Assessing the Risk That Phytophthora melonis Can Develop a Point Mutation (V1109L) in CesA3 Conferring Resistance to Carboxylic Acid Amide Fungicides

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Abstract

The risk that the plant pathogen Phytophthora melonis develops resistance to carboxylic acid amide (CAA) fungicides was determined by measuring baseline sensitivities of field isolates, generating resistant mutants, and measuring the fitness of the resistant mutants. The baseline sensitivities of 80 isolates to flumorph, dimethomorph and iprovalicarb were described by unimodal curves, with mean EC50 values of 0.986 (±0.245), 0.284 (±0.060) and 0.327 (±0.068) μg/ml, respectively. Seven isolates with different genetic background (as indicated by RAPD markers) were selected to generate CAA-resistance. Fifty-five resistant mutants were obtained from three out of seven isolates by spontaneous selection and UV-mutagenesis with frequencies of 1×10⁻⁷ and 1×10⁻⁶, respectively. CAA-resistance was stable for all mutants. The resistance factors of these mutants ranged from 7 to 601. The compound fitness index (CFI = mycelial growth × zoospore production × pathogenicity) was often lower for the CAA-resistant isolates than for wild-type isolates, suggesting that the risk of Phytophthora melonis developing resistance to CAA fungicides is low to moderate. Among the CAA-resistant isolates, a negative correlation between EC50 values was found for iprovalicarb vs. flumorph and for iprovalicarb vs. dimethomorph. Comparison of the full-length cellulose synthase 3 (CesA3) between wild-type and CAA-resistant isolates revealed only one point mutation at codon position 1109: a valine residue (codon GTG in wild-type isolates) was converted to leucine (codon CTG in resistant mutants). This represents a novel point mutation with respect to mutations in CesA3 conferring resistance to CAA fungicides. Based on this mutation, an efficient allelic-specific PCR (AS-PCR) method was developed for rapid detection of CAA-resistance in Phytophthora melonis populations.

Introduction

The oomycete Phytophthora melonis Katsura, which is conspecific with P. sinensis, causes a severe disease of cucumber (Cucumis sativus) which has been reported in China, Japan, Egypt, Turkey, Korea, India and Iran [1]. In addition to cucumber, P. melonis infects other cucurbits including zucchini (Cucurbita pepo L.), hami melon (Cucumis melo L.), wax gourd (Benincasa hispida (Thunb.) Cogn.) [2–5], and pointed gourd (Trichosanthes dioica Roxb.) [6]. It also infects pistachio (Pistacia vera L.) [7], causing blight, dieback, root rot, foot rot and crown rot. The use of resistant cultivars and chemical fungicides are two efficient control methods [2,5,8]. Phenylamides (e.g. metalaxyl) have been widely used for P. melonis disease control. However, metalaxyl-resistance of P. melonis has been reported in China [9]. Since the early 1980s, the efficacy of phenylamides has declined due to the emergence of resistant populations of oomycete pathogens in fields [10,11].

The current study concerns resistance of P. melonis to the carboxylic acid amide (CAA) fungicides, which are divided into three different chemical groups based on differences in structure: the cinamic acid amides (e.g., dimethomorph and flumorph), the valine amide carbamates (e.g., benthiavalicarb, benthiavalcab-carbaryl and iprovalicarb) and the mandelic acid amides (e.g., mandipropamid) (FRAC Code List, www.frac.info). These fungicides are used to control the pathogens in the families Peronosporaceae (e.g., Plasmopara viticola and Bremia lactuca) and Pythiaceae (e.g., Phytophthora spp., but not Phytophthora spp.) [12]. All CAA fungicides strongly inhibit all asexual stages of susceptible pathogens but do not inhibit zoospore release and mobility [13–16]. Inhibition by CAA fungicides results from the interruption of cellulose biosynthesis and the disruption of cell wall structure [17].

istance to phenylamide fungicides, resistance to CAA fungicides is an important problem. Since dimethomorph’s introduction in the 1980s, CAA-resistant isolates of P. viticola have been detected in most areas of Europe (FRAC web). In China, flumorph-resistant isolates of Pseudoperonospora cubensis were obtained after successive applications of flumorph in a greenhouse [18]. P. viticola and P. cubensis are classified by FRAC as being at high risk to develop resistance to CAA fungicides, but P. infestans is considered to have a low risk of developing such resistance (FRAC pathogen risk list, www.frac.info). No resistant isolates of P. infestans have been detected in field since the introduction of CAA fungicides over 15 years ago (CAA Minutes

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2010 final RG, www.frac.info). Using UV- and EMS-mutagenesis, researchers obtained stable CAA-resistant mutants of *P. infestans* only with difficulty [19–21] and an isolate of *P. capsici* failed to develop dimethomorph-resistance after repeated exposure to the fungicide [19]. However, *P. capsici* mutants with high CAA-resistance were obtained by mass selection from zoospores and oospores, and the resistance risk was considered low to moderate to CAAIs in *P. capsici* [22,23].

Cross-resistance among CAA fungicides has been reported in *P. viticola* [24] and *P. capsici* [22]. The resistance mechanism of some pathogens to CAA fungicides has been elucidated in recent reports. Amino acid substitutions at codon position 1105 (G1105V or G1105A) in cellulose synthase (CesA3) were responsible for resistance to the CAA fungicide mandipropamid in EMS-generated mutants of *P. infestans* [17]. Changes of G1105V or G1105W led to CAA-resistance in *Ps. cubensis* [25]. In *P. viticola*, changes of G1105V in PvCesA3 conferred CAA-resistance [26]. Based on the point mutation, a PCR-RFLP method has been developed for detecting CAA-resistant isolates in *P. viticola* populations [27].

