Detection and Characterization of Carboxylic Acid Amide-Resistant *Plasmopara viticola* in China Using a TaqMan-MGB Real-Time PCR

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Abstract

Grape production is increasing globally and so are problems with downy mildew, one of the main constraints in grape production. Downy mildew on grape is caused by *Plasmopara viticola*, an obligate biotrophic pathogen belonging to the oomycetes. Control of the disease is usually performed by fungicide applications, of which carboxylic acid amide (CAA) fungicides represent one of the most widely used groups of fungicides. Our previous research showed that the extensive application of CAA fungicides can result in fungicide resistance and in China, CAA-resistant isolates of *P. viticola* were collected from the field in 2014. To monitor the distribution and spread of CAA fungicide resistance, we developed a TaqMan-minor groove binder (MGB) real-time PCR-based method designed on a functional mutation in the *PvCesA3* gene that allows efficient identification of CAA fungicide resistant and sensitive genotypes. The assay was validated on 50 isolates using Sanger sequencing and fungicide bioassays and exploited in a comprehensive

survey comprising 2,227 single-sporangiotheca isolates from eight major grapevine regions in China. We demonstrate that CAA fungicide resistance in *P. viticola* is widespread in China. On average, 53.3% of the isolates were found to be resistant, but marked differences were found between locations with percentages of resistant isolates varying from 0.3 to 96.6%. Furthermore, the frequency of CAA-resistant isolates was found to be significantly correlated with the exposure to CAA fungicides ($P < 0.05$). We further discussed the possibilities to apply the TaqMan-MGB real-time PCR assay to assess the frequency of fungicide-resistant *P. viticola* isolates in each region or vineyard, which would facilitate the correct choice of fungicide for grape downy mildew and resistance management strategies.

Keywords: *Plasmopara viticola*, TaqMan-MGB real-time PCR, carboxylic acid amide fungicides, resistance

Grape (*Vitis vinifera* L.) is one of the earliest domesticated and major economic fruit crops in the world (Myles et al. 2011). Since 2010, China is the largest grape producer in the world, and the total area of grape cultivation increased to 870,000 ha in 2017, comprising table, wine, and dried grape (Tian et al. 2019). Table grape including *V. vinifera*, *V. labrusca*, and interspecific hybrids is most popular, covering 83% of the grape acreage (OIV 2018; Tian et al. 2019), followed by wine grape (11%). Most wine grape varieties are *V. vinifera*, as well as some Chinese wild species, such as *V. davidii* and *V. amurensis*. One of the main constraints in grape production is downy mildew. This devastating disease is caused by *Plasmopara viticola* and has been implicated in major economic losses on a global scale (Emmett et al. 1992). In China, yield losses caused by grape downy mildew were reported ranging from 20 to 80% (Hu et al. 2013).

P. viticola is an obligate biotrophic pathogen that belongs to the oomycetes. Although they share a range of morphological features with fungi, oomycetes possess various distinct characteristics including a diploid vegetative mycelial stage and cellulose as a major

component of their cell wall. Downy mildew was first reported in China in 1899 and can be observed in almost all Chinese grape vineyards (reviewed by Yin et al. 2014). Although some American and Chinese wild species show different levels of resistance, all major commercial *V. vinifera* cultivars are highly susceptible to *P. viticola* (Yu et al. 2012) and chemical control is currently the most effective measure used to protect grapes from downy mildew disease (Chen et al. 2007; Waard et al. 1993).

Carboxylic acid amide (CAA) fungicides (FRAC code no. 40) are widely used to control the pathogens in the families Peronosporaceae (e.g., *P. viticola* and *Bremia lactucae*) and Pythiaceae (e.g., *Phytophthora* spp., but not *Pythium* spp.) (Gisi and Sierotzki 2008; Gisi et al. 2007a). CAA fungicides comprise a series of popular fungicides, such as dimethomorph, mandipropamid, pyrimorph, iprovalicarb, benthialacarb, valifenalate, and flumorph, all targeting the cellulose synthase 3 (*CesA3*) enzyme (Blum et al. 2010a). Resistance in *P. viticola* field populations to CAAs has been reported in Europe for almost 10 years, with cross-resistance shown between representatives of this class of fungicides (FRAC 2017; Gisi et al. 2007b). The implementation of effective management strategies impeding the evolution of resistance requires an accurate understanding of the emergence and development of resistance in pathogen populations (REX Consortium 2013; Schnabel et al. 2012). In China, dimethomorph was widely used to control *P. viticola* in the last 20 years, and CAA-resistant *P. viticola* isolates were first collected in 2014 (Zhang et al. 2017). However, the information on frequency of resistant isolates in China is still largely unknown.

