Rapid Detection of *SdhB*^{P225F} and *SdhB*^{H272R} Mutations in Boscalid Resistant *Botrytis cinerea* Strains by ARMS-PCR

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SdhB^{P225F} and *SdhB*^{H272R} mutations have been found associated with boscalid resistance in *Botrytis cinerea* from strawberry in Shanghai, China. For rapid detection of two mutations, tetra-primers were designed and optimized to gain the relatively high accuracy and specificity based on the ARMS-PCR technique, by which resistance can be identified with different lengths of products on agarose gels. The tetra-primer ARMS-PCR systems for *SdhB*^{P225F} and *SdhB*^{H272R} were validated by 9 *SdhB*-sequenced strains repeatedly. Then, sensitivity of 30 more strains were also tested by the methods, which were accordant with genotypes by sequencing and the sensitivity of conidial germination to boscalid by 100%. Thus, the methods developed in this study are proved to be rapid, inexpensive, accurate and practical for resistance detection of *Botrytis cinerea* caused by *SdhB*^{P225F} and *SdhB*^{H272R} mutations.

Keywords : ARMS-PCR, boscalid resistance, detection of point mutations, grey mould, PASA

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Botrytis cinerea Pers., the causal agent of grey mould disease, is a ubiquitous fungus and could infect more than 200 plant species worldwide, including vegetables, ornamental

plants, and fruits, such as strawberry in particular, which is prone to grey mould diseases during various growth stages in greenhouses or open field (Fernández-Ortuño et al., 2012; Williamson et al., 2007). In a disease cycle, *B. cinerea* can survive and grow on dead and decayed plant tissues. After that, a great mass of conidia is produced on these sources under appropriate humidity and temperature conditions. As airborne inocula, these abundant conidia of *B. cinerea* give the chance to cause infection for many times within one season (Williamson et al., 2007). Thus, this pathogen is closely associated with heavy economic losses of strawberry production every year in China. At present, the control of the grey mould disease often depends on appropriate fungicide applications.

The fungicide boscalid, a succinate-ubiquinone dehydrogenase inhibitor (SDHI), is highly effective against *B. cinerea* (Stammler et al., 2008) and has been registered for grey mould control in China. SDHIs are single-site inhibitors and target succinate dehydrogenase (Sdh) complex, the mitochondrial respiratory complex II in the electron transport chain. The site-specific mode of action makes the fungus easier to gain resistance to boscalid, especially for *B. cinerea* with high genetic variability, short life cycle, and prolific reproduction (FRAC, 2018, <http://www.frac.info/publications>). In general, a single-base mutation at the codon 225 and 272 of *SdhB*, leading to the substitution of P225L, P225F, P225T, H272Y, and H272R, confer different levels of resistance to boscalid (Angelini et al., 2010; Leroux et al., 2010; Yin et al., 2011). So far, field resistance to boscalid of *B. cinerea* from strawberry, grapevine, lettuce, and many other crops has been reported in many countries, including the United States, France, Germany, Greece et al. (Chatzidimopoulos et al., 2013; Kim and Xiao, 2010; Leroux et al., 2010). According to our previous investigation of boscalid resistance of 195 *B. cinerea* strains from 6 strawberry-production districts (13 orchards,

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totally) in Shanghai in 2017, over 25% of strains collected were found to be resistant to boscalid and there were two kinds of natural mutation in *SdhB* detected by sequencing, *SdhB*^{P225F} and *SdhB*^{H272R}.

