1 h, the mycelial respiration inhibition rates of the five groups of *sdhC* replacement mutants were determined. The results showed that the oxygen consumption rates of R-G1, R-G2, R-G3, and R-G4 were significantly inhibited compared with the reference strain R-GB05.10. In addition, the inhibition rate of R-G1 was significantly lower than that of R-G2, R-G3, and R-G4 (Fig. 4A).

Effect of the SdhC amino acid polymorphism on utilizing carbon sources.

To assess the roles of SdhC amino acid polymorphism in assimilation and utilization of different carbon sources, mycelia growth rates of the five groups of replacement mutants were measured on four carbon source media (glucose, glycerol, acetate, and succinate). The results indicated that, compared with the reference strain G-B05.10, the mycelia growth rate of R-G1

was decreased on glucose medium; growth rates of R-G1, R-G2, and R-G4 were decreased on glycerol medium; and growth rates of R-G3 and R-G4 were decreased on acetate medium and succinate medium, respectively. (Fig. 4B).

Effect of the SdhC amino acid polymorphism on oxidative stress response.

To analyze the sensitivity of the five groups of *sdhC*-replacement mutants to oxidative stress generated by paraquat, the mycelia growth inhibition rates of the *sdhC*-replacement mutants in response to paraquat were determined. Compared with the reference strain R-G-B05.10, the sensitivity of R-G1, R-G2, R-G3, and R-G4 was decreased in response to paraquat at 10 µg/ml, and that of R-G2 and R-G4 was decreased when responding to paraquat at 50 µg/ml. The five groups of replacement mutants exhibited no difference in sensitivity to the oxidative stress when responding to paraquat at 100 µg/ml (Fig. 4C).

Fig. 1. Intrinsic sensitivity of G-B05.10, G1, G2, G3, and G4 against the succinate dehydrogenase inhibitors (SDHIs). EC₅₀ = effective concentration that provides 50% growth reduction. Bars denote the standard deviation of four repeated experiments. Values on the bars followed by the same letter are not significantly different at $P = 0.05$.

Table 2. Distribution of substitutions at the codons of SdhC of succinate dehydrogenase from 52 *Botrytis cinerea* strains

Type	Strains	Number of isolates	Corresponding codon changes	Frequency distribution (%)
G-B05.10	Beside G1-4	33	No mutation (reference B05.10)	63.46
G1	TM01, TM03, TM04, LD02, LY04, LY05, HLD08, YC-6, YC-7, BT13-15, NJ5-1, B29, BT9-12, BT3-13, BT2-9	15	G85A, I93V, M158V, V168I	28.85
G2	YCHMOY, BCHM	2	G37S	3.85
G3	TM02	1	I93V	1.92
G4	Onion76	1	I79V, G85A, L151I	1.92

Homology modeling.

C-score is a confidence score for estimating the quality of predicted models by I-TASSER. It is calculated based on the significance of threading template alignments and the convergence parameters of the structure assembly simulations. C-score is typically in the range of -5 to 2 , where a C-score of higher value signifies a model with a high confidence and vice versa. In this model, the value of C-score was -2.02 ; therefore, the quality of the homology model is suitable as a molecular docking receptor.

Model docking.

Molecular docking of fluopyram, boscalid, and carboxin into the homology models according to the binding site was performed. Boscalid docked into the SDH complex from five groups of strains of *B. cinerea* (Figs. 5 and 6). Total scores are shown in Table 3. Boscalid was well bound to the receptor proteins via hydrogen bond (Fig. 5). Boscalid was bound to proteins with its amide oxygen interacting with hydrogen of TYR144 (Fig. 6A and D) or TRP81 (Fig. 6C). The N atom from the pyridine ring of boscalid can also form a hydrogen bond with residue HIS272 (Fig. 6B and E).