As *P. viticola* is an obligate biotrophic pathogen, screening for fungicide-resistant isolates is traditionally performed using a sensitivity assay on grape leaf disks (Wong and Wilcox 2000). However, this method is laborious and time consuming. In 2010, Blum et al. first described the molecular mechanism underlying resistance to a CAA fungicide. Resistance to CAA is conferred by recessive mutations of the nuclear gene encoding cellulose synthase 3 (*PvCesA3*). If an SNP leading to a substitution of a glycine (GGC) to a serine (AGC) residue at position 1105 (G1105S) in the deduced amino acid sequence is present in both alleles, isolates are resistant to CAA

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(Blum et al. 2010b). A second mutation at the same codon in *PvCesA3* gene, G1105V, was also found associated with CAA resistance (Sierotzki et al. 2011), but it is detected at a low frequency and at a small number of sites (reviewed by Zhang et al. 2017). Based on the mutation G1105S of the *PvCesA3* gene, several molecular methods have been developed for the detection of resistant isolates (Aoki et al. 2011, 2013; Zhang et al. 2017).

In our previous research, a tetra-primer amplification refractory mutation system (tetra-primer ARMS PCR) was established (Zhang et al. 2017). A set of four primers was designed to distinguish the different allelic variants (the homozygous and heterozygous alleles) in a single assay, which was demonstrated to be more rapid, accurate, and practical to detect *P. viticola* resistant to CAA fungicides compared with the traditional bioassays and some other molecular methods, such as PCR-RFLP (Zhang et al. 2017). Nevertheless, two-pair primers can affect the amplification specificity (Zhang et al., unpublished data)—such difference in primer melting temperature (T_m) was previously reported (Hamajima et al. 2002). A new strategy for diagnostics that is more practical for high throughput screening is therefore required. A TaqMan minor groove binder (MGB) probe real-time PCR assay was selected to enhance the specificity and accuracy of detection.

Application of a TaqMan-MGB probe was first reported in 2000 (Kutyavin et al. 2000). The MGB stabilizes A/T rich duplexes and allows use of shorter probes with higher melting temperature compared with ordinary DNA probes, hence increasing the specificity of an MGB probe and allowing discrimination of single-base mismatches (Ma et al. 2013; Van Hoeyveld et al. 2004). Furthermore, different TaqMan-MGB probes can be labeled with different fluorescence reporter dyes, which allows detection of multiple allelic variants in a single assay (Fan et al. 2014). In order to efficiently and simultaneously detect the two alleles of diploid organisms such as *P. viticola*, real-time PCR based on TaqMan-MGB probes is a particularly appropriate method.

In this study, a TaqMan-MGB real-time PCR based method was developed to detect the CAA fungicide resistant allele of *P. viticola*, based on a previously reported functional point mutation at codon

1105 of the *PvCesA3* gene. Using this new method, a comprehensive survey of 2,227 isolates from eight grape-growing regions in China was performed to detect the frequency of resistance genotype to CAA fungicides. Its application to improve the integrated pest management strategies against *P. viticola* in grapevine fields in China is also discussed.

Materials and Methods

Isolates. The dimethomorph resistant isolate YQ1 and the sensitive isolate LF1, previously characterized by Zhang et al. (2017) using the leaf disc bioassay and Sanger sequencing, were used to test the specificities of the primers and probes of the newly developed TaqMan-MGB based assay. During the growing season in June to August of 2016, at the veraison and fruit maturation stage, *P. viticola* infected leaves were collected at random in each vineyard from plants along two crossing diagonal transects, and a total of 3,026 samples were obtained in eight major grape-growing regions, namely Beizhen, Qingxu, Wei, Binchuan, Penglai, Gongan, Huaihua, and Yongren, in China (Fig. 1). Single-sporangium isolations were conducted according to the method described by Zhang et al. (2017). Single-sporangium isolates were propagated on the sterilized leaf discs of *V. vinifera* ‘Lizamate’ on a water agar (0.8%) medium in a Petri dish and incubated at 21°C with 16 h day and 8 h night at 100% humidity to ensure leaf wetness. After 7 days, leaf discs with sporangia and sporangiaophores were immediately frozen in liquid nitrogen and stored at -80°C for CAA resistance testing. A total of 2,277 single-sporangium strains were successfully isolated (Table 1). Fifty *P. viticola* isolates from Wei County were selected to validate the method (Table 2).

DNA extraction. DNA of the reference strains (YQ1 and LF1) was extracted using the SP Fungal DNA isolation kit (Omega, Norcross, GA) according to the manufacturer’s protocol. DNA extraction of leaves with sporangia and sporangiaophores of 2,227 strains was performed using the method described by Xin et al. (2003). DNA concentrations were determined by NanoVue Plus (GE Healthcare Life Sciences, Pittsburgh, PA).

