JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

pubs.acs.org/JAFC Article

# Activity and Resistance Assessment of a New OSBP Inhibitor, R034-1, in *Phytophthora capsici* and the Detection of Point Mutations in PcORP1 that Confer Resistance

Dong Lin,[†] Zhaolin Xue,[†] Jianqiang Miao, Zhongqiao Huang, and Xili Liu*

Cite This: *J. Agric. Food Chem.* 2020, 68, 13651−13660 Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

**ABSTRACT:** R034-1 is a new member of the piperidinyl thiazole isoxazoline class of fungicides that shows high activity against most plant-pathogenic oomycetes and could effectively inhibit several developmental stages of *Phytophthora capsici*. Here, the potential resistance risk for R034-1 was evaluated in *P. capsici*. The baseline sensitivities of 135 isolates to R034-1 showed a unimodal curve, with a mean $EC_{50}$ value of 0.004 μg/mL. Twelve resistant mutants were generated by fungicide adaptation and displayed lower fitness compared to parental isolates, which suggests that the resistance risk of *P. capsici* to R034-1 is low. R034-1 and oxathiapiprolin are structurally related, and resistant isolates display cross-resistance to both compounds, suggesting that these fungicides may target the same oxysterol binding protein. Comparison of *PcORP1* genes in the resistant mutants and their parental isolates revealed (N767S, N767I, and G700V) amino acid substitutions in the R034-1 resistant mutant. Causality was functionally validated using site-directed mutagenesis of the target gene using the CRISPR/Cas9 system.

**KEYWORDS:** *Phytophthora capsici, R034-1, high activity, resistance assessment, point mutation*



## INTRODUCTION

Oomycetes are morphologically funguslike filamentous organisms with a closer phylogenetic relationship to brown algae and diatoms than the true fungi.[1,2] The most important plant pathogenic oomycetes are *Phytophthora* spp., *Pythium* spp., and *Peronospora* spp., which represent significant threats to agricultural production and food quality.[3,4] For instance, *P. capsici* can cause crown, root, and fruit rot in a wide range of hosts, including vegetable crops such as peppers, eggplants, and cucurbits, leading to severe yield declines and concomitant economic losses.[5−7]

Fungicides play an important role in the comprehensive management of oomycetes to prevent diseases and ensure yields.[7,8] However, due to many physiological and biochemical differences between oomycetes and fungi, as well as increasingly serious resistance problems, effective antioomycete fungicides are very limited and face huge challenges.[9−12] Therefore, there is a pressing need to create new fungicides with high activity, low toxicity, and environmental friendliness.[13]

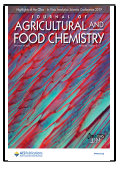
Systematic and comprehensive resistance risk assessment of new fungicides is particularly important and has been listed as a necessary regulatory process for product approval in China since 2012 (NY/T1859.2-2012).[14,15] This process mainly includes the following aspects: the establishment of the baseline sensitivity of a large number of pathogens, determination of the survival fitness for stable resistant mutants, and the measurement of cross-resistance with other fungicides.[13,14]

Oxathiapiprolin was discovered and developed by Du Pont in 2007 and then registered in China in 2016.[16] It is the first member of the piperidinyl thiazole isoxazoline class of fungicides and has been shown to have excellent activity against various plant pathogenic oomycetes, such as *P. capsici*, *P. nicotianae*, and *Pseudoperonospora cubensis*.[16−22] Its molecular target protein has been demonstrated to be the oxysterol binding protein (OSBP) by affinity chromatography assays[17,23] and referred to as PcORP1 in *P. capsici*.[13]

Resistance to the fungicide often involves point mutations or overexpression of the target protein, synthesis of alternative proteins to the target protein of the fungicide, detoxification through efflux or metabolism of the fungicide, and other mechanisms that remain to be elucidated.[24−27] Recent research has identified amino acid changes in PcORP1 and PsORP1 that confer resistance to oxathiapiprolin in *P. capsici* and *P. sojae*.[28]

R034-1 and its precursor compound R031-1 are new fungicides closely related to oxathiapiprolin (Figure 1),[18] developed by Beijing Deerle Agricultural High-tech R&D Center. R034-1 is derived by replacing a trifluoromethyl substituted pyrazole ring in oxathiapiprolin with a tetrazole ring and has a relatively lower cost of synthesis. We speculated that R034-1 has a similar resistance mechanism to oxathiapiprolin. However, its activity against plant-pathogenic oomycetes, potential resistance risk, and the R034-1-resistance

Received: August 28, 2020
Revised: October 27, 2020
Accepted: October 30, 2020
Published: November 16, 2020







https://dx.doi.org/10.1021/acs.jafc.0c05531
J. Agric. Food Chem. 2020, 68, 13651−13660

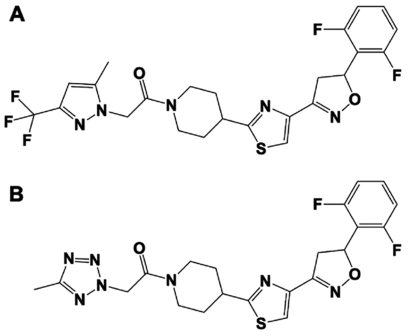



**Figure 1.** Chemical structure of fungicide R034-1 and oxathiapiprolin: (A) oxathiapiprolin; (B) R034-1.

mechanism at the molecular level in *P. capsici* have not been elucidated.

The objective of the present study was therefore to (i) evaluate the in vitro activity of R034-1 and R031-1 against seven species of plant-pathogenic oomycetes and *Magnaporthe oryzae*; (ii) evaluate the sensitivity of *P. capsici* to R034-1 and R031-1 at different developmental stages; (iii) determine the baseline sensitivity of *P. capsici* to R034-1 and assess the potential risk that *P. capsici* can develop resistance to R034-1; (iv) investigate the R034-1 resistance mechanism in *P. capsici*; and (v) verify the point mutations in target protein PcORP1 conferring R034-1 resistance by genome editing using the CRISPR/Cas9 system in *P. capsici*.