Resistance monitoring, which is the basis of resistance management, always requires plenty of time and labour to complete the tests for mycelial growth or conidial germination on fungicide-amended media with enough quantity of samples. More efficient methods for resistance detection are required to be explored and tested. Amplification refractory mutation system (ARMS-PCR) is a method in which PCR is adapted for rapid detection of single-base changes in genomic DNA by using specifically designed primers (Liu et al., 1997). It is known as PCR amplification of specific alleles (PASA), as well. Ye et al. (1992) were the first to describe the tetra-primer ARMS-PCR method, in which four primers are needed, including two outer primers, and two inner specific primers. For this technique, an inner primer is designed to match one allele perfectly but mismatch the other allele at or near the 3' end. By changes on the 3' primer ends, two inner primers can specifically amplify one allele over another with their outer primers in pair (Liu et al., 1997). Then, the results are expected by amplification products with different length on agarose gels. Therefore, ARMS-PCR is a rapid, simple and inexpensive method, and it is well suitable for high-throughput detections of point mutations.

In this study, we developed accurate methods based on ARMS-PCR for identification of boscalid-resistance strains of *B. cinerea* by identifying two types of mutations in *SdhB* gene leading to the P225F or H272R substitution. Furthermore, the methods were applied in 30 more strains to guarantee the universality of this method.

A total of 39 single-spore isolates of *B. cinerea* were in-

involved in this study. All of them were isolated from strawberry fruits with grey mould in Shanghai in 2017. Among them, 9 *SdhB*-sequenced strains of 3 genotypes were used for ARMS-PCR validation (Table 1), and 30 other strains were randomly selected from 3 different districts for application of this methods. The process of single-spore isolation was conducted with the method described by Fernández-Ortuño et al. (2012). All the strains were cultured on potato dextrose agar (PDA) at 24°C, and stored on PDA slants at 4°C in the dark. The genomic DNA of *B. cinerea* strains was extracted and purified from mycelia grown on PDA for 3 days. An SDS lysis method for DNA extraction and purification was used according to Harju et al. (2004). Tetra-primers for ARMS-PCR were designed and selected. The original tetra-primers with appropriate GC content and product sizes were selected using the Primer-Blast tool on the NCBI website. Then, extra mismatches were introduced near the 3' end of primers by trial and error. In order to avoid the interference of other latent mutations at the position of 225 or 272 codon, the first and the second letters of the 225 and 272 codons remained unchanged at 3' end of inner primers. The final primer sets of 225-EX-F6, 225-EX-R, 225-M-F9 and 225-S-R3 are used for *SdhB*^{P225F} detection, and primer 272-EX-F, 272-EX-R2, 272-M-F2 and 272-S-R2 are used for *SdhB*^{H272R} detection (Table 2). The PCR reaction was performed in a final volume of 20 µL, including 10 µl of 2 × EasyTaq PCR SuperMix (Trans, Beijing), 0.25 µM of each 4 primers, 20 ng of fungal DNA. PCR program consisted of an initial denaturation step of 94°C by 3 min; 32 cycles for *SdhB*^{P225F} and 34 cycles for *SdhB*^{H272R} of 94°C by 20 s, 55°C by 20 s, and 72°C by 30 s; and a final elongation of 5 min at 72°C. The PCR products were electrophoresed on 2% agarose gels. Tests were replicated three times. The resistance phenotypes were de-

Table 1. *Botrytis cinerea* strains for ARMS-PCR validation

Strains	Origin	Codon numbers in <i>BcSdhB</i> by sequencing ^a		Resistance phenotype ^b
		225	272	
B1	Pudong, Shanghai	TTC, Phe	CAC, His	R
B2	Pudong, Shanghai	TTC, Phe	CAC, His	R
B3	Pudong, Shanghai	TTC, Phe	CAC, His	R
B4	Jiading, Shanghai	CCC, Pro	CGC, Arg	R
B5	Qingpu, Shanghai	CCC, Pro	CGC, Arg	R
B6	Jinshan, Shanghai	CCC, Pro	CGC, Arg	R
B7	Pudong, Shanghai	CCC, Pro	CAC, His	S
B8	Jinshan, Shanghai	CCC, Pro	CAC, His	S
B9	Qingpu, Shanghai	CCC, Pro	CAC, His	S

^aBold characters are the mutant codon.