TaqMan-MGB real-time PCR based method for detection of dimethomorph resistance. *Design of TaqMan probes and primers.*

Fig. 1. The carboxylic acid amide (CAA) resistance and sensitive genotype frequency of *P. viticola* in the eight grape growing areas of China. The orange and green piece represents the percentage of CAA fungicide resistant and sensitive genotype strains, respectively, which were detected by the newly developed TaqMan-minor groove binder (MGB) real-time PCR based method. The black dot represents the sampling site. The map was generated in R (version 3.6.1).

The G to A mutation at codon 1105 of the *PvCesA3* gene causing a substitution of serine to glycine in the protein confers CAA resistance in *P. viticola* isolates (Blum et al. 2010b). Based on the alignment of *PvCesA3* gene sequences (GQ258975.1, KU860541.1, KU860539.1, KU860536.1, KU860542.1, KU860540.1, KU860535.1, and KU860534.1), a pair of specific primers (Cesa3F/Cesa3R) was developed to amplify a region of *PvCesA3* gene including codon 1105 using the Primer 5.0 software (PREMIER Biosoft, Palo Alto, CA). Two specific TaqMan probes (Cesa3Pr/Cesa3pS) targeting the G and A alleles, respectively, were designed by Primer Express 3.0 software (Applied Biosystems, Foster City, CA). Figure 2 shows the sequence targeted by the specific TaqMan MGB probes and primers. Information on the primers and probes used in this study is given in Table 3. The specific MGB TaqMan probes for the resistant (Cesa3pR) and the sensitive allele (Cesa3pS) were labeled with FAM (carboxyfluorescein) and VIC (Applied Biosystems, Carlsbad, CA) reporter dyes, respectively, at the 5' end, and a nonfluorescent quencher (NFQ) with MGB ligands was used as the quencher at the 3' end. The primers and probes were synthesized by Sangon Biotech (Shanghai, China) and Applied Biosystems (Loughborough, UK), respectively.

Specificity test for primers and probes. The DNA of CAA-resistant strain YQ1 and the CAA-sensitive strain LF1 were used as templates to test the specificities of the primers (Cesa3F/Cesa3R) and probes (Cesa3pR/Cesa3pS). To simulate the detection of the heterozygote genotype, *P. viticola* DNA of the CAA-resistant strain was mixed with sensitive strain LF1 in a 1:1 ratio. The fluorescence of Cesa3pR (FAM signal) was expected only from the resistant strain YQ1 and mixed DNA sample (YQ1-LF1), while the fluorescence of Cesa3pS (VIC signal) was expected only from sensitive strain and mixed DNA (YQ1-LF1). TaqMan-MGB real-time PCRs were performed in a 20 µl volume containing 20 ng of template DNA, 0.2 µM of the forward and reverse primer, 1 µM of Cesa3pR probe, 1 µM of Cesa3pF probe, 10 µl of Probe qPCR Mix (Takara, Otsu, Japan), and finally 0.2 µl of ROX reference dye II was added as a passive internal reference. In each run, a negative amplification control (double distilled water, ddH₂O) was included. Amplifications were conducted in an ABI 7500 real-time PCR instrument (Applied Biosystems, Carlsbad, CA) programmed for 1 min at 60°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, and 1 min at 60°C. The data were subsequently analyzed

Table 1. The detection of dimethomorph resistance in *P. viticola* isolates from main grape-growing areas in China by the TaqMan-minor groove binder (MGB) real-time PCR based method

Origin	No. of isolates	Resistant genotype isolate	Sensitive genotype isolate		Resistance frequency (%)	Resistance allele frequency (%)
			Homozygous	Heterozygous		
Beizhen, Liaoning	327	316	8	3	96.6	97.1
Qingxu, Shanxi	324	1	316	7	0.3	1.4
Wei, Hebei	332	297	28	7	89.5	90.5
Penglai, Shandong	315	278	30	7	88.3	89.4
Gongan, Hubei	150	78	66	6	52.0	54.0
Huaihua, Hunan	309	34	267	8	11.0	12.3
Binchuan, Yunnan	216	19	192	5	8.8	10.0
Yongren, Yunnan	254	164	88	2	64.6	65.0
Total	2,227	1,187	995	45	53.3	54.3

Table 2. Comparison among TaqMan-minor groove binder (MGB) real-time PCR, minimum inhibitory concentration (MIC), and sequencing method in evaluation of carboxylic acid amide (CAA) fungicide resistance in *P. viticola* isolates from Hebei Province