DISCUSSION

According to previous reports, *sdhB* substitution plays a critical role in developing resistance of *B. cinerea* to SDHIs (Konstantinou et al. 2015; Lalève et al. 2014), and H132R in the

sdhD was also reported to be involved in the resistance of *B. cinerea* to carboxin (Fan et al. 2018; Leroux et al. 2010). The roles of *sdhC* substitutions in the sensitivity of *B. cinerea* against SDHIs have not been reported. Compared with the *sdhC* sequence of B05.10, we first found that isolates with substitution at *sdhC* comprised 36.54% of all of the fields isolated, and these substitutions might play supportive roles in developing the resistance of *B. cinerea* to SDHIs. Amino acid polymorphism of the SdhC conferred not only intrinsic sensitivity of *B. cinerea* against SDHIs but also other biological fitness characteristics, which were confirmed by a reverse genetic method. To our knowledge, this is the first report about the intrinsic sensitivity of plant pathogens against fungicides, which enhances the understanding of the interaction of SDHIs with *sdhC* in *B. cinerea*. Numerous studies have recently shown that the rapid evolution of particular genes could play an important role in enhancing the ability of pathogens to adapt to new environments or to infect new host species (Croll and McDonald 2012; Matute et al. 2008), and the advantageous substitutions will be rapidly fixed in the population (King et al. 2016). To date, 25 genes in *B. cinerea* have been determined to undergo positive selection evolution (Aguileta et al. 2012). In the current study, 52 *B. cinerea* isolates without a history of exposure to the SDHIs were used to analyze gene diversity of SDH. Interestingly, the amino acid polymorphism only occurred in SdhC of these isolates.

In *Ustilago maydis*, substitution of a highly conserved site H257Y/L in SdhB is correlated with carboxin resistance (Broomfield and Hargreaves 1992; Xiong et al. 2015), and the resistance of *Sclerotinia sclerotiorum* to boscalid is conferred by the point mutation H132R in *sdhD* (Fan et al. 2018). In *B. cinerea*, the *sdhB* mutations confer differential levels of resistance to the SDHIs (Hu et al. 2016). In this study, groups R-G1 (G85A, I93V, M158V, and V168I), R-G2 (G37S), R-G3

Fig. 2. Succinate dehydrogenase inhibitor (SDHI) sensitivity and mycelia growth. **A**, Comparison of sensitivity to the SDHIs among the strains of the five groups R-G-B05.10, R-G1, R-G2, R-G3, and R-G4 after incubation for 3 days on potato dextrose agar modified with various concentration of the SDHIs. EC₅₀ = effective concentration that provides 50% growth reduction. **B**, Colony diameter of R-G-B05.10, R-G1, R-G2, R-G3, and R-G4 strains incubated for 3 days at 25°C. Bars denote the standard deviation of four repeated experiments. Values on the bars followed by the same letter are not significantly different at $P = 0.05$.

Fig. 3. Conidial and virulence assay. **A**, Comparison of conidiation among the R-G-B05.10, R-G1, R-G2, R-G3, and R-G4 strains after 12 days of incubation on potato dextrose agar. **B**, Virulence analysis on various plant tissues following inoculation with the R-G-B05.10, R-G1, R-G2, R-G3, and R-G4 strains. Agar plugs were used as negative controls. Disease symptoms were analyzed 60 h after inoculation. Bars denote the standard deviation of four repeated experiments. Values on the bars followed by the same letter were not significantly different at $P = 0.05$.

(I93V), and R-G4 (I79V, G85A, and L151I) exhibited decreased sensitivity to SDHIs (boscalid and fluopyram). These results indicated that substitutions in SdhC are involved in the sensitivity of various fungi to the SDHIs. In the previous study,

the *S. sclerotiorum*, the SdhB V11A boscalid-resistant strain exhibited lower mycelia growth rate and weaker virulence, and lost the ability to produce sclerotia (Wang et al. 2015). In *A. alternata*, the SdhD D123E boscalid-resistant isolate grew

Fig. 4. Respiratory rate, carbon source utilization, and oxidative stress response. **A**, Comparison of mycelial respiration inhibition among the R-G-B05.10, R-G1, R-G2, R-G3, and R-G4 strains stressed by fluopyram. **B**, Mycelial growth rate of the R-G-B05.10, R-G1, R-G2, R-G3, and R-G4 strains on the four carbon sources. YSS = 2 g of KH_2PO_4 , 1.5 g of K_2HPO_4 , 1 g of $(\text{NH}_4)_2\text{SO}_4 \cdot 7 \text{H}_2\text{O}$, 2 g of yeast extract, and 12.5 g of agar in 1 liter of deionized water, Glc = glucose, Gly = glycerol, Ac = acetate, and Suc = succinate. **C**, Inhibition of mycelial growth was analyzed after each strain was incubated for 3 days on potato dextrose agar supplemented with paraquat at the content indicated in the figure. Bars denote standard deviation from three experiments. Values on the bars followed by the same letter were not significantly different at $P = 0.05$.