## MATERIALS AND METHODS

**Fungicides.** Technical grade R034-1 and R031-1 [98%, active ingredient (a.i.)] were kindly provided by Beijing Deerle Agricultural High-tech R&D Center (Beijing, China); oxathiapiprolin (96.7% a.i.) was kindly provided by Dupont Crop Protection (Wilmington, DE, USA). Other fungicides used were sourced commercially, including fluopicolide (97.2% a.i., Bayer Crop Science, Co., Ltd., Shanghai, China), zoxamide (97% a.i., Gowan Company, United States), dimethomorph (95% a.i., Gowan Company, United States), azoxystrobin (98% a.i., Syngenta Biotechnology, Co., Ltd., Shanghai, China), fluazinam (95% a.i., Japan Ishihara, Co., Ltd.), cyazofamid (98.4% a.i., Mingde Lida Agricultural Technology, Co., Ltd., Beijing, China), chlorothalonil (98% a.i., Henan Chunguang Agrochemical, Co., Ltd., Henan, China), and cymoxanil (98% a.i., Xinyi Agrochemical, Co., Ltd., Jiangsu, China). All fungicides were dissolved in dimethyl sulfoxide (DMSO) for stock solutions ($10^4$ μg/mL) at 4 °C for all the in vitro assays, except for cymoxanil, which was dissolved in DMSO at $10^5$ μg/mL.

**Plant Pathogens and Plant Cultivation.** *P. capsici*, *P. sojae*, *P. nicotianae*, *P. litchi*, *P. ultimum*, *P. delicense*, *P. aphanidermatum*, and *M. oryzae* were used in this study and cultured on the potato dextrose agar (PDA) medium,[29,30] and *P. sojae* was cultured on the V8 juice agar medium for mycelial growth. Additionally, *P. capsici* isolates were cultured on the V8 juice agar medium for sporangial production.[14,31] The *P. capsici* isolates HNJZ10-E and LP3-M included in this study are oxathiapiprolin-resistant mutants obtained previously (resistance factor > 1000).[13]

Pepper seeds (cv. Xichengdaniujiao) were sown in seedling trays (540 mm × 280 mm × 50 mm) using a peat and vermiculite mixture (1:1 v/v) supplemented with 0.1% chicken manure and grown in a greenhouse (27 ± 2 °C, 80% relative humidity, and 12 h photoperiod). Pepper seedlings were cultivated to the four-true-leaf stage.[13,14]

**In Vitro Activity of R034-1 and R031-1 against Plant-Pathogenic Oomycetes and *Magnaporthe oryzae*.** *P. capsici*, *P. sojae*, *P. nicotianae*, *P. litchi*, *P. ultimum*, *P. delicense*, *P. aphanidermatum*, and *M. oryzae* were cultured in the potato dextrose broth (PDB) medium, and *P. sojae* was cultured in the V8 juice liquid medium for 72−96 h at 25 °C. The fresh mycelium was drained and weighed for 0.1 g. Then, the mycelium was put into 50 mL of PDB liquid medium or V8 juice liquid medium and was broken into small hyphal segments using a tissue disrupter. The mixtures of 100 μL of hypha suspension and 100 μL of fungicide diluent (a series of concentrations of 0, 0.00001, 0.0001, 0.001, 0.01, 0.1, and 1 μg/mL) were incubated in 96-well microplates at 25 °C for 3−4 days. The mixture of 100 μL of sterile culture medium and 100 μL of $H_2O$ with 0.1% DMSO was used as the reference control, and the mixture of 100 μL of hypha suspension and 100 μL of $H_2O$ with 0.1% DMSO was used as the blank control. The $OD_{600}$ value of each treatment was detected with a microplate reader, and each treatment was repeated three times. The inhibition rate (%) = ($OD_{600}$ of blank treatment − $OD_{600}$ of fungicide treatment)/($OD_{600}$ of blank treatment − $OD_{600}$ of reference treatment). The effective concentration for 50% inhibition ($EC_{50}$) was calculated according to a previous description.[29]

**Sensitivity of *P. capsici* to R034-1 and R031-1 at Different Developmental Stages.** The sensitivities of *P. capsici* isolates LP3-1 and BYA5 to R034-1 and R031-1 were determined at different developmental stages, and the final concentrations of all fungicides used in the experiment are listed in Table S1. Mycelial growth inhibition was tested as described above. Sporangial production and zoospore release of *P. capsici* isolates were induced as described in previous studies[11,31] with some modifications. To assess fungicidal effects on sporangial production, 10 mycelial plugs (5 mm) from the culture edge of colonies on the V8 juice agar medium were placed into a Petri dish, containing 15 mL of sterile water that has been mixed with the fungicide in advance. Then, the Petri dishes were placed at 25 °C for 3 days under a 12 h photoperiod. After this incubation period, the number of sporangia was counted, and the inhibition of sporangium formation was calculated.

For inducing zoospore release, 10 mycelial plugs (5 mm) that had produced sporangia were placed in a centrifuge tube containing 5 mL of sterile distilled water amended with fungicide. Then, the tubes were maintained at 4 °C for 30 min before incubation at 25 °C for 30 min, and the numbers of empty sporangia were counted under a light microscope.

The zoospore suspensions were shaken using a vortex for 30 s to 1 min in order to complete cystospore encystment. To assess fungicidal effects on cystospore germination, cystospore suspensions were diluted to a concentration of 1 × $10^5$ individuals/mL with a hemocytometer. The mixture of 100 μL of suspension and 100 μL of fungicide diluent was incubated at 25 °C for 6 h in the dark. More than 100 cystospores were examined under the microscope and considered as germinated if the length of the germ tube was greater than the cystospore diameter.

Each treatment consisted of three replicate plates. The effective concentration for 50% inhibition ($EC_{50}$) and 90% inhibition ($EC_{90}$) of mycelial growth, sporangial production, zoospore release, and cystospore germination for each isolate were estimated using probit analysis.[22]

**Baseline Sensitivity of *P. capsici* Isolates to R034-1.** A total of 135 *P. capsici* isolates obtained from 28 provinces of China during 2010−2015 were assessed for sensitivity to R034-1. Final concentrations of 0, 0.001, 0.002, 0.004, 0.006, 0.008, and 0.01 μg/mL R034-1 were added to PDA media, and the $EC_{50}$ of mycelial growth for each isolate was calculated as above. Each concentration consisted of three replicate plates.