Strain no.	MIC ^a	TaqMan ^b	Sequencing ^c	Strain no.	MIC ^a	TaqMan ^b	Sequencing ^c
HeB1	R	R	A/A	HeB26	S	S	G/A
HeB2	S	S	G/A	HeB27	R	R	A/A
HeB3	S	S	G/G	HeB28	S	S	G/A
HeB4	R	R	A/A	HeB29	R	R	A/A
HeB5	R	R	A/A	HeB30	R	R	A/A
HeB6	R	R	A/A	HeB31	R	R	A/A
HeB7	R	R	A/A	HeB32	S	S	G/G
HeB8	R	R	A/A	HeB33	R	R	A/A
HeB9	S	S	G/G	HeB34	R	R	A/A
HeB10	S	S	G/G	HeB35	R	R	A/A
HeB11	R	R	A/A	HeB36	S	S	G/G
HeB12	R	R	A/A	HeB37	S	S	G/G
HeB13	R	R	A/A	HeB38	S	S	G/G
HeB14	R	R	A/A	HeB39	R	R	A/A
HeB15	S	S	G/G	HeB40	R	R	A/A
HeB16	R	R	A/A	HeB41	R	R	A/A
HeB17	R	R	A/A	HeB42	R	R	A/A
HeB18	S	S	G/G	HeB43	S	S	G/G
HeB19	S	S	G/G	HeB44	S	S	G/G
HeB20	R	R	A/A	HeB45	R	R	A/A
HeB21	R	R	A/A	HeB46	R	R	A/A
HeB22	S	S	G/A	HeB47	R	R	A/A
HeB23	R	R	A/A	HeB48	R	R	A/A
HeB24	R	R	A/A	HeB49	S	S	G/G
HeB25	S	S	G/G	HeB50	R	R	A/A

^a MIC > 1.6 µg ml⁻¹ was regarded as a resistant isolate (R), and MIC < 1.6 µg ml⁻¹ was regarded as a sensitive isolate (S) (Sun et al. 2010).

^b S and R represent sensitive and resistant genotypes identified by TaqMan-MGB.

^c G/G, A/A, and G/A represent sensitive homozygote, resistant homozygote, and sensitive heterozygote genotypes at the position in the *PvCesA3* genes identified by sequencing.

with 7500 Software Version 2.0.6 (Applied Biosystems, Carlsbad, CA).

Sensitivity of the new method. The sensitivity of the TaqMan-MGB RT-PCR based method was determined using a 10-fold serial dilution of total DNA over a five-log scale. For YQ1, 0.126 pg to 12.6 ng; for LF1, 0.205 pg to 20.5 ng; and for the 1:1 mixture of the both strains, 5 pg to 50 ng was used as the template. The correlated cycle threshold (C_T) values were used to set the standard curves for respective absolute quantifications (Decaro et al. 2005). The double distilled water was used as negative amplification control. The tests were performed in three replicates.

Leaf disc assay. The conventional leaf disc method was used to evaluate the dimethomorph resistance of *P. viticola* (Wong and Wilcox 2000). Briefly, 50 purified strains from Wei County were inoculated on the leaf discs using the method described above. Seven days later, the sporangia and sporangiophores were collected in a tube with sterile water. After the suspension was filtered through a 30- μ m sterile mesh, the sporangia were counted under a microscope (BX41, Olympus, Tokyo, Japan) using a hemocytometer and adjusted to 10^4 sporangia ml^{-1} to serve as the *P. viticola* inoculum. The dimethomorph (97% active ingredient) used in this study was kindly provided by the pesticide laboratory of the Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China. According to the report of Sun et al. (2010), the fungicide was dissolved in methanol and diluted with water to a concentration of $1.6 \mu g ml^{-1}$. Fully expanded leaves of *V. vinifera* 'Rizamat,' four to six leaf positions distal from the shoot tip, were washed with tap water thoroughly, surface disinfested for 90 s in sterile water containing 0.5% sodium hypochlorite and 0.1% Tween 20 (Hushi, Shanghai, China), triple rinsed in sterile water, and blotted dry between paper towels. Discs (diameter 15 mm) from leaves were incubated for 1 h in the fungicide solution and transferred onto rifampicin-ampicillin-pimaricin (RAP) agar medium (Wong and Wilcox 2000; Zhang et al. 2017). Ten discs per plate were used for each isolate and the abaxial side of each disc was inoculated with a 10- μ l drop of a *P. viticola* suspension. The dimethomorph resistant isolate YQ1 and the sensitive isolate LF1 were used as positive and negative controls, respectively. Leaf discs treated with a solution without dimethomorph were used as a blank control. After 8 days of incubation at 21°C and a 16-h photoperiod, downy mildew symptoms on each leaf disc were recorded. If the symptoms were present at the minimum inhibitory concentration (MIC, $1.6 \mu g ml^{-1}$) in the treated discs of experimental and positive control groups, and not in the negative and blank controls, the isolate was regarded as resistant; if they infected the discs of the positive control, but not the experimental, negative, and blank control groups, isolates were designated as sensitive (Sun et al. 2010). The test was repeated three times.