The objectives of the present study were to (i) determine the baseline sensitivity of *P. melonis* to the CAA fungicides flumorph, dimethomorph and iprovalicarb; (ii) assess the risk of resistance to the three CAA fungicides; (iii) investigate the CAA-resistance mechanism in *P. melonis*; and (iv) develop a rapid and reliable method for detection of CAA-resistant isolates in populations of *P. melonis*.

**Results**

**Baseline Sensitivity of *P. melonis* to Flumorph, Dimethomorph and Iprovalicarb**

For the 80 *P. melonis* isolates investigated, the frequency distribution of EC50 values for each of the three CAA fungicides were described by unimodal curves (Figure 1), indicating the absence of CAA-resistant subpopulations among these isolates. The mean and range of EC50 values were 0.986±0.245 μg/ml and 0.410–1.577 μg/ml for flumorph, 0.284±0.060 μg/ml and 0.171–0.590 μg/ml for dimethomorph, and 0.327±0.068 μg/ml and 0.100–0.482 μg/ml for iprovalicarb. The highest EC50 value was 3.45-, 3.85-, and 4.82-times greater than the smallest value for flumorph, dimethomorph and iprovalicarb, respectively.

**Development of CAA-resistant Isolates of *P. melonis* in vitro**

**Random amplified polymorphic DNA (RAPD).** RAPD analysis was used to identify isolates with different genetic backgrounds; these isolates would be used as the parents for the generation of CAA-resistant mutants. Sixteen primers (Table 1) that produced easily recognizable and consistent banding patterns were used for RAPD analysis of 16 isolates from different geographic origins (Table 2). RAPD analysis using primer combinations clearly separated these isolates. A dendrogram based on UPGMA analysis indicated that the 15 isolates of *P. melonis* formed three major groups (Figure 2). One isolate of *P. drechsleri* was separated from *P. melonis*. RAPD groups were not related to the geographic origins or sensitivity to CAA fungicides of these isolates. Seven isolates (TJ-38, TX-11, TJ-99, TJ-104, TJ-114, 63 and 70) from different RAPD groups were selected for generation of CAA-resistant mutants.

**Generation of CAA-resistant isolates.** When the seven isolates were exposed to the CAA fungicides, spontaneous mutation resulted in five isolates with flumorph-resistance, one with dimethomorph resistance and three with iprovalicarb resistance. These nine isolates with spontaneous mutations were obtained from TJ-38, 63 and 70, and the survival frequency was approximately $1 \times 10^{-6}$ for mutants exposed to the three fungicides (Table 3). No resistant isolates were derived from the other parent isolates. Following the UV treatment, however, the survival frequency was increased to approximately $1 \times 10^{-5}$, and 19 flumorph-resistant, 17 dimethomorph-resistant and 10 iprovalicarb-resistant mutants were obtained from the parent isolates TJ-38, 63 and 70 (Table 3).

**Characteristics of CAA-resistant Mutants**

**Resistance stability and resistance factor.** After 10 transfers on non-amended medium, the mutant isolates grew as well on fungicide-amended medium as on non-amended medium, indicating that the resistance to CAA fungicides was stable. The level of resistance, as indicated by the resistance factor (RF = EC50 of mutant at the 10th transfer/EC50 of its parent), ranged from 7 to 601 (Table 3).

**Mycelial growth, sporulation and virulence in vitro.** Compared to the mycelial growth of the corresponding parents (TJ-38, 63 and 70), mycelial growth was faster for some resistant isolates and slower for others. For example, the mycelial growth rate relative to the parent TJ-38 was significantly decreased for F58-1, F58-3, F58-4 and I58-2 (p<0.05), but significantly increased for D58-2 and I58-1 (p<0.05) on the non-amended medium (Table 4). Virulence also increased or decreased, depending on the resistant isolate. Lesions were significantly larger for the resistant isolates D58-3, D58-2, I58-1, I58-2 and F56-8 than for their parent TJ-38 (p<0.05) (Table 4). The resistant isolates F58-3, D58-3, D58-5 and I58-1, however, produced fewer zoospores than the wild-type isolate (Table 4). A compound fitness index (CFI) was calculated: CFI = in *vitro* mycelia growth × zoospore production × lesion area on cucumber leaves. CFI values of resistant isolates were significantly lower for two of nine mutants derived from parent isolate TJ-38, for five of nine mutants derived from parent isolate 63, and all eight isolates derived from parent isolate 70 (Table 4). CFI values were never significantly greater for the mutants than for the parents and were frequently lower but without statistical significance.

**Cross-resistance.** There was a high level of cross-resistance among all three CAA fungicides: the values of Spearman’s rho (r) were all ≥0.8000 (p<0.0001) (Figure 3 A to C). Examination of the EC50 values (those for CAA-resistant isolates and clustered on the right side of Figure 3 A, B and C) once again indicated a positive correlation between resistance to dimethomorph and flumorph (Figure 3 D), but a negative correlation between resistance to iprovalicarb and flumorph (Figure 3 E) and between resistance to iprovalicarb and dimethomorph (Figure 3 F). No cross-resistance was detected between CAA fungicides and non-CAA fungicides such as metalaxyl, cytoxanil, azoxystrobin and cyazofamid (p>0.05) (data not shown).