more rapidly and produced fewer spores, while the SdhB H277Y boscalid-resistant isolate exhibited increased virulence (Landschoot et al. 2017). In *B. cinerea*, the isolate with mutation H272Y showed a lower mycelia growth rate and produced fewer spores, while the H272L mutant caused smaller lesion diameters (Veloukas et al. 2013). In this study, R-G-05.10 (G85A, I93V, M158V, and V168I) and R-G2 (G37S) mutants exhibited decreased mycelia growth rate while the R-G3 (I93V) mutant showed increased mycelia growth rate, and the sporulation of R-G4 (I79V, G85A, and L151I) mutants was significantly decreased. R-G2 (G37S) and R-G4 (I79V, G85A, and L151I) produced larger lesions on strawberry leaves, and the R-G2 (G37S) and R-G3 (I93V) mutants exhibited larger lesions on tomato leaves. The R-G1 (G85A, I93V, M158V, and V168I) mutants exhibited lower virulence on apple fruit, while R-G4

(I79V, G85A, and L151I) mutants produced smaller lesions on apple fruit but caused larger lesions on onion. These results indicated that substitutions in SdhC were involved in the vegetative development and virulence in different fungi. In addition, the different host plant preferences may be related to the selection of the SdhC amino acid polymorphism of *B. cinerea* in the field, which needs to be explored in a future study.

In connecting oxidative phosphorylation and energy synthesis, SDH provides electrons to the aerobic respiratory chain and SDH activity is an indicator of the TCA cycle in mitochondria (Mahaboob Basha and Saumya 2013). Additionally, the *S. sclerotiorum* SdhB V11A boscalid-resistant mutant exhibited a higher mycelia respiration rate than that of the wild-type strain (Wang et al. 2015). In this study, under fluopyram treatment conditions, the respiration inhibition rates of R-G1

Fig. 5. Pocket figure of boscalid to the binding site of the succinate dehydrogenase complex. **A**, wild-type G-B05.10; **B**, G1 (G85A, I93V, M158V, and V168I); **C**, R-G2 (G37S); **D**, R-G3 (I93V); and **E**, R-G4 (I79V, G85A, and L151I).

Fig. 6. Flat figure of boscalid to the binding site of the succinate dehydrogenase complex. **A**, Wild-type G-B05.10; **B**, G1 (G85A, I93V, M158V, and V168I); **C**, G2 (G37S); **D**, G3 (I93V); and **E**, G4 (I79V, G85A, and L151I). Magenta color indicates the mutational residues and purple color indicates the interacted residues.