Sequence analysis. DNA sequencing of part of the *PvCesA3* gene of 50 *P. viticola* isolates was conducted to confirm the point

mutations at codon 1105 of isolates in the present study. PCR primers for DNA sequencing, *PVcesA3F* and *PVcesA3R*, were designed to amplify a 603 bp fragment including codon 1105 using the software Primer Premier 5.0 (PREMIER Biosoft, Palo Alto, CA). Primer information is shown in Table 3. PCR amplification was performed in a total volume of 20 μ l including 20 ng of *P. viticola* DNA template, 0.5 μ M of each primer, 10 mM of Tris-HCl (pH 8.3), 50 mM of KCl, 1.5 mM of $MgCl_2$, 250 μ M of each dNTP, and 0.5 units of Taq polymerase (Tiangen, Beijing, China). PCR amplification was performed in a Veriti 96-well thermal cycler (Applied Biosystems, Carlsbad, CA) with the following temperature profile: an initial denaturation at 95°C for 5 min; followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 30 s; and a final extension at 72°C for 10 min. PCR products were sequenced using Sanger sequencing by a Sangon Biotech Company sequencer (Shanghai, China). Subsequently, the partial *PvCesA3* sequences were examined and aligned with other reference sequences from GenBank database (GQ258975.1, KU860534.1~542.1) using Geneious 11.1.5 (Biomatters, Ltd., Auckland, New Zealand).

Monitoring of dimethomorph resistance of *P. viticola*. To understand the frequency of CAA fungicide resistance in *P. viticola* samples in China, the genotype of *PvCesA3* of 2,227 *P. viticola* isolates (Table 1) obtained from vineyards located in the eight main grapevine growing areas (Fig. 1) were detected by the TaqMan-MGB real-time PCR based method.

Two or three large vineyards were selected as representatives in each sampled region where the dimethomorph treatment including frequency of applications and total amounts used were surveyed from 2014 to 2016 (Supplementary Table S1). The correlation between the total frequency of CAA-resistant isolates and annual average dimethomorph dosage and average fungicide application frequency,

Table 3. Primers and probes used in this study

Name	Sequence (5'-3')	Product length (bp)
Cesa3pR ^a	FAM-AGCAACGAGCTGAA-MGB	
Cesa3pS ^a	VIC-CAGCAACGAGCCGA-MGB	
Cesa3F ^b	CGGCTGCTACCTTTACGGCAAA	66
Cesa3R ^b	GCACAAACACGACAATGTAGACAA	
PVcesA3F ^c	CCGATTACGCTCGTGGTACCA	603
PVcesA3R ^c	CTACGTCACAGTCGTGGCAGCA	

^a The specific TaqMan-minor groove binder (MGB) probes for resistant (Cesa3pR) and sensitive allele (Cesa3pS), respectively. FAM (carboxy-fluorescein) and VIC (Applied Biosystems, Carlsbad, CA) at the 5' end represent the reporter dye; MGB at the 3' end represents a nonfluorescent quencher (NFQ).

^b The specific primer for TaqMan-MGB real-time PCR.

^c PCR primers for DNA sequencing.

Fig. 2. Minor groove binder (MGB) probe and primer map for TaqMan-MGB real-time PCR analysis of *PvCesA3*. They were designed from *PvCesA3* of *P. viticola* (complete cds: accession no. GQ258975 and partial cds: accession nos. KU860541.1, KU860539.1, KU860536.1, KU860542.1, KU860540.1, KU860535.1, and KU860534.1). The black arrow and black box respectively indicate the point mutation and codon 1105 of the *PvCesA3* gene conferring carboxylic acid amide (CAA) fungicide resistance, of which GGC (Gly) represents the sensitive allele, AGC (Ser) represents the resistant allele. The red box indicates the probes (Cesa3pS: positions 3409–3422; Cesa3pR: positions 3410–3423); the green box indicates the primer (Cesa3F: positions 3383–3404; Cesa3R: positions 3425–3448).

respectively, in each sample region was determined using the generalized linear model (GLM) and Spearman's correlation coefficient, which were performed in the software package R (version 3.6.1) with glm and cor function.

Results

Establishment of a TaqMan-MGB real-time PCR based method.

Specificity of primers and probes. Using the DNA of resistant (YQ1) and sensitive (LF1) isolates and their mixture, the specificity of the TaqMan-

MGB probes and primers were tested. The results showed that the FAM signals were detected only when a sample contained the *PvCesA3* resistant allele (YQ1 and the mixture of YQ1 and LF1). VIC signals were detected only when a sample contained DNA with the sensitive allele (Fig. 3). The allelic discrimination of YQ1 and LF1 was in agreement with the leaf disc test and DNA sequencing and this new method can detect heteroallelic *PvCesA3* isolates.