**Analysis of the CesA3 Gene in *P. melonis***

The full-length of *PmCesA3* gene contained 3550 bp, with one intron of 130-bp located after nucleotide 143 (Figure 4 A). The *PmCesA3* gene coded for a polypeptide chain of 1139 amino-acids and had a predicted molecular weight of 126,5 kDa. The analysis of identities between the *PmCesA3* amino acid sequence and those of the closest organisms found in the NCBI GenBank database revealed that homologies were higher with the CesA3 in oomycetes than with CesA3 in *Arabidopsis thaliana* (Table 5). Compared to the CesA3 in sensitive isolates, only one amino-acid substitutions was detected in the CesA3 in the CAA-resistant isolates: this substitution (a GTG to CTG mutation) occurred at
Figure 1. Frequency distributions of EC\textsubscript{50} values (the effective concentration causing 50\% inhibition of mycelial growth of \textit{Phytophthora melonis}) for flumorph, dimethomorph and iprovalicarb. In total, 80 isolates of \textit{P. melonis} were collected from areas never exposed to carboxylic acid amide fungicides.
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Figure 2. Genetic relationships among 15 isolates of \textit{Phytophthora melonis}. The denrogram (UPGMA) shows the relationships among the isolates of \textit{P. melonis} based on randomly amplified polymorphic DNA (RAPD) analysis with 16 decamer primers. Scale at the bottom depicts the genetic distance.
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codon 1109 and resulted in the replacement of a valine residue with a leucine residue (Figure 4B).

AS-PCR for Rapid Detection of CAA-resistant Isolates of *P. melonis*

Four pairs of allele-specific primers, designed according to the single mutation in the *PmCesA3* gene, were used for PCR with DNA template from CAA-resistant and -sensitive isolates. Using the primer pair PmA3F1 + PmA3S1, a 500-bp fragment was amplified at different annealing temperatures whether the template DNA was from resistant or sensitive isolates (Figure 5A), indicating that primers designed by the traditional method could not discriminate between sensitive and resistant alleles. The introduction of an artificial mismatch base at the second nucleotide at the 3'-end of the primers improved specificity at various annealing temperatures (Figure 5A). As the annealing temperature increased, the reverse primer with artificial mismatch 'T' at the second nucleotide showed more specificity than the primers with mismatch 'C' or 'G'. At the annealing temperature of 68.5°C, the primer PmA3F1 was optimal for distinguishing the mutation at codon 1109. With the primer pairs PMF + PMR1109B, the 500-bp fragment was amplified from CAA-resistant isolates F58-4, I63-2, D63-1, F63-11 and D70-3 but not from CAA-sensitive isolates TX21, TX33, TJ90 and TJ12 (Figure 5B).

**Discussion**

The sensitivity of 80 *P. melonis* isolates (collected from 13 fields in China) to the CAA fungicides flumorph, dimethomorph and iprovalicarb was determined by measuring EC50 values. The frequency distributions of the EC 50 values were described as unimodal curves with a narrow range for each fungicide, indicating the absence of CAA-resistant subpopulations among the 80 isolates. Therefore, these results can be used as baselines for tracking future sensitivity shifts of *P. melonis* populations to these three CAA fungicides. Mycelial growth was inhibited more strongly by dimethomorph than by iprovalicarb or flumorph. Similar results were reported for *P. capsici* [22,23,28], *Bremia lactucae* [29], *P. infestans* [21,30] and *Peronosphyra nighotii* [15], indicating that dimethomorph is generally more effective than iprovalicarb or flumorph for control of oomycete plant pathogens.

Table 1. Nucleotide sequences and characteristics of primers used in this study.

| Primers | Sequence (5’–3’) | Description | Source or reference |
|---------|------------------|-------------|---------------------|
| ABA3    | AGTCAGCCAC       | Primer for RAPD analysis | [42] |
| ABA7    | GAAACGAGGTG      | Same as for ABA3 | [42] |
| ABA9    | GGATACCGGCC      | Same as for ABA3 | [42] |
| ABA10   | GTGATCGCAG       | Same as for ABA3 | [42] |
| ABA13   | CAGACCCACAC      | Same as for ABA3 | [42] |
| ABA17   | GACCGCTTGT       | Same as for ABA3 | [42] |
| ABA18   | AGGTGACCGT       | Same as for ABA3 | [42] |
| ABA20   | GTTCGAGATCC      | Same as for ABA3 | [42] |
| Y11     | AGACGATGGG       | Same as for ABA3 | [43] |
| Y04     | GGCTGCAAATG      | Same as for ABA3 | [43] |
| OPG-16  | ACCTCCCTCC       | Same as for ABA3 | [44] |
| OPS-14  | AAGGGGTCC        | Same as for ABA3 | [44] |
| OPX-12  | TCAGCCACCA       | Same as for ABA3 | [44] |
| OPG-11  | TGCCGCTGCT       | Same as for ABA3 | [44] |
| OPG-14  | GGATGAGACC       | Same as for ABA3 | [44] |
| OPG-15  | ACTGGGACTC       | Same as for ABA3 | [44] |
| PmA3F1  | TCTCGTGTCGGACGGACAA | Primer for amplification and partial sequencing of *PmCesA3* gene | This study |
| PmA351  | ATCATCGGTCTACCTTGCC | Sequencing primer for *PmCesA3* gene | This study |
| PmA352  | CGGTCTTGTGTGGATCCGACTG | Same as for PmA351 | This study |
| PmA353  | TCGAGCTACTTGATCCGACCA | Same as for PmA351 | This study |
| PmA354  | TCATACATGGAACCGTGACG | Same as for PmA351 | This study |
| PmA355  | TGACCGTGAGGTGGTGGCTCAG | Same as for PmA351 | This study |
| PmA356  | AGACCTCGTCTGGGCTC | Same as for PmA351 | This study |
| PmA3R1  | TCTGAGTGGTCAGGCTTCC | Same as for PmA3F1 | This study |
| PMF     | ATCTACGTCGGCTGCCGTAACAG | Primer for rapid detection of resistance | This study |
| PMR1109A | CGAACCCACATGACCCCA | Same as for PMF | This study |
| PMR1109B | CGAACCCACATGATACCCCA | Same as for PMF | This study |
| PMR1109C | CGAACCCACATGACCCCA | Same as for PMF | This study |
| PMR1109D | CGAACCCACATGACCCCA | Same as for PMF | This study |