**Selection of R034-1-Resistant Mutants of *P. capsici*.** A total of 15 wild-type isolates (LP3, BYA5, HNJZ10, JA8, Pc1723, 12-11, HD3, CG1, HD11, HX18, Pc482, Pc467, Pc485, Pc945, and A1) were used for inducing resistant mutants. The isolates were initially assessed at a concentration of 0.01 μg/mL, which was approximately the minimum inhibitory concentration and tentatively considered to identify resistant isolates effectively. After incubation at 25 °C in the dark for 5 days, mycelial plugs excised from faster-growing colonies were transferred to new PDA plates containing higher fungicide concentrations (a series of stepwise increased concentrations of 0.02, 0.05, 0.1, 0.2, 0.5, 1, 5, 10, and 50 μg/mL). The selection procedure


https://dx.doi.org/10.1021/acs.jafc.0c05531
J. Agric. Food Chem. 2020, 68, 13651−13660

**Table 1. Activity of R034-1 and R031-1 against Eight Kinds of Plant Pathogens**

| isolate | $EC_{50}$ (μg/mL) | | | |
|---|---|---|---|---|
| | R034-1 | R031-1 | oxathiapiprolin | dimethomorph |
| *phytophthora capsici* (HNJZ10) | $1.50 \times 10^{-3}$ | 0.09 | $1.80 \times 10^{-4}$ | 0.13 |
| *phytophthora capsici* (HNJZ10-E) | >1[a] | >1[a] | 0.14 | 0.25 |
| *phytophthora capsici* (LP3-1) | $1.80 \times 10^{-3}$ | 0.11 | $3.50 \times 10^{-4}$ | 0.17 |
| *phytophthora capsici* (LP3-M) | 0.01 | 0.09 | 0.21 | 0.25 |
| *phytophthora sojae* (Ps6) | $1.80 \times 10^{-3}$ | >1 | $3.30 \times 10^{-4}$ | 0.06 |
| *phytophthora nicotianae* | $3.70 \times 10^{-3}$ | 0.59 | $2.40 \times 10^{-4}$ | 0.98 |
| *phytophthora litchi* (FJ9-4) | $2.10 \times 10^{-3}$ | 0.08 | $3.60 \times 10^{-4}$ | 0.19 |
| *pythium ultimum* (A) | 0.55 | 0.56 | 0.07 | >1 |
| *pythium deliense* | >1[a] | >1[a] | >1[a] | >1[a] |
| *pythium aphanidermatum* (C) | >1[a] | >1[a] | >1[a] | >1[a] |
| *magnaporthe oryzae* (SCD3-4) | >1[a] | >1[a] | >1[a] | >1[a] |

[a]This means that at the highest concentration of 1 μg/mL set in this experiment, the inhibitory rate of the fungicides on pathogens is significantly lower than 50%, especially for the *Pythium* spp. and *Magnaporthe oryzae* as listed above, with almost no inhibition.

was repeated until the resistant colonies exhibited normal growth on PDA plates with or without the fungicide.

**Resistance Factor and Stability of R034-1-Resistant Mutants.** The mycelial growth of R034-1-resistant mutants and their parental isolates was measured on PDA plates amended with two series of R034-1 concentrations (Table S2). The resistance factor (RF) was calculated as the ratio of the $EC_{50}$ of the mutant relative to the $EC_{50}$ of its parental wild-type isolate. The resistance stability of mutants was assessed after 10 successive transfers on fungicide-free PDA plates. The factor of sensitivity change (FSC) was calculated as the ratio of the RF of the 10th subculture relative to the RF of the first.

**Mycelial Growth of R034-1-Resistant Mutants and Their Parental Isolates at Various Temperatures.** The mutants and their corresponding parents were incubated on fungicide-free PDA media at a temperature series of 10, 18, 25, 30, and 37 °C in the dark for 5 days. Two perpendicular diameters of each colony were measured, and each treatment consisted of three replicate plates.

**Sporangium and Zoospore Production and Cystospore Germination In Vitro.** Sporangial production and zoospore release of resistant and parental isolates were measured according to the protocol described above.[11,30] Ten mycelial plugs (5 mm) from the culture edge and ten mycelial plugs from the area near the initial inoculum plug were placed into a centrifuge tube containing 5 mL of sterile distilled water to produce zoospores. The numbers of sporangia and zoospores, as well as cystospore germination, were assessed under a light microscope. Each treatment was conducted three times.

**Virulence on Pepper Seedlings In Vivo.** Zoospore inoculation on pepper seedlings and disease scoring were performed as previously described,[32−34] with minor modifications. The concentration of the zoospore suspensions was adjusted to $2 \times 10^4$ zoospores/mL, and 3 mL of zoospore suspensions were inoculated on the soil surface around each seedling. Each treatment consisted of 10 to 20 seedlings, and the disease severity of all seedlings was rated after 7 days. This experiment was conducted three times.

**Cross-Resistance with Other Antioomycete Fungicides.** Sensitivities of the 12 R034-1-resistant mutants, 7 oxathiapiprolin-resistant mutants,[13] and 11 wild-type *P. capsici* isolates to 10 kinds of antioomycete fungicides (Table S2) were determined by mycelial growth inhibition as described above. $EC_{50}$ was calculated as previously described.[29] Each combination of isolate and concentration consisted of three replicate plates, and the experiments were performed three times.

**Cloning and Analysis of the *PcORP1* Gene.** Total DNA was extracted from the mycelia of *P. capsici* isolates grown on the PDA medium for 4 days using the method described by Ristaino et al.,[35] with some modifications, and frozen at −20 °C until required. The primers and PCR steps used for amplification of the full-length *PcORP1* sequence were performed as in the report of Miao et al.[13] The PCR products were sequenced by Beijing Tsingke Co. Ltd., and DNAMAN v.8.0 and Snapgene software were used to analyze and compare the sequence of *PcORP1* from the R034-1 resistant mutants and the wild-type isolates.

**Verification of Point Mutation Transformants Using the CRISPR/Cas9 System.** The CRISPR/Cas9 system in *P. capsici* was used to investigate whether point mutations in PcORP1 confer resistance to R034-1. According to the protocol of a previous study,[36] a single guide RNA (sgRNA) (sg2358-GCTATGCTCAACACCAA-CAA) was designed and cloned into all-in-one plasmid PYF515.[28,37,38] Flanking sequences containing 1000 bp upstream and downstream of the mutation site were amplified and infused into the basic donor plasmid pBS-SK+ using the primers listed in Table S3 and the In-Fusion HD Cloning Kit (Clontech, Mountain View, CA, USA). To prevent the sgRNA-guided Cas9 from cutting the homology-directed repair donor, the sgRNA2358 recognition site was mutated synonymously using the primers listed in Table S3. All DNA fragments were amplified using TransStart® FastPfu DNA Polymerase (TransGen Biotech, Beijing, China).