(G85A, I93V, M158V, and V168I), R-G2 (G37S), R-G3 (I93V), and R-G4 (I79V, G85A, and L151I) were obviously increased. These results indicated a substitution in the SdhC effect on mycelial respiration treated with or without SDHIs. We inferred that SdhC substitution may be related to the binding activity of SDHs with the SDH complex, which then affected the respiratory rate of *B. cinerea*. Although electron transfer chain complex II (succinate:ubiquinone oxidoreductase) does not contribute to the proton motive force, it is an essential enzyme in the TCA cycle and the oxidation of succinate to fumarate, which is coupled to the reduction of ubiquinone to ubiquinol in the mitochondrial inner membrane, and the carbon source plays important roles in the TCA cycle (Dahal et al. 2014; Xekouki and Stratakis 2012). In this study, the carbon source utilization assay showed that, compared with R-G-B05.10, the mycelia growth rate of R-G1 mutants (G85A, I93V, M158V, and V168I) was decreased on glucose medium, while that of R-G1 (G85A, I93V, M158V, and V168I), R-G2 (G37S), and R-G4 (I79V, G85A, and L151I) on glycerol medium and R-G3 (I93V) on acetate medium were all decreased, suggesting that the SdhC substitution can regulate the carbon source utilization ability of *B. cinerea*. In addition, we inferred that the SdhC substitution may be involved in the selection of *B. cinerea* by their favorite host plants owning different preponderant carbon sources, which can contribute to optimizing the populations of *B. cinerea*. Previous studies demonstrated that mutations in the *sdh* gene were correlated with hypersensitivity to oxidative stresses (Fan et al. 2015). The *A. alternata* SdhD D123E boscalid-resistant mutant exhibited increased sensitivity to paraquat (Landschoot et al. 2017). In *Saccharomyces cerevisiae*, mutation in SdhC resulted in hypersensitivity to oxidative stress (Fan et al. 2015; Szeto et al. 2007). In this study, the sensitivity of R-G1 (G85A, I93V, M158V, and V168I), R-G2 (G37S), R-G3 (I93V), and R-G4 (I79V, G85A, and L151I) to paraquat (10 µg/ml) were decreased when compared with that of R-G-B05.10. The results showed that the SdhC substitution is involved in the tolerance of various fungi to oxidative stress. It is well known that reactive oxygen species play a significant role in the interaction of pathogens with host plants (Yin et al. 2018a). Therefore, the SdhC substitution effect on oxidative stress sensitivity might be related to the interaction of *B. cinerea* with the host plant, then regulate the adaption of *B. cinerea* to the environment. In the model docking assay, we found that substitutions of R-G1 (G85A, I93V, M158V, and V168I), R-G1(G37S), R-G3 (I93V), and R-G4 (I79V, G85A, and L151I) were all located in the SDHI fungicide-binding site of SdhC and involved in binding activity of the SDHs with SdhC, which caused the difference in the intrinsic sensitivity among the five groups of strains responding to SDHIs. The results indicated that amino acid polymorphism of SdhC plays an important role in the intrinsic sensitivity of *B. cinerea* to SDHI fungicides.

In conclusion, amino acid substitutions were identified in SdhC of *B. cinerea* which was never exposed to SDHIs;

therefore, amino acid polymorphism in SdhC of *B. cinerea* is the result of natural biological evolution. The SdhC amino acid polymorphism of *B. cinerea* might play important roles in vegetative development, SDHI intrinsic sensitivity, oxidative stress response, carbon source utilization, and virulence. Furthermore, amino acid polymorphism of SdhC may contribute to the population evolution of *B. cinerea* in the natural environment. These results undoubtedly enhance the understanding of intrinsic sensitivity of plant pathogens against SDHIs, functions of SDH, and the interaction of SDHs with the target.

MATERIALS AND METHODS

Fungal isolates and fungicides.

In total, 52 *B. cinerea* isolates were collected from eight host plants, including strawberry, celery, tomato, onion, phalaenopsis, garlic, oilseed rape, and cabbage, and different geographical districts in China (Table 1). All of the isolates were obtained by a single-spore method and used to analyze the sequence of four SDH subunits (SdhA, SdhB, SdhC, and SdhD) of *B. cinerea*. The wild-type isolate BT8-12 was used as the recipient isolate for transformation. The SdhC of B05.10, used as reference, was named G-B05.10; the natural isolate B29 carrying G85A, I93V, M158V, and V168I in SdhC was named genotype G1; the natural isolate YCHMOY carrying G37S in SdhC was named genotype G2; the natural isolate TM02 carrying I93V in SdhC was named genotype G3; and the natural isolate Onion 76 carrying I79V, G85A, and L151I in SdhC was named genotype G4 (Table 2). All of these isolates were grown on PDA (200 g of potato, 20 g of dextrose, and 20 g of agar power per liter of distilled water) for mycelial growth assays and stored in slants at 4°C.

Fluopyram (96% active ingredient [a.i.]; Sigma), boscalid (95% a.i.; Sigma), and carboxin (98% a.i.; Uniroyal Chemical Company) were dissolved in methanol to obtain 10⁴ µg/ml for the stock solution and stored at 4°C.

Standard molecular methods.