^bThe resistance phenotypes were determined based on conidial germination at discriminatory doses of 50 mg/l

Table 2. Primers used in this study

Primers ^a	Sequences (5'-3') ^b	Uses
225-M-F9	CATGCTGCTCGACATCT <u>AACTTC</u>	<i>SdhB</i> ^{P225F} detection
225-S-R3	CCTCACTGTTCCACCAGT <u>TGCAGG</u>	
225-EX-F6	CTTCAACACACCGAC <u>GCAAC</u>	
225-EX-R	ACCGCCCAAAACACCACAAC	
272-M-F2	AGCATGAGTTTGTACAGAT <u>CGCGC</u>	<i>SdhB</i> ^{H272R} detection
272-S-R2	CCTCGAGCAGTTGAGAATAG <u>ACTG</u>	
272-EX-F	AGACGCTAAGCACGAAACGA	
272-EX-R2	TAACCGCCCAAAACAC <u>GTCA</u>	
SdhB-F	TCGAACCTACTCGCCCTATCCAATT	<i>SdhB</i> sequencing
SdhB-R	GACTTCTTAGAAAGCCATTTCTTTC	

^aPrimers for P225F and H272R detections in this table were the final sets by optimization.

^bBold characters are the positions of point mutations. Italic characters with underlines are the extra mismatch bases.

terminated based on conidial germination at discriminatory doses of 50 mg/l, as described by Fernández-Ortuño et al. (2012). 30 *B. cinerea* strains were randomly selected for mutation detection with the above ARMS-PCR systems. The mutation types were determined by band patterns on agarose gels, and furtherly confirmed by *SdhB* sequencing and resistance phenotype test on conidial germination to boscalid.

Based on the results of sequencing, Strain B1, B2 and B3 were *SdhB*^{P225F} mutant, and strains B4, B5 and B6 were *SdhB*^{H272R} mutant. It is confirmed that B1, B2, B3, B4, B5 and B6 were all resistant to boscalid according to the sensitivity of conidial germination. Strain B7, B8 and B9 were wild-type strains, which are sensitive to boscalid. For the P225F detection, as shown in Fig. 1A and Fig. 2A, a 426 bp control fragment was successfully amplified for all tested strains as a positive control. As expected, this system produces an amplification product of 330 bp for P225F mutant (B1, B2, B3), and a 142 bp product for the native Pro²²⁵ codon (B4~B9). For the H272R detection, as shown in Fig. 1B and Fig. 2B, a 540 bp control fragment was successful-

ly amplified for all tested strains. As expected, this system produced an amplification product of 192 bp for H272R mutant (B4, B5, B6), and a 394 bp products for the native His²⁷² codon (B1~B3, B7~B9). When a DNA mixture of the mutative and native codon was used as the template, the two specific fragments were both observed, indicating that this method can detect combinations of strains from populations with different resistance types. The application of ARMS-PCR for *SdhB*^{P225F} and *SdhB*^{H272R} in 30 isolates from 3 districts in Shanghai showed the same result as it of sequencing and conidial germination sensitivity (Table 3). These methods appeared to be suitable and perfect in practice to monitoring boscalid resistance caused by *SdhB*^{P225F} and *SdhB*^{H272R}.

According to Fungicide Resistance Action Committee (FRAC), *B. cinerea* is at a high risk of resistance development to fungicides. Resistance of *B. cinerea* has been quite frequently reported in commercial situations for many important fungicide classes, including methyl benzimidazole carbamates (MBC), N-phenyl carbamates, quinone outside Inhibitor (QoI), dicarboximides, demethylation

Fig. 1. Details of mutations and ARMS-PCR primers (A: *SdhB*^{P225F}, B: *SdhB*^{H272R}). Mutations in genomic sequences are indicated by dotted box. Bold characters in primers are the positions of point mutations. Lower italic letters are the extra mismatch bases. Direction of primers are indicated by arrows.