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Obtained by mass selection from zoospores and oospores, and no CAA-resistant isolates of *P. infestans* but not among field populations of *P. viticola*, considered high risk pathogens, while *P. infestans*, *P. capsici*, *P. viticola* isolates that are stable and competitive have been detected are also based on field observations. Thus, CAA-resistant *www.frac.info*). Assessments of the risk of fungicide resistance have been considered low risk pathogens (FRAC, *www.frac.info*), indicating a high risk of resistance to CAAs in *P. melonis*.

Isolates with resistance to CAA fungicides were generated from *P. melonis* isolates that are stable and competitive have been detected. Mutants resistant to one of the CAA fungicides in the current study were resistant to other CAAs but not non-CAA fungicides, indicating that there was cross-resistance among flumorph, dimethomorph and iprovalicarb but not between the CAA and non-CAA fungicides. Similar results have been reported for *P. viticola* [24], *P. capsici* [22,23,32]. Although the cross-resistance suggests that the CAA-resistant isolates have a similar resistance mechanism, the negative correlation between higher EC50 values for iprovalicarb and flumorph and between higher EC50 values for iprovalicarb and dimethomorph but not between those for flumorph and dimethomorph suggests that the resistance mechanism may differ somewhat between the cinnamic acid amides (dimethomorph and flumorph) and the valine amide carbamates (iprovalicarb).

We amplified and sequenced the CesA3 gene of *P. melonis*. Analysis of the CesA3 amino acid sequence revealed that the wild-type and CAA-resistant isolates of *P. melonis* differed only in the V1109L substitution (Figure 5B). Previous studies reported that resistance to CAA fungicides was conferred by G1105V or G1105W in CesA3 of *Ps. cubensis* [18] and *P. capsici* [22,23,32]. The substitution of V1109L suggests that the resistance mechanism may differ somewhat between the cinnamic acid amides (dimethomorph and flumorph) and the valine amide carbamates (iprovalicarb).

### Table 2. Isolates of *Phytophthora melonis* used for RAPD analysis and their sensitivities to flumorph, dimethomorph and iprovalicarb.

| Isolate | EC50 | Origin                  |
|---------|------|------------------------|
| TX-9    | 1.240| Xiqing 1°, Tianjin      |
| TX-11   | 1.080| Xiqing 2, Tianjin       |
| TX-13   | 1.193| Xiqing 3, Tianjin       |
| TX-15   | 0.983| Xiqing 4, Tianjin       |
| TX-17   | 1.161| Xiqing 5, Tianjin       |
| TX-20   | 0.857| Xiqing 6, Tianjin       |
| TJ-3    | 1.091| Hexi 1, Tianjin         |
| TJ-18   | 1.182| Hexi 2, Tianjin         |
| TJ-42   | 0.760| Hexi 3, Tianjin         |
| TJ-58   | 1.010| Hexi 4, Tianjin         |
| TJ-99   | 0.552| Nankai 1, Tianjin       |
| TJ-104  | 0.775| Nankai 2, Tianjin       |
| TJ-114  | 1.022| Nankai 3, Tianjin       |
| 63      | 0.440| UC Riverside, USA       |
| 70      | 1.410| UC Riverside, USA       |
| 0206a   | 0.710| Nanjing                 |

Although RAPD analysis revealed a high degree of genetic diversity in *P. melonis* collected from different geographical regions, the groups defined by RAPD markers did not share CAA-sensitivity. A likely reason for this lack of correlation is that RAPD markers could not reflect the defined loci responding to sensitivity to fungicides [31]. The RAPD results, however, made it possible to select isolates with different genetic backgrounds for resistance generation.

Isolates with resistance to CAA fungicides were generated from three of the seven isolates used, suggesting that the risk of *P. melonis* resistance to CAA fungicides may be associated with an isolate’s genetic background. This would explain why dimethomorph-resistant mutants of *P. capsici* could not be obtained from only one isolate by taming [21], but why CAA-resistance could be obtained by mass selection from zoospores and sexual progeny [22,23].

The risk of fungicide resistance also depends on the pathogen species and its biological characteristics. Based on disease cycles, dispersal ability, frequency of sexual recombination and the competitive ability, *P. viticola* and *P. cubensis* have been considered high risk pathogens, while *P. infestans*, *P. capsici* and *P. melonis* have been considered low risk pathogens (FRAC, *www.frac.info*). Assessments of the risk of fungicide resistance are also based on field observations. Thus, CAA-resistant isolates that are stable and competitive have been detected among field populations of *P. viticola* [24] and *P. cubensis* [18] but not among field populations of *P. infestans* [20] (FRAC, *www.frac.info*), indicating a high risk of resistance to CAAs in *P. viticola* and *P. cubensis* but a low risk in *P. infestans*. Until now, no CAA-resistant isolates of *P. capsici* have been reported in the field, but *P. capsici* mutants with high CAA-resistance were obtained by mass selection from zoospores and oospores, and the risk of resistance to CAAs was considered low to moderate in *P. capsici* [22,23]. For *P. melonis* in the current study, CAA-resistant mutants were generated in *vitro* with a frequency of $1 \times 10^{-7}$ by spontaneous selection and $1 \times 10^{-6}$ by UV-mutagenesis of zoospores. That the frequency was higher with UV-mutagenesis than with spontaneous selection suggests that UV radiation can increase the probability of CAA fungicide resistance in *P. melonis*. The CFIs (compound fitness indices) were often lower for the CAA-resistant isolates than the wild-type isolates, indicating that CAA-resistance in this study was generally associated with reduced fitness. This supports our inference that the risk of resistance to CAAs in *P. melonis* is low to moderate.