Polyethylene glycol-mediated protoplast transformation was conducted using a previously described protocol.[36] All-in-one plasmid PYF515-sg2358 and the donor plasmid were co-transformed into protoplasts of *P. capsici* BYA5. Transformants were transferred to the V8 agar medium containing 50 μg/mL G418 and incubated for 3 days at 25 °C. For verification of the transformants, their genomic DNA was extracted to amplify the full length of *PcORP1* gene, and the resultant PCR product was sequenced for verifying homozygous or heterozygous mutation.

**Sensitivity of Point Mutation−Positive Transformants to R034-1 and Oxathiapiprolin.** Fungicide sensitivity was determined by the mycelial growth inhibition method as described above, and $EC_{50}$ was calculated as previously described.[29] The concentrations of R034-1 and oxathiapiprolin are listed in Table S2. Each treatment was replicated three times per experiment, and the experiment was performed three times.

**Statistical Analysis.** Statistical analyses were conducted using IBM SPSS Statistics v.20.0. Differences between the means were determined by the least significance difference multiple range test at $P = 0.05$. Spearman's rank correlation analysis was carried out to test sensitivity associations between R034-1 and each of the other nine fungicides.

## RESULTS

**In Vitro Activity of R034-1 and R031-1 against Plant-Pathogenic Oomycetes and *Magnaporthe oryzae*.** Compared to dimethomorph and the precursor compound R031-1, R034-1 had a stronger inhibitory activity on mycelial growth of wild type *Phytophthora* spp. in vitro, with $EC_{50}$ values of $1.50 \times 10^{-3}$ to $3.70 \times 10^{-3}$ μg/mL (Table 1). R034-1 and R031-1 also effectively inhibited mycelial growth of *P.*


https://dx.doi.org/10.1021/acs.jafc.0c05531
J. Agric. Food Chem. 2020, 68, 13651−13660

Table 2. Sensitivity of *Phytophthora capsici* to R034-1 and R031-1 at Different Developmental Stages

| | | mycelial growth | | sporangial production | | zoospore release | | cystospore germination | |
|---|---|---|---|---|---|---|---|---|---|
| fungicide | isolate | $EC_{50}$ ($\mu$g/mL) | $EC_{90}$ ($\mu$g/mL) | $EC_{50}$ ($\mu$g/mL) | $EC_{90}$ ($\mu$g/mL) | $EC_{50}$ ($\mu$g/mL) | $EC_{90}$ ($\mu$g/mL) | $EC_{50}$ ($\mu$g/mL) | $EC_{90}$ ($\mu$g/mL) |
| R034-1 | LP3-1 | $3.60 \times 10^{-3}$ | $8.60 \times 10^{-3}$ | $9.00 \times 10^{-4}$ | $3.10 \times 10^{-3}$ | >20 | >20 | 0.04 | 0.60 |
| | BYA5 | $3.20 \times 10^{-3}$ | $5.10 \times 10^{-3}$ | $7.00 \times 10^{-4}$ | $3.60 \times 10^{-3}$ | 0.03 | >20 | 0.03 | >0.05 |
| R031-1 | LP3-1 | 0.17 | 0.48 | $3.60 \times 10^{-3}$ | 0.09 | >50 | >50 | 21.42 | 59.23 |
| | BYA5 | 0.18 | 0.54 | $6.40 \times 10^{-3}$ | 0.15 | 0.61 | >50 | 9.84 | >25 |
| oxathiapiprolin | LP3-1 | $7.00 \times 10^{-4}$ | $2.20 \times 10^{-3}$ | $1.00 \times 10^{-5}$ | $2.00 \times 10^{-4}$ | 4.38 | >10 | $6.00 \times 10^{-3}$ | 0.20 |
| | BYA5 | $4.00 \times 10^{-4}$ | $7.00 \times 10^{-4}$ | $4.00 \times 10^{-5}$ | $4.00 \times 10^{-4}$ | 1.33 | 9.19 | $1.00 \times 10^{-3}$ | >0.005 |
| dimethomorph | LP3-1 | 0.29 | 0.78 | 0.01 | 0.15 | >10 | >10 | 0.24 | 2.57 |
| | BYA5 | 0.16 | 0.31 | 0.01 | 0.11 | >10 | >10 | 0.24 | 0.49 |

*ultimum* in vitro, with $EC_{50}$ values of 0.55 and 0.56 $\mu$g/mL, respectively, but had only a slight effect on *P. aphanidermatum*, *P. deliense,* and *M. oryzae* (Table 1). The oxathiapiprolin-resistant mutants HNJZ10-E and LP3-M were also less sensitive to R034-1 and R031-1.

**Sensitivity of *P. capsici* to R034-1 and R031-1 at Different Developmental Stages.** R034-1 was found to inhibit mycelial growth and sporangial production of *P. capsici* most effectively, with $EC_{50}$ values of $7.00 \times 10^{-4}$ to $3.60 \times 10^{-3}$ $\mu$g/mL and $EC_{90}$ values of $3.10 \times 10^{-3}$ to $8.60 \times 10^{-3}$ $\mu$g/mL. Cystospore germination of isolates LP3-1 and BYA5 was also effectively suppressed by R034-1, with $EC_{50}$ values of 0.04 and 0.03 $\mu$g/mL, respectively. However, the inhibitory activity of R034-1 on zoospore release was less pronounced and varied between the tested isolates (Table 2). R031-1 also effectively inhibited sporangial production and mycelial growth with $EC_{50}$ values of $3.60 \times 10^{-3}$ to 0.18 $\mu$g/mL, while the inhibitory activities against zoospore release and cystospore germination were relatively unremarkable (Table 2). Overall, R034-1 had stronger inhibitory activity at different developmental stages of *P. capsici* than dimethomorph and R031-1 and was slightly inferior to oxathiapiprolin.

**Baseline Sensitivity of *P. capsici* Isolates to R034-1.** The sensitivity of 135 *P. capsici* collected from 28 provinces throughout China was tested, and $EC_{50}$ values ranged from $2.5 \times 10^{-3}$ to $8.1 \times 10^{-3}$ $\mu$g/mL. The frequency distribution of $EC_{50}$ values showed a unimodal curve, with a mean $EC_{50}$ of $4.0 \times 10^{-3}$ $\mu$g/mL (Figure 2). The extremely low $EC_{50}$ values and their low variability indicated that none of the tested *P. capsici* wild-type isolates were resistant to R034-1.