Efficiency and sensitivity. The efficiency and sensitivity of each assay was determined using the amplification plots and standard

Fig. 3. Specificity of the primer pair and TaqMan probes of TaqMan-minor groove binder (MGB) real-time PCR. **A**, Amplification of the DNA from the dimethomorph-resistant isolate YQ1 (curve 1: resistant allele; curve 2: negative control). **B**, Amplification of the DNA from the dimethomorph-sensitive isolate LF1 (curve 1: sensitive allele; curve 2: negative control). **C**, Amplification of the mix DNA of YQ1 and LF1 (curve 1: resistant allele; curve 2: sensitive allele; curve 3: negative control).

curves. As shown by the results in Figure 4, the slopes of these assays were between -3.562 and -3.415 (90.9–96.3% efficiency), with a correlation coefficient exceeding 0.99 (Fig. 4B, D, F). For the resistant strain, TaqMan-MGB RT-PCR detected the resistant genotype in all three replicates from 0.126 pg to 12.6 ng. No signal was obtained with the sensitive allele, even at 12.6 ng (Fig. 4A). The detection limit was determined at 0.126 pg and the specificity for target versus nontarget of $>10,000$ -fold. The sensitive allele was detected in all three replicates from 0.205 pg to 20.5 ng. No signal was observed for the resistant allele, even at 20.5 ng (Fig. 4C). This indicates a detection limit of 0.205 pg and a specificity for target versus nontarget of $>10,000$ -fold. For the mixture DNA of the two strains, the resistant and sensitive alleles were simultaneously detected from 5 pg to 50 ng (Fig. 4E). In all the reactions, the negative PCR control did not generate any signal (Fig. 4).

Verification of the TaqMan-MGB real-time PCR based method. The TaqMan-MGB real-time PCR method was validated by subjecting 50 single-spore isolates to the leaf disc assay and Sanger sequence analysis of partial *PvCesA3* gene. The TaqMan-MGB

real-time PCR identified 32 isolates with the CAA-resistant genotype, which were homozygous for the resistant allele of *PvCesA3*. The other 16 isolates showed the CAA-sensitive genotype; 14 of them were homozygous for the sensitive allele and four were heterozygotes including both the sensitive and resistant allele. The genotypes determined by the TaqMan-MGB real-time PCR were in accordance with the DNA sequencing of the mutated positions in *PvCesA3* genes (Table 2). All homozygous resistant isolates, as determined by molecular methods, infected the leaf disks in the in vivo bioassay, even when $1.6 \mu\text{g ml}^{-1}$ dimethomorph was applied. In contrast, dimethomorph completely inhibited infection by the sensitive genotype isolates, including both the homozygous and heterozygotes (Table 2). This verified that the present TaqMan-MGB real-time PCR based method can specifically detect the resistant and sensitive alleles of *PvCesA3* gene and assess the CAA fungicide resistance found in *P. viticola* populations.

Detection of dimethomorph resistant and sensitive genotypes of *P. viticola* in China. The TaqMan-MGB real-time PCR was used to survey the frequency of the dimethomorph-resistant *P. viticola* isolates from a collection of 2,227 *P. viticola* single-spore isolates

Fig. 4. Amplification plots and standard curves of the TaqMan-minor groove binder (MGB) real-time PCR based method with 10-fold dilutions of DNA from resistance allele (**A** and **B**) of the *PvCesA3* gene (lines 1–6: 12.6 ng to 0.126 pg; line 7: negative control); or sensitive allele (**C** and **D**) of the *PvCesA3* gene (lines 1–6: 20.5 ng to 0.205 pg; line 7: negative control) or mixture of the resistant and sensitive allele (**E** and **F**) of the *PvCesA3* gene (lines 1–5: 50 ng to 5 pg; line 6: negative control). For all the amplification plots, the red lines represent the resistant allele, and blue lines represent the sensitive allele.

isolations originating from eight locations in China (Table 1). The CAA-resistant genotype was present in 1,187 isolates (53.3%), and 1,040 isolates showed the genotype sensitive to CAA fungicide, of which 45 were heterozygotes. The frequencies of resistance from high to low was Beizhen (96.6%), Wei County (89.5%), Penglai (88.3%), Yongren (64.6%), Gongan (52%), Binchuan (8.8%), and Qingxu (0.3%) (Fig. 1). In addition, the frequencies of resistant alleles were higher than the frequencies of resistance genotypes in all the regions, but the trend was consistent (Table 1). Furthermore, the total resistant genotype frequency in each region was significantly correlated with the local average fungicide dosage (Spearman's correlation: $\rho = 0.90$, $P = 0.0045$; GLM: $P = 0.0295$) and average application frequency (Spearman's correlation: $\rho = 0.95$, $P = 0.0011$; GLM: $P = 0.0009$), respectively (Supplementary Fig. S1).