Mutants resistant to one of the CAA fungicides in the current study were resistant to other CAAs but not non-CAA fungicides, indicating that there was cross-resistance among flumorph, dimethomorph and iprovalicarb but not between the CAA and non-CAA fungicides. Similar results have been reported for *P. viticola* [24], *P. cubensis* [18] and *P. capsici* [22,23,32]. Although the cross-resistance suggests that the CAA-resistant isolates have a similar resistance mechanism, the negative correlation between higher EC50 values for iprovalicarb and flumorph and between higher EC50 values for iprovalicarb and dimethomorph but not between those for flumorph and dimethomorph suggests that the resistance mechanism may differ somewhat between the cinnamic acid amides (dimethomorph and flumorph) and the valine amide carbamates (iprovalicarb).

We amplified and sequenced the CesA3 gene of *P. melonis*. Analysis of the CesA3 amino acid sequence revealed that the wild-type and CAA-resistant isolates of *P. melonis* differed only in the V1109L substitution (Figure 5B). Previous studies reported that resistance to CAA fungicides was conferred by G1105V or G1105W in CesA3 of *Ps. cubensis* [18], G1105S in CesA3 of *P. viticola* [20] and G1105V or G1105W in CesA3 of *Ps. cubensis* [25]. The substitution of V1109L in PmCesA3 would therefore represent a novel mutation causing resistance to CAA fungicides. The finding of only one mutation and the detailed cross-resistance results suggest that other genes might also be involved in CAA resistance. In addition, CAA resistance was considered to be controlled by a recessive gene in *P. infestans* [17] and *P. viticola* [26], but by two dominant genes in *P. capsici* [23]. In this study, we did not find any CAA-resistant isolates with a heterozygous mutation at codon position 1109 on PmCesA3, suggesting that CAA resistance in *P. melonis* may also be controlled by a recessive gene(s). Confirming this will require further genetic experiments, but genetic manipulation of *P. melonis* is difficult because it is homothallic.

Several methods such as AS-PCR and PCR-RFLP have been developed for detecting isolates with mutations associated with fungicides resistance [39]. A recent study described a PCR-RFLP method that rapidly detects CAA resistance in *P. viticola* populations [27]. In our study, AS-PCR primers were designed (based on the mutation of V1109L); these primers effectively identified CAA resistance in *P. melonis*. Compared with the traditional AS-PCR primers, the new reverse primer contained an additional mismatch at the second nucleotide of the 3' end; the introduction of this mismatch was previously reported to increase specificity of the allele-specific primer [34–36]. In our trial, the mismatch nucleotide ‘T’ was more optimal than the mismatch nucleotides of ‘C’ and ‘G’. However, different mismatches can increase or decrease the specificity of the primer, indicating that the most suitable mismatch must be tested in different cases [37]. The AS-PCR primers described...
here will be useful for detecting CAA-resistant isolates of *P. melonis* from field populations.

**Materials and Methods**

**Isolates and Culture Conditions**

Roots and stems of cucumber (*Cucumis sativus* Linn.) with typical signs and symptoms of infection by *P. melonis* were collected from 15 fields in Xiqing, Hexi and Nankai districts in Tianjin of China in 2005, where CAA fungicides had never been used. Tissue plugs were cut from the margin of lesions on stems and roots. The plugs were disinfested for 3 min in 0.5% (vol/vol) NaClO. After being rinsed three times with sterile water, these plugs were placed on white kidney bean agar (WKB) (60 g of white kidney bean, 7 g of agar and distilled water up to 1 liter) plates amended with 50 µg/ml of ampicillin (98% a.i., Tuoyingfang Biotech Co., Ltd., Beijing), 50 µg/ml of rifampicin (90% a.i., Tuoyingfang Biotech Co., Ltd., Beijing) and 50 µg/ml of pentachloronitrobenzene (PCNB) (40% a.i., Sanli Chemical Industry Co., Ltd., Shenzhen, China). After being incubated at 25°C for 24 h, single germinated zoospores and associated agar were transferred to fresh WKB agar medium. For long-term storage, each culture was transferred to WKB agar slants, covered with sterile mineral oil, and stored at room temperature.