Fungal genomic DNA was extracted as described by a previous study (McDonald and Martinez 1990). Plasmid DNA was isolated using a plasmid miniprep purification kit (Omega Bio-Tek). Southern blot hybridization analysis of homologous recombination in *B. cinerea* was conducted using a 576-bp downstream fragment probe (Supplementary Fig. S2). The probe was labeled with digoxigenin using the high prime DNA labeling and detection starter kit II according to the protocol of the manufacturer (Roche). DNA isolated from *B. cinerea* was digested with *Xba*I and used for Southern hybridization analyses.

PCR amplification and DNA sequence.

The primers used for fragment amplification of *sdhA*, *sdhB*, *sdhC*, and *sdhD* are listed in Supplementary Table S1. Amplification reactions were performed in a 25-µl reaction mixture containing 0.25 µl of *LA Taq* polymerase, 2.5 µl of 10× LAPCR buffer, 2.5 µl of MgCl₂ (2.5 mM), 4 µl of dNTP mixture (2.5 mmol), 1 µl each of primers F and R (20 µmol), 1 µl of template DNA (1 µg ml⁻¹), and 12.75 µl of double-distilled H₂O (Takara). PCR was performed in a DNA Engine System PT-200 (Bio-Rad) with the following conditions: an initial preheating at 94°C for 5 min; followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 52 or 56°C for 30 s, and extension at 72°C for 50 or 70 s; with a final extension at 72°C for 10 min. The PCR products were purified by a gel extraction kit (Omega), cloned into the pMD18-T simple vector (Takara), and sequenced by Springen Biotech. For comparison of *sdhA*, *sdhB*, *sdhC*, and *sdhD* in different isolates, the sequences were analyzed using Bioedit software (version 7.0.5.3) for manual editing and translation.

Table 3. Total score obtained via docking of three succinate dehydrogenase inhibitors with the protein structure model of different type succinate dehydrogenase complex

Inhibitor	Binding energies of each complex with different fungicides				
	G-B05.10 (WT)	G1 (G85A, I93V, M158V, and V168I)	G2 (G37S)	G3 (I93V)	G4 (I79V, G85A, and L151I)
Fluopyram	2.31	2.53	2.91	2.13	2.86
Boscalid	4.05	3.39	4.60	4.07	3.54
Carboxin	2.93	2.71	2.95	2.68	2.90

Construction of replacement constructs for *SdhC*.

The replacement constructs upstream-*SdhC*-PNeo-downstream of the *B. cinerea* *sdhC* gene were generated by fusion PCR (Yu et al. 2004) (Supplementary Fig. S1). The upstream and downstream of *B. cinerea* was amplified from isolate BT8-12 with primer pairs P1/P2 and P3/P4, respectively. The entire *sdhC* gene of *B. cinerea* (BC1G_14785) was amplified from B05.10, B29, YCHMOY, TM02, and Onion76 with the primer pair P5/P6. A 1.1-kb *Neo*-resistant cassette containing the *Aspergillus nidulans* *trpC* promoter was amplified from pII99-Pro (DOHH)-GFP plasmid vector (Ge et al. 2013) with the primer pair P7/P8. Then, the *sdhC* fragment was mixed with the *Neo* resistance cassette in a molar proportion of 1:1 and fused by double-joint PCR. The fused PCR product was used as DNA template to amplify the *SdhC*-PNeo fragment using the primer pair P5/P8. Finally, the two *sdhC* flanking fragments were mixed with the *SdhC*-PNeo in a molar proportion of 1:1:3 and fused by double-joint PCR again. The fused PCR product was used as DNA template to amplify replacement constructs with primer pair P1/P4 (Supplementary Fig. S1; Supplementary Table S1). The PCR production was gel purified, sequenced, and used for protoplast transformation of *B. cinerea*.

Transformation of *B. cinerea*.