Fig. 2. Electrophoretogram of tetra-primer ARMS-PCR system for detection of the *SdhB*^{P225F} and *SdhB*^{H272R} mutation associated with boscalid resistance. (A) The 426 bp and 330 bp fragments are directed to the P225F mutant (B1~B3). The 426 bp and 142 bp fragments on agarose gel are directed to the native Pro²²⁵ codon (B4~B9). The mixture of the mutant and the native were shown behind, with three fragments (B1+B4 and B1+B7). (B) The 540 bp and 192 bp fragments are directed to the H272R mutant (B4~B6). The 540 bp and 394 bp fragments are directed to the native His²⁷² codon (B1~B3, B7~B9). The mixture of the mutant and the native were shown behind, with three fragments (B1+B4 and B4+B7).

inhibitor (DMI), succinate-ubiquinone dehydrogenase inhibitor (SDHI) et al. (FRAC, Publication: list of resistant plant pathogens). Boscalid, a relatively new SDHI, is also classed as ‘high risk’, due to its single target site, rapid increase of resistance, significant decrease of disease control under field conditions, and other traits. The combined resistance risk of *B. cinerea* to boscalid has been estimated to be at the highest level among dozens of pathogen/fungicide combinations by FRAC (Publication: pathogens risk list).

Our results showed the high frequency of boscalid resistance of *B. cinerea* from strawberry in Shanghai. These conformed to our earlier investigation and monitoring which gained the resistance frequency of over 25%. Among all the sampling sites, Pudong, Qingpu and Jinshan are the districts with the highest resistance frequencies in Shanghai, 2017. Compared to the previous results, we used a newly designed ARMS-PCR method to test newly collected samples and also detected fairly high resistance frequencies in these three districts (9/10, 3/10 and 4/10). Besides, *SdhB*^{P225F} resistant mutants were only found in Pudong, and *SdhB*^{H272R} resistant mutants were the only mutation type found in other districts, which indicated that there were the specific dominant resistant mutation types in each region. Yin et al. (2011) found H272R and H272Y in *BcSdhB* are the dominant genotypes in field boscalid resistance in Washington from apple. Fernández-Ortuño et al. (2012) showed the H272R mutation in *BcSdhB* was much more common occurred in resistant isolates from strawberry fields in the Carolinas. Therefore, molecular detection based on the genomic characteristics of dominant mutants

might be the direct way to identify rapid developed resistance.

Tests for sensitivity by mycelial growth or conidial germination is the common approaches to monitor resistance now, which require plenty of time and labour to make pathogens mycelium growth or sporulation on the proper culture mediums. Besides, the operations and observations of thousands of petri dishes are considerably labour-consuming, as well. According to Fernández-Ortuño et al. (2012), discriminatory doses can be used to distinguish the sensitive and the resistance spores by the significant difference of the germination rates. However, the method might also be not perfect to detect spores directly collected from the field, because of the lower germination activity of over-stayed spores and the interfering microexamination with other mixed microorganisms.

In consideration of the rapid resistance development of *B. cinerea* to boscalid and the mutant genotypes detected responsible for resistance, more rapid methods of molecular biological detection are required to be explored and tested. Previously, several molecular methods have been applied in detection of fungicide resistance offered by single-base mutations in the genome of *B. cinerea*. Luck and Gillings (1995) identified benomyl resistant *B. cinerea* strains by PCR-restriction fragment length polymorphism (RFLP) and allele specific amplification (ASA) of β -tubulin gene. Banno et al. (2008) recognized changes in the nucleotide sequence associated to benzimidazole and dicarboximide resistance by analyzing melting curve profiles on a real-time PCR instrument. Chatzidimopoulos et al. (2014)

Table 3. Boscalid resistance monitoring by tetra-primer ARMS-PCR, conidial germination, and sequencing