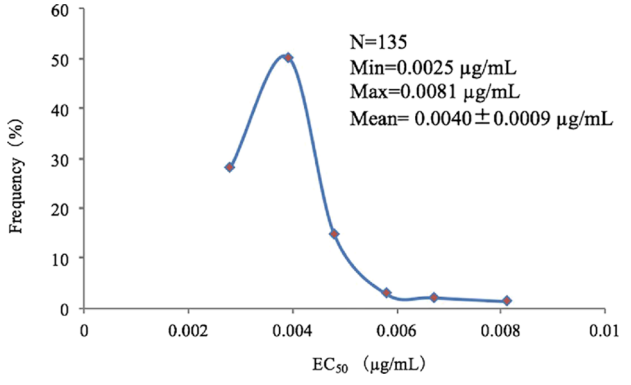



**Figure 2.** Frequency distribution of the $EC_{50}$ values for R034-1 in 135 *Phytophthora capsici* isolates collected from 28 provinces throughout China.

**Generation, RF, and Stability of *P. capsici* Mutants Resistant to R034-1.** Fifteen wild-type parental isolates were originally used to induce mutants resistant to R034-1 by 10 stepwise increased concentration cycles (0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 5, 10, and 50 $\mu$g/mL), but only HNJZ10, Pc1723, and A1 parental isolates contributed to the final total of 12 mutants. The mutation frequency was calculated as the number of obtained resistant mutants divided by the total number of inoculations, with approximately $1 \times 10^{-8}$. The RF values of the first generation mutant ranged from 55.93 to 2000 (Table 3). After a series of 10 subcultures on fungicide-free media, the RF values of all R034-1-resistant mutants were more than 100, except for M369, whose RF value dropped from 55.93 to 16.02. The fact that the majority of FSC values were above 0.76, indicating that the fungicide resistance of the mutants was relatively stable, except for M369, with a significantly lower FSC value (Table 3). Although the FSC values of M327, M328, M333, M355, and M356 cannot be calculated precisely, these mutants still displayed high and stable resistance to R034-1 after 10 generations. In addition, the *P. capsici* mutants that arose with resistance to R034-1 displayed resistance to oxathiapiprolin, and the resistance of most mutants was stably inherited in the 10th-generation isolates, with the FSC values over 1.38, except for M369 (Table S4).

**Mycelial Growth of *P. capsici* R034-1 Resistant Mutants and Their Parental Isolates at Various Temperatures.** The optimum temperature for mycelial growth of all R034-1-resistant mutants and their parental isolates was 25 °C (Figure 3). The mycelial growth rates of most mutants were significantly lower than those of their parents at all temperatures used in the experiment. Mutant M378 grew at the same rate as its parental isolate Pc1723 at 25 °C, and no significant differences in mycelial growth rates between mutant M369 and its parent A1 were observed at 10 and 37 °C (Figure 3).

**Sporangium and Zoospore Production and Cystospore Germination In Vitro.** No significant differences in sporangium production were found among the mutants derived from HNZJ10, while M378 and M369 produced more sporangia than their respective parental isolate Pc1723 and A1. The number of zoospores of all mutants decreased significantly compared to their parents. The cystospore germination rates of all mutants were not significantly different from the parents with the exception of M378, which produced significantly fewer germinated cystospores than its parental isolate Pc1723 (Table 4).

**Virulence on Pepper Seedlings.** The in vivo pathogenicity tests showed that the mutants had a significantly lower disease index than the wild-type parents, except for M369,


https://dx.doi.org/10.1021/acs.jafc.0c05531
J. Agric. Food Chem. 2020, 68, 13651−13660

**Table 3. Resistance Level and Stability of *Phytophthora capsici* R034-1-Resistant Mutant**

| | | $EC_{50}$ (μg/mL) | | RF[a] | | |
|---|---|---|---|---|---|---|
| isolate | origin | first | tenth | first | tenth | FSC[b] |
| HNJZ10 | parent | $5.00 \times 10^{-3}$ | $4.60 \times 10^{-3}$ | -[c] | -[c] | -[c] |
| M239 | mutant | 1.04 | 0.87 | 207.24 | 189.65 | 0.92 |
| M245 | mutant | 0.72 | 1.06 | 143.39 | 231.02 | 1.61 |
| M246 | mutant | 0.30 | 0.58 | 60.15 | 126.59 | 2.10 |
| M260 | mutant | 0.99 | 0.70 | 197.33 | 152.43 | 0.77 |
| M366 | mutant | 1.04 | 0.82 | 206.82 | 177.35 | 0.86 |
| M327 | mutant | >10 | >10 | >2000 | >2000 | -[c] |
| M328 | mutant | >10 | >10 | >2000 | >2000 | -[c] |
| M333 | mutant | >10 | >10 | >2000 | >2000 | -[c] |
| M355 | mutant | >10 | >10 | >2000 | >2000 | -[c] |
| M356 | mutant | >10 | >10 | >2000 | >2000 | -[c] |
| Pc1723 | parent | $4.70 \times 10^{-3}$ | $5.90 \times 10^{-3}$ | -[c] | -[c] | -[c] |
| M378 | mutant | 0.68 | 0.89 | 145.12 | 151.75 | 1.05 |
| A1 | parent | $8.60 \times 10^{-3}$ | $8.30 \times 10^{-3}$ | -[c] | -[c] | -[c] |
| M369 | mutant | 0.48 | 0.13 | 55.93 | 16.02 | 0.29 |

[a]RF, resistance factor, ratio of $EC_{50}$ of fungicide-resistant mutants relative to the $EC_{50}$ of the parental isolate. [b]FSC, factor of sensitivity change, the ratio of RF of the tenth transfer to the RF of the first transfer. [c]The dash indicates that the data cannot be calculated or cannot be calculated precisely.

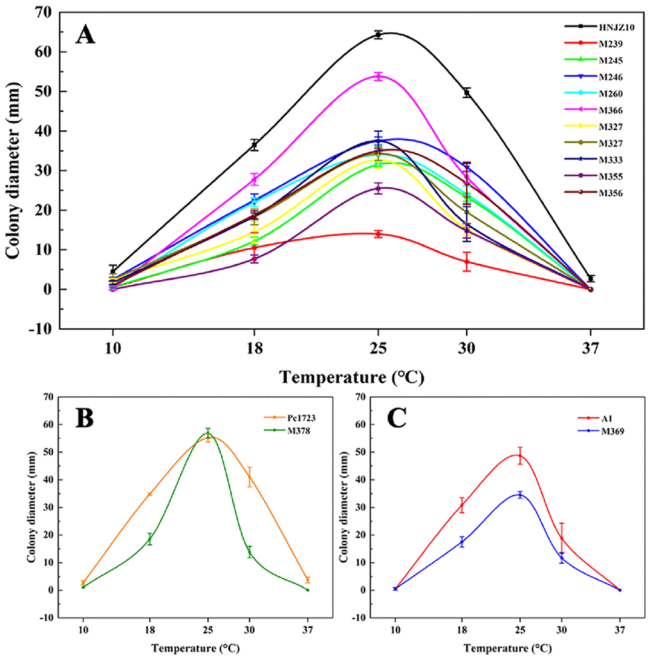



**Figure 3.** Mycelial growth of *Phytophthora capsici* R034-1-resistant mutants and wild-type isolates at various temperatures.

which had a slightly higher disease index than its parent A1 (Table 4).