Protoplasts were prepared from *B. cinerea* isolate BT8-12 and transformed according to a previously described method (Duan et al. 2013; Maier et al. 2005), with some modifications. To generate protoplasts, mycelial plugs cut from a 3-day-old colony were placed into 250-ml flasks containing 100 ml of potato dextrose broth (PDB) (200 g of potato and 20 g of dextrose per liter of distilled water). The flasks were kept on a rotary shaker (175 rpm, 25°C) for 36 h. Mycelia were collected using a sterile nylon filter and washed with distilled water twice to remove medium. Then, 0.1 g of mycelium was resuspended in 20 ml of KCl buffer (0.6 M KCl and 50 mM CaCl₂) containing 0.4 g of lysing enzymes (Sigma-Aldrich) and incubated for 2 h at 30°C and 85 rpm. Protoplasts were separated from cell debris with filtration through four layers of lens tissue and washed twice with KCl buffer. They were isolated via centrifuging at 1200 rpm and 4°C for 5 min and resuspended in 10 ml of STC (0.8 M sorbitol, 50 mM Tris-HCl, and 50 mM CaCl₂, pH 8.0). After centrifuging at 1,200 rpm for 5 min, protoplasts were suspended in SPTC (STC with 40% [wt/vol] PEG 6000). For transformation, 2×10^8 protoplasts in 200 µl of SPTC buffer, 5 µg of heparin, and 3 µg of replacement vector were mixed and incubated on ice for 30 min; then, 1 ml of SPTC was mixed with the suspension and incubated at 25°C for 20 min. Transformed protoplasts were mixed into 200 ml of RM (yeast extract at 0.5 g/liter, casein hydrolysate at 0.5 g/liter, 0.7 M sucrose, and agar powder at 16 g/liter) (Ge et al. 2013; Lalève et al. 2014; Malonek et al. 2004) at 43°C, poured into petri plates, and incubated at 25°C in darkness. After 12 h, RM plates were overlaid with 10 ml of SRM (yeast extract at 0.5 g/liter, casein hydrolysate at 0.5 g/liter, 1 M sucrose, agarose at 12 g/liter, and G-418 at 100 µg/ml), and incubated at 25°C. After 3 to 7 days, geneticin-resistant colonies appeared and individual transformants were transferred on PDA plates containing G-418 (100 µg/ml) and used in subsequent experiments.

Sensitivity to fluopyram, boscalid, and carboxin.

Sensitivity to SDHIs (including fluopyram, boscalid, and carboxin) was determined in the different group isolates (wild-type isolates and replacement mutants) by mycelial growth inhibition according to a previous study (Matheron and Porchas 2004). The medium was amended with fluopyram, boscalid, and carboxin to obtain a gradient of five concentrations (fluopyram: 0.125, 0.25, 0.5, 1, and 2 µg ml⁻¹; boscalid: 0.5, 1, 2, 4,

and 8 µg ml⁻¹; and carboxin: 0.01, 0.05, 0.1, 0.5, and 1 µg ml⁻¹). A mycelial plug (5 mm in diameter) cut from the edge of a 3-day-old colony of each isolate was transferred to 9-cm Petri dishes containing the amended medium, with three replicates per concentration. Each isolate was incubated at 25°C in the dark for 3 days. Mean colony diameter (minus the diameter of the inoculation plug) was measured for each treatment and expressed as a percentage of growth inhibition (the percentage of growth inhibition determined relative to the fungicide-free check). The EC₅₀ values of fungicides for each isolate were calculated by regressing the percentage growth inhibition against the log of fungicide concentration (Zhou et al. 1994). The experiment was repeated twice.

Colony growth, sporulation, and pathogenicity.

For colony growth and sporulation assays, mycelial plugs (5 mm in diameter) of 10 *sdhC* replacement mutants (two isolates chosen from each group) were cut from the edge of a 3-day-old colony and inoculated on PDA plates with four replicates. After 3 days at 25°C, the mean diameter of each isolate was measured. After 12 days, spores were collected from plates by pouring 5 ml of sterile water with 0.2% Tween-80 onto each plate and filtering the suspension through two layers of lens tissue. Conidia were counted with a hemocytometer (Zhang et al. 2014).

Pathogenicity of the *sdhC* replacement mutants was analyzed on tomato leaves, apple fruit, onion fruit, and strawberry leaves according to previous studies (Duan et al. 2013; Zhang et al. 2014), with slight modifications. Mycelial plugs (5 mm in diameter) cut from a 3-day-old colony of each isolate were placed on the surface of fruit and leaves. Then, inoculated fruit and leaves were placed at 20°C with a 16-h photoperiod and 85% relative humidity for 3 days; and, finally, two orthogonal diameters of each lesion were measured. There were five fruit and eight leaf replicates for each replacement mutant. The experiment was repeated twice.