Origin	Isolates	Tetra-primer ARMS-PCR ^a		Resistance phenotype ^b	Codon numbers in <i>SdhB</i> by sequencing ^a	
		<i>SdhB</i> ^{P225F}	<i>SdhB</i> ^{H272R}		225	272
Pudong, Shanghai	PD1	F	H	R	TTC (F)	CAC (H)
	PD2	F	H	R	-	-
	PD3	F	H	R	-	-
	PD4	F	H	R	-	-
	PD5	F	H	R	-	-
	PD6	P	H	S	CCC (P)	CAC (H)
	PD7	F	H	R	-	-
	PD8	F	H	R	TTC (F)	CAC (H)
	PD9	F	H	R	-	-
	PD10	F	H	R	-	-
Qingpu, Shanghai	QP1	P	R	R	CCC (P)	CGC (R)
	QP2	P	H	S	-	-
	QP3	P	R	R	-	-
	QP4	P	R	R	CCC (P)	CGC (R)
	QP5	P	H	S	CCC (P)	CAC (H)
	QP6	P	H	S	-	-
	QP7	P	H	S	-	-
	QP8	P	H	S	-	-
	QP9	P	H	S	-	-
	QP10	P	H	S	-	-
Jinshan, Shanghai	JS1	P	H	S	-	-
	JS2	P	R	R	-	-
	JS3	P	H	S	CCC (P)	CAC (H)
	JS4	P	H	S	CCC (P)	CAC (H)
	JS5	P	R	R	CCC (P)	CGC (R)
	JS6	P	R	R	-	-
	JS7	P	H	S	-	-
	JS8	P	H	S	-	-
	JS9	P	H	S	-	-
	JS10	P	R	R	CCC (P)	CGC (R)

^aBold characters are the mutant codon. Strains with Pro²²⁵ (CCC) and His²⁷² (CAC) in *SdhB* genes are the wild-type sensitive. The P225F (TTC) or H272R (CGC) mutants are resistant to boscalid. A dash (–) denotes not identified by sequencing.

^bThe resistance phenotypes were determined based on conidial germination at discriminatory doses of 50 mg/l.

detected and differentiated the substitutions associated with resistance to fenhexamid and boscalid (*SdhB*^{H272R} & *SdhB*^{H272Y}) by high resolution melting (HRM-PCR) assay. Compared to PCR-RFLP or real-time PCR methods, the ARMS-PCR has various advantages, such as easiness to manipulate with fewer steps, less time and labour consumed, fewer instruments and less expense required (Muñoz et al., 2009). To be specific, with the same procedure as ordinary PCR, the ARMS-PCR assay can be completed within 2h. Besides, ARMS-PCR is stable and accurate, with the function to distinguish between homozygotes and heterozygotes in one PCR reaction (Zhang et al., 2017). In this study, ARMS-PCR systems were developed for

detection of *SdhB*^{P225F} and *SdhB*^{H272R} mutations associated with boscalid resistance. There was a 100% correlation with fungicide sensitivity assays of conidial germination. In addition, a mixture of mutants and wide types could be identified by this method, in case the ‘single-spore isolate’ came from several adjacent conidia with different genetic background.

Nevertheless, the flaw of the methods developed in this study is the incomplete mutation types included. There are some other mutations in 225 and 272 codons of *SdhB* worldwide, such as P225L, P225T and H272Y. Even one strain with N230I substitution in *SdhB*, and another with H132R in *SdhD* were also reported to be resistant

to boscalid (Leroux et al., 2010). Besides, new resistance mechanisms will be likely to emerge with boscalid continuous application in the future. However, extra mismatch of tetra-primers developed in this study were designed with the first and second letters unchanged of codon 225 and 272. Thus, an added mismatch caused by new mutations at codon 225 or 272 of *SdhB* may be possibly identified by the only positive control fragment on agarose gels because of no specific allele matched by inner primers in our ARMS-PCR systems. In addition, detection for new mutations might be realized by a few modifications of the available primers developed in this study. In view of the rapid resistance increase of *B. cinerea*, the strategies of fungicide application and resistance management of boscalid need to be established and practiced to delay the development of resistance, and at the same time, as the base of resistance management, studies on efficient and accurate resistance monitor are urgently needed, to adapt to new conditions and mechanisms of field resistance of *B. cinerea* to boscalid.

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