**Cross-Resistance with Other Antioomycete Fungicides.** A strong positive cross-resistance was detected between R034-1 and oxathiapiprolin, while R034-1 showed no cross-resistance with the other conventional oomycete fungicides tested, including azoxystrobin, dimethomorph, zoxamide, chlorothalonil, and cyazofamid ($P > 0.05$, Figure 4). Although a weak or moderate correlation existed between R034-1 and three fungicides (fluazinam, fluopicolide, and cymoxanil) in rank correlation analysis, R034-1-resistant mutants were sensitive to these fungicides (Table S5), indicating that R034-1 actually did not have cross-resistance to the fungicides.

**Cloning and Analysis of the *PcORP1* Gene of *P. capsici*.** The full-length nucleotide sequence of the *PcORP1* gene was cloned from R034-1-resistant mutants and their corresponding parental isolates, and single nucleotide polymorphisms were sought that occurred only in the mutants. Sequence analysis revealed three types of heterozygous point mutations, including base changes A2374A/G, A2374A/T, and G2173G/T, which essentially caused amino acid changes N767S, N767I, and G700V in the PcORP1 protein (Table 5). It is noted that the resistance level of the mutants with the G700V amino acid change is exceedingly high, manifested as RF values all greater than 2000. The resistance caused by the amino acid mutation at position 767 was at a relatively lower level, with RF values less than 300 (Table 3; Table 5).

**Verification of Point Mutation Transformants Obtained by the CRISPR/Cas9 System in *P. capsici*.** According to the sequencing analysis, two positive heterozygous transformants 4-T-2 and 4-T-X were recovered for N767I in this study (Table 6). However, no positive transformant was obtained for N767S, either heterozygous or homozygous. However, one amino acid deletion (ΔN767) transformant and three heterozygous transformants for G700V were obtained in a previous study,[37] which can be used to determine the resistance mechanism to R034-1.

**Sensitivity of Point Mutation Transformants to R034-1 and Oxathiapiprolin.** All the point mutation transformants tested were resistant to R034-1 and oxathiapiprolin. The RF values of N767I heterozygous transformants to R034-1 were over 450, and the RFs to oxathiapiprolin were over 50. The ΔN767 transformants and three G700V heterozygous transformants showed high resistance to both fungicides, with RF values of more than 1000 (Table 6).

## DISCUSSION

R034-1, a new member of the piperidinyl thiazole isoxazoline class of fungicides, effectively controlled all of the *Phytophthora* plant-pathogenic oomycetes tested, as well as *P. ultimum*. However, it had weak inhibitory activity against *P. aphanidermatum* and *P. deliense*, as well as *M. oryzae*. R034-1 and oxathiapiprolin likely affected a similar spectrum of target


https://dx.doi.org/10.1021/acs.jafc.0c05531
J. Agric. Food Chem. 2020, 68, 13651−13660

Table 4. Fitness of *Phytophthora capsici* R034-1 Resistant Mutants and their Parental Isolates[a]

| isolate | origin | no. sporangia ($\times 10^4$/cm$^2$) | no. zoospores ($\times 10^4$ /mL) | cystospore germination (%) | disease index[c] |
|---|---|---|---|---|---|
| HNJZ10 | parent | 0.37 | 200.00 | 99.50 | 83.85 |
| M239 | mutant | 0.39 | 4.00* | 81.50 | 7.27* |
| M245 | mutant | 0.32 | 2.25* | 96.00 | 18.57* |
| M246 | mutant | 0.41 | 26.50* | 92.00 | 2.31* |
| M260 | mutant | 0.54 | 3.50* | 92.00 | 18.26* |
| M366 | mutant | 0.20 | 5.00* | 84.61 | 7.50* |
| M327 | mutant | 0.20 | 1.25* | 90.45 | 12.31* |
| M328 | mutant | 0.29 | 55.00* | 92.50 | [b] |
| M333 | mutant | 0.26 | 1.25* | 71.43 | 9.17* |
| M355 | mutant | 0.28 | 2.00* | 97.50 | 22.31* |
| M356 | mutant | 0.48 | 5.00* | 92.00 | 19.31* |
| Pc1723 | parent | 0.26 | 69.00 | 61.50 | 86.45 |
| M378 | mutant | 0.48* | 40.00* | 33.50* | 9.17* |
| A1 | parent | 0.07 | 4.00 | 100.00 | 6.86 |
| M369 | mutant | 0.14* | 1.25* | 90.00 | 11.25 |

[a]The asterisk in each column indicates that the mutant is significantly different from the parent ($P < 0.05$). [b]The dash indicates that the data have not been measured. [c]Disease index = $\sum$(number of plants allocated to an individual disease scale × disease scale) × 100/(total number of plants investigated × highest disease scale recorded).

organisms due to their structural similarity.[18,22] R034-1 had a significantly lower inhibitory activity to *Pythium* spp. than *Phytophthora* spp., which may be caused by differences in amino acid positions of the target protein, which should be investigated in further detailed studies.

R034-1 was found to effectively inhibit mycelial growth, sporangial production, and cystospore germination of *P. capsici*, with a relatively weaker inhibitory activity against zoospore release. It seems to affect the different life stages of *P. capsici* in a similar manner to oxathiapiprolin[21,22] but has a slightly lower inhibitory activity. However, R034-1 has a lower cost of synthesis than oxathiapiprolin and does have a superior activity to its precursor compound R031-1 and thus may represent an economical advantage in many situations.

In order to monitor the potential for development of resistance to R034-1, its resistance risk was assessed in this study. The baseline sensitivity of 135 *P. capsici* collected from 28 provinces throughout China during 2010−2015 was established; the frequency distribution of $EC_{50}$ values showed a unimodal curve, with a mean $EC_{50}$ of $4.0 \times 10^{-3}$ $\mu$g/mL. These results provide strong evidence that no R034-1-resistant subpopulations exist in the wild populations of *P. capsici* used in this study, and this baseline can be used for monitoring sensitivity changes of *P. capsici* to R034-1 in China.