Discussion

In this study, TaqMan RT-PCR primers and MGB probes specific to allele G1105S that conferred resistance of *P. viticola* to dimethomorph were successfully designed, validated, and applied. Compared with the conventional leaf disc method, the molecular methods used to detect fungicide resistance are much quicker (Miao et al. 2016). Therefore, a number of PCR-based methods had been developed to monitor the shift in the CAA fungicide sensitivity of *P. viticola* field populations (Aoki et al. 2011, 2013; Zhang et al. 2017). Among them, the multiplex allele-specific primer (ARMS) PCR assay was developed to simultaneously detect QoI and CAA fungicide resistance alleles in *P. viticola* (Aoki et al. 2013). This method was simple and rapid, but it can just detect the existence of the resistant allele and is unable to distinguish the homo- and heterozygote genotype of *PvCesA3*. Previously, we developed a tetra-primer ARMS PCR to detect the CAA-resistant *P. viticola* (Zhang et al. 2017). It not only detected the resistant alleles, but also could discriminate between homo- and heterozygote genotypes. However, the presence of two pairs of primers in one reaction might result in cross-amplification and false positive results (Hamajima et al. 2002), which was also observed in our experiments (Zhang et al., unpublished data). Due to significantly improved hybridization properties of MGB probes (Kutyavin et al. 2000), we opted to employ TaqMan-MGB probes, allowing a shorter probe and higher specificity of the probe (Ma et al. 2013). The use of TaqMan-MGB real-time PCR enabled specific distinction of resistant and sensitive genotypes of *P. viticola* to dimethomorph and other CAAs and enhanced specificity, sensitivity, and throughput.

Two-point mutations at the same codon (G1105S and G1105V) have been reported to result in CAA resistance (Blum et al. 2010b; Sierotzki et al. 2011). The G1105S mutation was predominant in most areas of Europe (Blum et al. 2010b; Sierotzki et al. 2011) and Japan (Aoki et al. 2015). In our previous study we also only detected this genotype in Guangxi Province in China (Zhang et al. 2017). So far, the G1105V mutation is only detected in a few locations worldwide and at low frequency (reviewed by Zhang et al. 2017). Therefore, the resistant frequency based on identification of G1105S mutation is currently sufficient to assess CAA fungicide resistance in China. Nevertheless, we suggest that in addition to the genotypic surveys, phenotypic surveys should also be performed to monitor other possible mutations and to establish a living collection that can be stored at -20°C (Delmotte et al. 2014). This will help to gain a more comprehensive understanding of mutations conferring CAA resistance of *P. viticola* in China.

As *P. viticola* is an obligate biotrophic pathogen, genotyping is performed in the background of the host and low concentrations of *P. viticola* DNA from samples are expected. The TaqMan-MGB real-time PCR assay developed in this study could detect the resistant homozygous allele in 0.126 pg total DNA and homozygous sensitive allele in 0.205 pg total DNA; the heterozygote genotype could be detected in 5 pg DNA. The sensitivity was much higher than the RFLP PCR (2 ng) (Aoki et al. 2011). Subsequently, Aoki et al. (2013) developed a multiplex allele-specific primer PCR assay (ARMS), which greatly improved the detection sensitivity, but could not

distinguish between homo- and heterozygote genotype required for the correct identification of resistant isolates. The high sensitivity was further exploited to use the assay on infected leaf discs. Using the TaqMan-MGB real-time PCR assay, the homozygote and heterozygote genotypes of the mutation sites (G1105S) in *PvCesA3* gene conferring CAA resistance were successfully detected in the DNA of infected leaf discs of the 50 isolates from Wei County. The results were in agreement with the direct sequencing and fungicide sensitivity assays in vitro, suggesting that the new method is robust and reliable for infected leaf disc samples in the laboratory. This would omit the step of isolation of the isolates and further enhance the throughput. However, we did not test the specificity of this method; therefore, further research is needed for the potential application of direct screening of infected leaf samples from the field, which would allow identification of CAA fungicide resistance in a high density grid required to assess spatial and temporal distributions and assist decision support during the season.