Mycelial respiration rate assay.

To explore the effect of SDHIs on respiration of *SdhC* replacement mutants, fluopyram was used to measure mycelial oxygen consumption. The experiment was performed according to previous studies (Feng et al. 2009; Markoglou et al. 2006; Zhang et al. 2014), with minor modifications. Mycelial plugs of different group replacement mutants were put into conical flasks containing 50 ml of PDB and incubated under shaking (175 rpm, 25°C). After 36 h, fluopyram solutions were added to flasks at concentrations of 0 and 5 µg/ml. The flasks were incubated for 1 h; then, oxygen consumption was measured with an oxygraph system (F12-ED; Julabo) and the inhibition ratio of respiration rate was calculated (Zhang et al. 2014). The experiment was repeated twice.

The effect of carbon sources on mycelial growth.

To investigate the effect of carbon on mycelial growth, YSS (2 g of KH₂PO₄, 1.5 g of K₂HPO₄, 1 g of (NH₄)₂SO₄·7 H₂O, 2 g of yeast extract, and 12.5 g of agar in 1 liter of deionized water) supplemented with different 0.05-M carbon sources (glucose, glycerol, acetate, and succinate) was used to determine the mycelial growth of each group of replacement mutants according to Lalève et al. (2014), with slight modifications. The mycelial plug (5 mm in diameter) of each isolate was cut from a 3-day-old colony and inoculated on YSS cultivated at 20°C for 3 days. The mean diameter of two replacement mutants per group was calculated. The experiment was repeated twice.

Sensitivity to oxidative stress.

Paraquat is an herbicide inducing oxidative stress through the generation of superoxide ions in either the cytoplasm or

mitochondria. According to a previous study (Yin et al. 2018b), the sensitivity of each *sdhC* group replacement mutants to paraquat was determined on PDA plate amended with paraquat to obtain final concentrations of 10, 50, and 100 µg/ml. The mycelial plugs (5 mm in diameter) cut from the edge of a 3-day-old colony of each isolate were transferred onto 9-cm Petri dishes containing the amended PDA with three replicates per concentration. Each isolate was incubated at 25°C in darkness for 3 days. Mean colony diameter was measured for each treatment and expressed as a percentage of growth inhibition as compared with the untreated control. The experiment was repeated twice.

Homology modeling.

The three-dimensional experimental structure of *B. cinerea* SdhC is not yet available and, hence, we modeled its homology protein. The NCBI protein database was used to search the SdhC amino acid of *B. cinerea*, and the hypothetical protein sequences were analyzed. The BLAST server was used to search the templates for these four chains. We used the SDH from porcine heart (PDB code 1ZOY) and avian heart (PDB code 1YQ3) as two templates. The amino acid sequence of SdhC of each isolate was uploaded to the online server platform of I-TASSER software, which builds a homology crystal structure model of SdhC referring to templates.

Model docking.

The surflex-Dock module of SYBYL software was used for the molecular docking study. Surflex-Dock is a fully automatic, flexible molecular docking algorithm that combines the scoring function from the Hammer-head docking system with a search engine that relies on a surface-based molecular similarity method as a means to rapidly generate suitable putative poses for molecular fragments (Xie et al. 2015). Chain B residues P225, S226, W228, W229, H272, and I274 form the major part of the binding pocket. Chain C (L73, W81, S84, and R88) and chain D (D143 and Y144) form the remainder of the fungicide-binding cavity. Protein structure minimization was performed by applying a Tripos force field, and partial atomic charges were calculated by the Gasteiger-Huckel method (Aparoy et al. 2011; Sang et al. 2018).

ACKNOWLEDGMENTS

We thank C. Bi from Southwestern University, Z. Qi from Shenyang Agricultural University, X. Wang from Shihezi University, and G. Li from Huazhong Agricultural University for generously offering the strains of *Botrytis cinerea*.

AUTHOR-RECOMMENDED INTERNET RESOURCES

NCBI protein database: <https://www.ncbi.nlm.nih.gov/protein>
BLAST server: <https://blast.ncbi.nlm.nih.gov>

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