A total of 12 R034-1-resistant mutants were obtained by screening mycelial plugs of wild-type isolates (HNJZ10, Pc1723, and A1) on fungicide-amended media. The resultant development of resistance to R034-1 is the first reported and may be representative of how resistance could develop naturally in fields. However, resistant mutants were only obtained from three of the 15 isolates tested in this study, which might be due to genotypic variation among different isolates.[13,39,40]

Biological characteristics of resistant isolates are also required for evaluating fungicide resistance risks. The fitness of most mutants was significantly impaired by their lower mycelial growth rates, decreased number of zoospores, and reduced disease index. Our results suggest that R034-1 resistant mutants may remain weaker competitors compared to wild-type isolates under natural field conditions. Miao et al.[13] tested the survival fitness of LP3-mutants and HNJZ10-mutants resistant to oxathiapiprolin and found that LP3-mutants exhibited strong adaptive traits in mycelial growth, sporangium production, cystospore germination, and pathogenicity, while HNJZ10-mutants showed weak fitness with the reduced number of sporangia and incapacity to produce zoospores. In addition, LP3-mutants and HNJZ10-mutants contained different amino acid substitutions of G769W and G700V, respectively, in the target protein PcORP1. Then, these two point mutations were confirmed by CRISPR/Cas9, and heterozygous resistant transformants at the G700V locus showed equivalent fitness compared with parental isolate BYA5.[28] The different effects of G700V in the two studies could possibly be caused by the genetic backgrounds of the parent isolates, HNJZ10 and BYA5. R034-1 resistant mutants generated from different backgrounds all displayed reduced fitness, suggesting that R034-1 may have a lower inherent resistance risk compared to oxathiapiprolin.

The molecular target protein of oxathiapiprolin has been shown to be the protein ORP1 in phytopathogenic oomycetes.[13,17,23] In previous research, amino acid changes of L733W, S768I/F/K/Y, G770A/I/P/V/L, N837I/F/Y, G839W, P861H, L863W/F, and I877F/Y in *P. infestans* ORP1 (PiORP1) conferred resistance to oxathiapiprolin.[23] Furthermore, *P. sojae* transformants containing amino acid changes of L733W, S768F/Y/K/I, G770V/L/P/A, N837Y/F/I, I877Y/F, G839W, P861H, L863W, and ΔG818/F819/837 in the target protein also showed oxathiapiprolin resistance to various extents.[28,41] A similarity of structure and host range, as well as strong cross-resistance, have been found between R034-1 and oxathiapiprolin. In addition, oxathiapiprolin-resistant mutants HNJZ10-E and LP3-M[13] were also less sensitive to the compound R034-1, and R034-1-resistant mutants also had stably inherited resistance to oxathiapiprolin.

Therefore, it was speculated that R034-1 may have the same protein target as oxathiapiprolin. For this reason, *PcORP1* genes of the R034-1-resistant mutants and wild-type parental isolates were compared, which revealed heterozygous mutations of N767S, N767I, and G700V in PcORP1 protein. These amino acid numbers are expressed relative to the PiORP1 as N837S, N837I, and G770V, according to the proposal of Mair et al.[42] to unify the labeling of amino acids in fungicide target


https://dx.doi.org/10.1021/acs.jafc.0c05531
J. Agric. Food Chem. 2020, 68, 13651−13660