Dimethomorph has been widely applied in Chinese vineyards since it was registered in 1996 for the control of *P. viticola* (Sun et al. 2010). In 2010, Sun et al. tested the sensitivity of 392 *P. viticola* isolates collected from 11 regions in seven provinces in China by the conventional leaf disk assay and no resistant isolates were detected (Sun et al. 2010). In 2014, dimethomorph-resistant isolates of *P. viticola* were first collected in Guangxi Province, China (Zhang et al. 2017). In this study, we investigate the CAA-fungicide resistance frequency of a large *P. viticola* population from eight main grape-growing areas in China. The resistance frequency is significantly correlated with the use of the CAA fungicides ($P < 0.05$). Beizhen, Penglai, and Wei (resistant frequency $>88\%$) are all traditional grape-growing areas since the 1950s and in these regions, CAA fungicides have been applied over a prolonged period of time. In the period from 2014 to 2016, the use of dimethomorph reached roughly 2.38 kg per hectare on average in those regions; the number of CAA treatments in those regions ranged from three to seven (Supplementary Table S1), and the average fungicide use frequency exceeded the maximum number recommended by FRAC. Those might result in a strong selection for a resistant population. This is in contrast to the situation in Qingxu, which is also a traditional grape growing region located in northern China; due to its relatively low level of economic development, the application of CAA fungicides was much lower (about 0.06 kg/ha; most winegrowers applied one time; Supplementary Table S1), and mainly copper products have been used to control grape downy mildew. This markedly lower selection pressure resulted in the lowest resistance frequency (0.3%), indicating dimethomorph is still an effective fungicide for grape downy mildew control in Qingxu. The CAA resistance frequency of Gongan, Yongren, Binchuan, and Huaihua ranged from 8.8 to 64.6%, being significantly lower than Beizhen, Penglai, or Wei ($>88\%$). These regions are located in southern China, where the humidity is high and the climate is conducive for grape downy mildew; therefore, the application of rain shelter cultivation is now popular, and this greatly reduced this disease as well as the dosage and application frequency of fungicides. The average dosage (1.09 kg/ha) and application frequency (3.2 times) in those regions are both significantly ($P < 0.001$, t test) less than in northern regions (2.38 kg/ha, 5.1 times) from 2014 to 2016 (Supplementary Table S1). In addition, those regions were new grape-growing areas since the early 1990s. The short-term and lower frequency and dosage of fungicide application may result in lower resistance frequencies of *P. viticola*. Finally of interest is the difference between Binchuan and Yongren, as they are located nearby and the climate is very similar; however, the CAA resistance frequencies in these regions are significantly different. This may be due to the more complex and larger variation in the fungicide application in Binchuan. This could result in a lower selective pressure of CAA fungicides and lower frequencies of resistance. In the regions with lower frequencies of CAA resistance, it is strongly recommended that CAA fungicides should be applied to control grape downy mildew disease according to FRAC recommendations (FRAC 2017), which include application of CAA fungicides preferably in a preventive manner (applying before the pathogen arrives or begins to develop),

and limiting the total number of CAA fungicide sprays to less than four per crop cycle. In the areas with high CAA resistance frequency, strategies should be implemented to prevent or decrease the further spread of resistant fungal subpopulations in the field, such as planting more resistant cultivars and applying CAA fungicides in a mixture with effective fungicides from other classes.

In addition, 4.3% of the sensitive strains were heterozygous, resulting in a higher resistance allele frequency. The resistance allele frequency may increase due to asexual spread of resistant strains as well as due to crossings in which the progeny segregates for the resistance allele and selection of resistant progeny. Limited information is available on the sexual cycle of *P. viticola* in the field in China. Yin et al. investigated the population diversity of a nationwide collection of *P. viticola* in China by SSR markers. They found high genotypic diversity in most regions and concluded that sexual reproduction contributes significantly to epidemics of *P. viticola* (Yin et al. 2014). However, linkage equilibrium among these loci was not analyzed, making it hard to evaluate the level of sexual reproduction. In this study, a low ratio of heterozygotes was found in all regions (0.79 to 4%); especially in Gonggan and Yongren, the frequency of heterozygotes is much lower than expected based on the allele frequencies ($P < 0.0001$). The lower fitness of the heterozygote when exposed to CAA fungicides could result in elimination of heterozygous progeny after the sexual cycle. Alternatively, the bias could be due to the asexual spread of resistant genotypes. Therefore, more population diversity and fitness studies are needed to identify the main drivers of the increase of resistance allele frequency.

In brief, we describe an easy and reliable TaqMan-MGB RT-PCR based method that could directly evaluate CAA resistance from infected plant tissue and simultaneously detect the resistant homozygote, the sensitive heterozygote, and the sensitive homozygote. Based on this method, CAA fungicide resistance frequency of a comprehensive collection of *P. viticola* from main grape-growing areas in China was monitored. To our knowledge, this collection of *P. viticola* isolates is the largest in China. The size and the sampling strategy in one season allowed detailed analyses of CAA fungicide resistance. The results provide important information on the resistant frequency of *P. viticola* to CAA fungicides in China and will improve the management of grape downy mildew in the future.

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