**Fungicides**

The following technical-grade fungicides were individually dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions (1 × 10⁻³ µg/ml) and stored at 4°C in the dark: flumorph (96% a.i., Research Institute of Chemical Industry, Shenyang, China), dimethomorph (95% a.i., Fery Agrochemicals Ltd), iprovalicarb (98% a.i.; Sigma-Aldrich Shanghai Trading Co. Ltd), metalaxyl (97% a.i., Agrole P. Ltd., Beijing), azoxystrobin (96% a.i., Syngenta Biotechnology Co. Ltd., Shanghai, China), cyazofamid (96% a.i., Sigma-Aldrich Shanghai Trading Co. Ltd) and cymoxanil (98% a.i., Xinyi Agrochemicals Company, Jiangsu, China). The final concentration of DMSO in the WKB agar medium was adjusted to 0.1% (vol/vol) throughout this study. WKB agar plates amended with fungicides were prepared by adding the same volume of serially diluted solutions to the molten agar medium at ≈50°C. WKB agar medium without fungicide but with the same volume of DMSO was used as a control.
Baseline Sensitivities of *P. melonis* to Flumorph, Dimethomorph and Iprovalicarb

The sensitivities of 80 *P. melonis* isolates to the CAA fungicides flumorph, dimethomorph and iprovalicarb were determined by measuring mycelium growth on fungicide-amended medium. Fresh mycelial plugs (5 mm in diameter) were cut from the edge of an actively growing colony and placed face up in the center of WKB agar medium plates, which were amended with flumorph (0, 0.50, 0.70, 0.90, 1.00, 1.25, 1.50 μg/ml), dimethomorph (0, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35 μg/ml) or iprovalicarb (0, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45 μg/ml). Each treatment was represented by four replicate plates. After incubation for 4 days at 25°C in darkness, colony diameter was measured at perpendicular angles, and the average of the two measurements (minus 5 mm for the mycelial plug) was used for data analysis. The percentage of inhibition was calculated for each concentration and the concentration of each fungicide causing 50% inhibition (EC50) was estimated from the regression of the probit of the percentage of growth inhibition against the logarithmic value of fungicide concentration. For each of the three CAA fungicides, the frequency distribution of 80 EC50 values was plotted as a representation of baseline sensitivity.

### Development of CAA-resistant Mutants of *P. melonis* in *vitro*

**RAPD.** To select *P. melonis* isolates with different genetic background for generation of CAA-resistant mutants, 15 isolates collected from different fields were randomly chosen for genetic relationship analysis by using RAPD, and one isolate of *P. drechsleri* was used as the outgroup control (Table 2). Mycelia were frozen in liquid nitrogen and ground into fine powder with mortar and pestle, which has been previously sterilized at 160°C for 2 h. Genomic DNA was extracted according to the modified Ristaino’s CTAB protocol [39]. About 100 mg of mycelial powder was placed in a 1.5-ml centrifuge tube. A 150-μl volume of extraction buffer (0.35 M sorbitol, 0.1 M Tris, 0.005 M EDTA [pH 7.5], and 0.02 M sodium bisulfite) was added, and the tube was then mixed with a vortex mixer. A 150-μl volume of nuclear lysis buffer

### Table 4. Fitness of CAA-resistant and -sensitive isolates of *Phytophthora melonis* in *vitro.*

| Isolates * | Mycelial growth (mm)* | Zoospore production (×10^5/cm²) | Lesion area on cucumber leaves (mm²) | CFI (×10⁶) |
|------------|-----------------------|---------------------------------|-------------------------------------|------------|
| TJ-S8      | 77 c                  | 1.53 a                          | 370 d                               | 43566 ab   |
| F58-1      | 73 e                  | 1.30 abcd                       | 398 bc                              | 37728 bc   |
| F58-3      | 72 e                  | 1.22 bcd                        | 380 cd                              | 33684 c    |
| F58-4      | 74 de                 | 1.44 ab                         | 383 cd                              | 40980 abc  |
| D58-2      | 81 a                  | 1.41 abc                        | 420 ab                              | 47483 a    |
| D58-3      | 76 cd                 | 1.20 bcd                        | 425 a                               | 38712 abc  |
| D58-5      | 77 c                  | 1.16 cd                         | 383 cd                              | 34205 c    |
| I58-1      | 80 ab                 | 1.13 d                          | 420 ab                              | 37743 bc   |
| I58-2      | 73 e                  | 1.28 abcd                       | 398 bc                              | 36977 bc   |
| I58-3      | 78 bc                 | 1.35 abcd                       | 368 d                               | 38660 abc  |
| 63         | 78 c                  | 0.76 a                          | 555 c                               | 32988 ab   |
| F63-1      | 73 f                  | 0.42 c                          | 532 c                               | 16296 e    |
| F63-3      | 76 de                 | 0.48 bc                         | 473 d                               | 17381 e    |
| F63-5      | 75 e                  | 0.75 a                          | 476 d                               | 27014 abc  |
| D63-1      | 76 de                 | 0.42 c                          | 462 d                               | 14893 e    |
| D63-2      | 77 de                 | 0.53 bc                         | 457 d                               | 18652 de   |
| D63-8      | 81 b                  | 0.51 bc                         | 476 d                               | 19648 cde  |
| I63-2      | 83 a                  | 0.39 c                          | 583 b                               | 18784 de   |
| I63-5      | 77 cd                 | 0.62 ab                         | 542 c                               | 25872 cde  |
| I63-9      | 72 f                  | 0.76 a                          | 621 a                               | 34020 a    |
| 70         | 87 c                  | 0.87 a                          | 575 d                               | 43719 a    |
| F70-1      | 82 e                  | 0.74 b                          | 497 e                               | 30125 cd   |
| F70-5      | 91 a                  | 0.58 de                         | 619 bc                              | 32809 bc   |
| F70-11     | 83 e                  | 0.70 bc                         | 561 d                               | 32795 bc   |
| D70-1      | 87 cd                 | 0.53 e                          | 639 b                               | 29280 cd   |
| D70-5      | 79 f                  | 0.68 bc                         | 681 a                               | 36690 b    |
| I70-1      | 89 b                  | 0.52 e                          | 567 d                               | 26310 d    |
| I70-5      | 85 d                  | 0.53 e                          | 564 d                               | 25505 d    |
| I70-9      | 87 cd                 | 0.63 cd                         | 607 c                               | 33155 bc   |

*Isolates in bold font are parents of the resistant isolates listed under them in regular font. Isolates starting with the letter F, D, and I, are flumorph-resistant mutants, dimethomorph-resistant mutants, and iprovalicarb-resistant mutants, respectively.*

*For each parent and its resistant progeny, means followed by same letters are not significantly different according to Fisher’s least significance difference (α = 0.05).*

*CFI (compound fitness index) = mycelial growth × zoospore production × lesion area on cucumber leaves.*

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Figure 3. Cross-resistance among flumorph, dimethomorph, and iprovalicarb. Log-transformed EC$_{50}$ values (the effective concentration for causing 50% inhibition of mycelial growth inhibition of Phytophthora melonis) for isolates of P. melonis were compared among the three carboxylic acid amide fungicides using Spearman's rank correlation coefficients. (A), (B), and (C) indicate positive cross-resistance among flumorph, dimethomorph, and iprovalicarb; (D-F) include only the higher EC$_{50}$ values from (A-C), i.e., EC$_{50}$ values from CAA-resistant isolates. (D) reveals a positive correlation between the EC$_{50}$ values for dimethomorph and flumorph among CAA-resistant isolates, while (E) and (F) reveals a negative correlation between iprovalicarb and flumorph and between iprovalicarb and dimethomorph among CAA-resistant isolates.

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(0.2 M Tris, 0.05 M EDTA \([\text{pH 7.5}]\), 2.0 M NaCl and 2% CTAB \([\text{pH 7.5}]\)) and 60 \(\mu\)l of 20% SDS (20 g SDS per 100 ml water) was added, and the tube was mixed again. After incubation at 65 °C for 30 min, an isopycnic mixture of chloroform-isoamyl alcohol (24:1, \(\text{v/v}\)) was added, and the tube was centrifuged for 15 min at 13,000 \(g\). The aqueous phase was transferred to a new tube, and the chloroform extraction was repeated. After adding 0.1 volume of 3 M sodium acetate (\(\text{pH 8.0}\)) and 0.6 volume of cold isopropyl alcohol, DNA was precipitated at -20 °C for 2 h. The tube was centrifuged at 13,000 \(g\) for 15 min, and the precipitate was washed with 75% ethanol and then dried at room temperature. DNA was resuspended using 50 \(\mu\)l of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA \([\text{pH 8.0}]\)) for PCR.

RAPD-PCR was performed with each of 16 decamer primers (Table 1). The primers were synthesized by Beijing Sunbiotech Co. Ltd. (Beijing, China). PCR was performed in a 25-\(\mu\)l volume containing 50 ng of template DNA, 1 \(\mu\)l of primer (10 \(\mu\)M), 2 \(\mu\)l of dNTP mixture (2.5 mM of each dNTP and 20 mM Mg\(^{2+}\)), 2.5 \(\mu\)l of EasyTaq DNA Polymerase Buffer (10 \(\times\)), and 2.5 \(\mu\)l of EasyTaq DNA Polymerase (TransGen Biotech, Beijing, China). Amplification was performed in a MyCycler™ Thermal Cycler (Bio-Rad) with the following parameters: 94 °C for 6 min; followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 36 °C for 1 min, and extension at 72 °C for 2 min and a final cycle of extension at 72 °C for 10 min. Amplification products were separated on 1.5% agarose gels in Tris-acetate (TAE) buffer at 110 V for 2 h and were visualized under UV light after being stained with ethidium bromide. All PCRs were repeated at least twice.

Differences in fingerprinting patterns among isolates were assessed based on the clear and reproducible bands. Presumed homologous bands were scored zero (absent) or one (present) and then transformed into a binary matrix. Genetic distance coefficients were calculated for all pairwise comparisons by Nei’s method [40]. The phylogenetic tree was generated based on the genetic distance coefficients by using UPGMA (unweighted pair-group method arithmetic averages) and MEGA (molecular evolutionary genetics analysis) software (version 5).

Generation of CAA-resistant isolates. Based on the genetic analysis, seven isolates of \(P.\) melonis were selected for generation of CAA-resistant mutants. In the case of flumorph, zoospores suspensions were prepared as described above, and 100 \(\mu\)l of each zoospore suspension (approximately 1.0 \(\times\)10\(^6\) zoospores/ml) was inoculated onto WKB plates amended with 10 \(\mu\)g/ml of flumorph (WKBF). After incubation at 25 °C in darkness for 5 days, the emergent colonies were transferred to a fresh WKBF plate. Single-zoospore isolates were obtained. The same procedure was used for generation of resistant mutants to dimethomorph (10 \(\mu\)g/ml of medium) and iprovalicarb (5 \(\mu\)g/ml of medium). This selection procedure was performed twice.

Ultraviolet (UV)-mutagenesis of zoospores. Zoospore suspensions were continuously agitated while they were exposed to UV irradiation (TUV Philips, 15 W, 254 nm) for 1 min at a distance of 30 cm. The suspensions were then spread on WKB
medium plates amended with the corresponding CAA fungicide as described in the previous section. These plates were incubated in the dark for 30 min to minimize light repair of DNA damage. This selection procedure was performed twice and included control plates that were not exposed to UV.

Biological Characteristics of CAA-resistant Mutants

Stability and level of resistance. For determination of the stability of CAA resistance of the mutants, the mutants were subjected to 10 successive transfers on fungicide-free medium before mycelium growth was measured on WKB agar medium amended with each corresponding fungicide at the concentrations described previously. The experiment was done twice. EC$_{50}$ values of mutants were estimated by measuring mycelium growth on fungicide-amended medium at 0, 5, 10, 20, 40, 80 and 100 mg/ml of each CAA fungicide. The level of resistance was described by the resistance factor: RF = EC$_{50}$ of mutant at the 10th transfer/EC$_{50}$ of its parent.

In addition, one spontaneous and one UV-induced mutant resistant to each of fungicide was randomly selected for determination of resistance stability of zoospore progeny. At least 20 single-zoospore isolates randomly sampled from each mutant were grown on WKBF medium. If these single-zoospore isolates grew on WKBF medium, their resistance was considered to be stable; otherwise their resistance was unstable. The same procedure was followed with dimethomorph at 10 mg/ml and with iprovalicarb at 5 µg/ml. This experiment was performed twice.

Mycelial growth and zoospores production. For determination of mycelial growth, the 26 CAA-resistant isolates and their parents were transferred to WKB medium in plates as described in the section concerning baseline sensitivities except that the medium did not contain fungicide. Each isolate was represented by three replicated plates. After incubation in the darkness at 25°C for 5 days, the colony diameter was measured at perpendicular angles, and the average of the two measurements was used to compare the mycelial growth of each resistant isolate and its parent. For comparison of zoospore production, 10 plugs (5-mm in diameter) from the colony margin and 10 plugs from the area near the initial inoculum plug were harvested, and zoospore production was induced as described above and quantified with a hemacytometer. The number of zoospores per cm$^2$ of culture was calculated. These experiments were conducted at least twice.

Virulence. The second or third true leaf from a cucumber plant (cv. Changchunnici) at the fifth true leaf stage was used for virulence tests. The leaves were harvested and rinsed three times with sterile-distilled water. Zoospore suspensions were prepared as described earlier for each of the 26 CAA-resistant mutants and their parent isolates. Four 10-µl droplets of a zoospore suspension (1.0×10$^7$ zoospores/ml) were placed on the upper surface of leaves. One half of each leaf was inoculated with a resistant mutant and the other half was inoculated with the corresponding parent. Ten replicate leaves were used for each combination of mutant and parent. After 5 days in a moist chamber at 20°C with 12 h of light and 12 h of darkness, the lesion areas on each leaf were measured. This experiment was conducted at least twice for each combination of mutant and parent.

Figure 5. Specificity of four allele-specific PCR primer pairs for the detection of carboxylic acid amide (CAA)-resistant isolates of Phytophthora melonis. (A) Specificity of the four primer pairs for the CAA-sensitive isolate TJ-58 (S) and the CAA-resistant isolate F58-4 (R) at gradient annealing temperatures. (B) Specificity of primer pair (PMF + PMR1109B) for four CAA-sensitive and five CAA-resistant isolates at 68.5°C. doi:10.1371/journal.pone.0042069.g005
**Cross-resistance.** The 55 CAA-resistant mutants and 20 wild-type isolates were cultured on WKB agar medium amended with the non-CAA fungicides metalaxyl (0, 0.01, 0.02, 0.05, 0.10, and 0.20 μg/ml), cyoxazole (0, 0.01, 0.02, 0.05, 0.10, and 0.30 μg/ml), or cyazofamid (0.01, 0.02, 0.05, 1.00, and 2.00 μg/ml) or with the CAA fungicides at the concentrations described above. After incubation in darkness at 25°C for 4 days, the colony diameters were measured and the E50 values were calculated as described above. Each treatment was represented by three replicate plates. The experiment was conducted at least twice for each isolate.

**Amplification of the CesA3 gene of P. melonis.**

Based on the conserved sequence of the CesA3 genes in *P. infestans* (ABP96904), *P. tannarius* (ABP96912) and *P. sojae* (ABP96908) in the Genbank/EMBL data libraries, homologous primers were designed for amplification of the partial *PmCesA3* gene fragment. The 5′and 3′end of the *PmCesA3* gene were acquired using SiteFinding-PCR [41]. The full-length *PmCesA3* gene was amplified and sequenced using primers listed in Table 1. All primers were synthesized by Beijing Sunbiotech Co. Ltd. (Beijing, China). Primers PmA3F1 and PmA3R1 were used to gene was amplified and sequenced using primers listed in Table 1.

Molecular Detection of Resistance Mutation in *PmCesA3* by Allele-specific PCR

According to the single mutation in the *PmCesA3* gene, allele-specific primers were designed with the match the nucleotide ‘C’ at the 3′-end of the reverse primers. The specificity of the primers was improved by introducing an artificial mismatch base at the second nucleotide at the 3′-end of the primers (Table 1). To test the specificity of the primers, all the primer pairs were used for gradient PCR using the DNA templates from wild-type isolate TJJ-58 and CAA-resistant mutant F58-4. PCR amplification was performed in a MyCyclerTM Thermal Cycler (Bio-Rad) with the following parameters: an initial preheating for 5 min at 95°C; followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 50 to 70°C for 30 s, and extension at 72°C for 30 s; and terminated with a final extension at 72°C for 10 min. A 5-μl volume of PCR product from each sample was analyzed by electrophoresis using a 1.5% agarose gel in TAE buffer.

**Statistical Analysis.**

Data were analyzed by using the general linear model (GLM) procedure with Statistical Analysis System software (version 9; SAS Inc., Cary, NC, USA). Means were separated using Fisher’s protected least significant difference (LSD, α = 0.05).

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**Author Contributions.**

Conceived and designed the experiments: LC SZ X. Liu. Performed the experiments: LC SZ X. Liu. Analyzed the data: LC SZ. Wrote the paper: LC SZ.

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