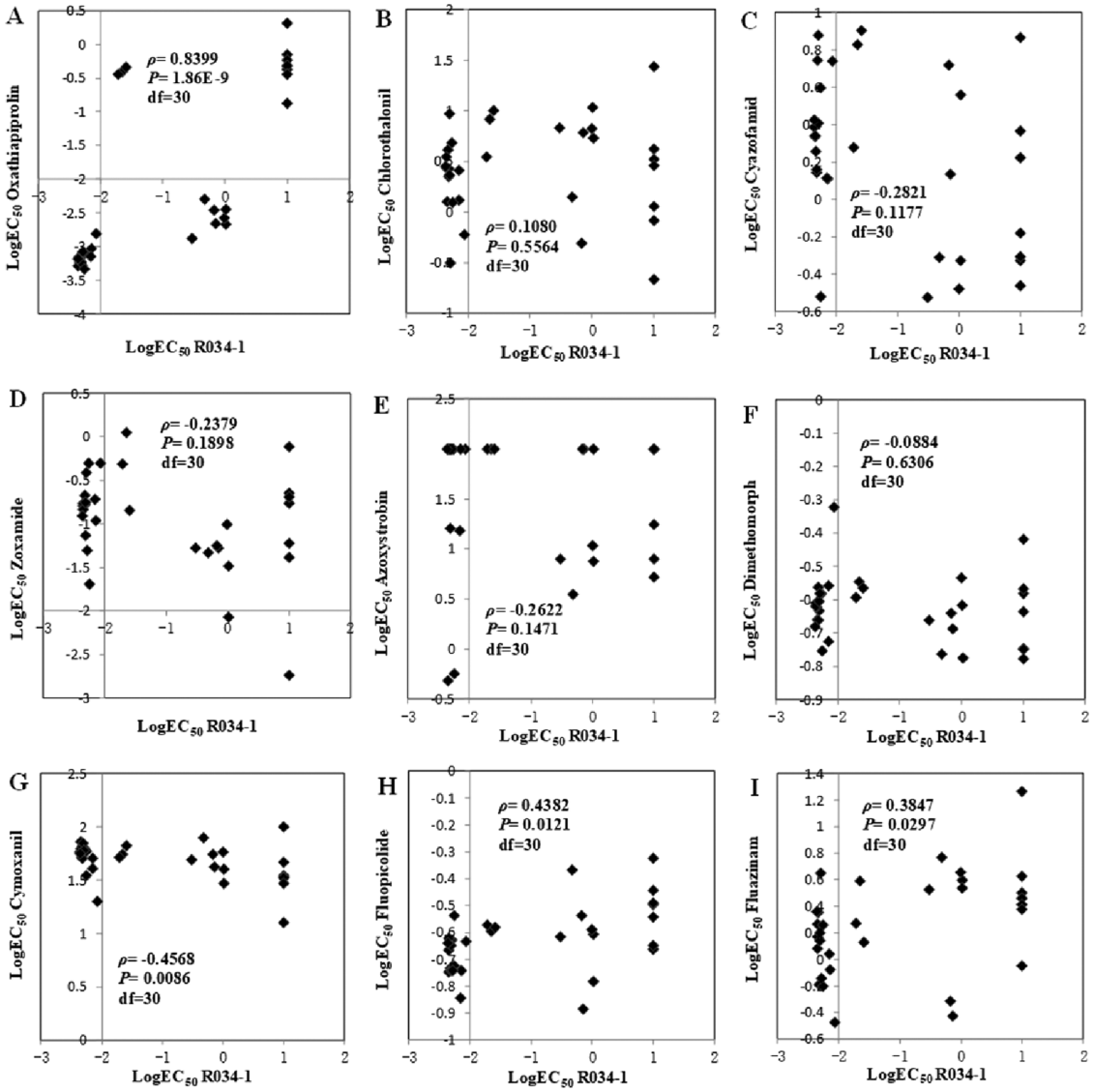



**Figure 4.** Spearman's rank correlation analysis for cross-resistance between R034-1 and other oomycete fungicides in *Phytophthora capsici* isolates: (A) oxathiapiprolin, (B) chlorothalonil, (C) cyazofamid, (D) zoxamide, (E) azoxystrobin, (F) dimethomorph, (G) cymoxanil, (H) fluopicolide, and (I) fluazinam.

proteins.[28] It was noted that N767S has not been reported before. Therefore, it will be important to characterize and compare the mutants' resistance to oxathiapiprolin and R034-1, which may be caused by different amino acid changes. Additionally, there may be new point mutations that can cause resistance to R034-1 because the subtle structural changes may affect the key binding sites of the compound with the target protein.

Heterozygous transformants containing G700V mutation and the homozygous transformants with a deletion of one amino acid (N767) in PcORP1 have been verified to confer high oxathiapiprolin resistance.[28] In this present study, the point mutation transformants of N767I in *P. capsici* were recovered by the CRISPR/Cas9 system. However, point mutation transformants of N767S were not obtained despite trying many different sgRNA sequences. The point mutation transformants of N767I, ΔN767, and G700V showed resistance to R034-1 and oxathiapiprolin, while the RF values of N767I transformants were lower than those of ΔN767 and G700V transformants to both fungicides. We speculate that different amino acid substitutions or a deletion at the same position could cause different changes in the protein structure, thereby affecting the binding of fungicide to the target protein. Miao et al.[41] also found that *P. sojae* transformants containing S768F/Y, G770V, N837Y/F, and I877Y in PsORP1 conferred high oxathiapiprolin resistance, but transformants containing S768I, G770L/P/A, N837I, and I877F exhibited low oxathiapiprolin resistance. The same phenomenon was also observed in other fungicides; for example, an H272L mutation in succinate dehydrogenase B (SdhB) in *Botrytis cinerea* led to higher resistance to boscalid than the H272R/Y mutation,[43] and A577T in myosin5 caused lower resistance to phenamacril in *Fusarium asiaticum* than A577G.[44]

In conclusion, a new member of piperidinyl thiazole isoxazoline fungicide, R034-1, has high activity against plant-pathogenic oomycetes, especially *Phytophthora* spp. It was found to effectively inhibit mycelial growth, sporangial production, and cystospore germination of *P. capsici*. The baseline sensitivity of 135 *P. capsici* isolates was established with a mean $EC_{50}$ of $4.0 \times 10^{-3}$ μg/mL, which can be used for


https://dx.doi.org/10.1021/acs.jafc.0c05531
J. Agric. Food Chem. 2020, 68, 13651−13660

**Table 5. Point Mutation Type of *Phytophthora capsici* R034-1-Resistant Mutants**

| isolate | origin | base change in *PcORP1*[a] | amino acid change in PcORP1 | homozygous or heterozygous in mutation position |
|---|---|---|---|---|
| HNJZ10 | parent | 2374A/ 2173G | | |
| M239 | mutant | 2374A/G | N767S | heterozygous |
| M245 | mutant | 2374A/G | N767S | heterozygous |
| M246 | mutant | 2374A/G | N767S | heterozygous |
| M260 | mutant | 2374A/G | N767S | heterozygous |
| M366 | mutant | 2374A/T | N767I | heterozygous |
| M327 | mutant | 2173G/T | G700V | heterozygous |
| M328 | mutant | 2173G/T | G700V | heterozygous |
| M333 | mutant | 2173G/T | G700V | heterozygous |
| M355 | mutant | 2173G/T | G700V | heterozygous |
| M356 | mutant | 2173G/T | G700V | heterozygous |
| Pc1723 | parent | 2374A | | |
| M378 | mutant | 2374A/G | N767S | heterozygous |
| A1 | parent | 2374A | | |
| M369 | mutant | 2374A/T | N767I | heterozygous |

[a]The base indicates the original base of wild-type and the corresponding changed base of mutants in the *PcORP1* full-length DNA sequence.

monitoring sensitivity changes of *P. capsici* to R034-1 in fields in China. Furthermore, point mutations at N767S, N767I, and G700V were detected in PcORP1 that confer resistance to R034-1. Combining the low fitness of most resistant mutants with the low inherent risk of *P. capsici* as a soil-borne pathogen according to the grading standards of Brent and Hollomon,[45] we speculated that the risk of *P. capsici* developing resistance to R034-1 is low. However, it is worth noting that oxathiapiprolin mutations successfully introduced in *P. capsici* using CRISPR/Cas9 were also conferring stronger resistance toward R034-1 compared with the ones generated during a forced selection. This suggests that additional resistance mutations could occur in nature. The fitness penalty encompassed by such mutations as well as further mutagenesis possibly using in planta selection may be performed to further strengthen our resistance assessment for R034-1. R034-1 has no strong positive cross-resistance with other antioomycete fungicides except for oxathiapiprolin, which suggests that it can be used alternately and mixed with other antioomycete fungicides (except for oxathiapiprolin) for avoiding the development of resistance in field populations. However, further monitoring for new mutation sites in the target protein that cause resistance to R034-1 is necessary.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c05531.

Information on concentrations used to determine the sensitivity of *P. capsici* to several fungicides at different developmental stages (Table S1), concentrations used to determine the sensitivities of R034-1-sensitive and R034-1-resistant *P. capsici* isolates to various fungicides (Table S2), PCR primers used in the current study (Table S3), resistance level and stability of R034-1 resistant *P. capsici* mutants to oxathiapiprolin (Table S4), and sensitivity and genotype of *P. capsici* used for testing the cross-resistance among 10 kinds of antioomycete fungicides (Table S5) (PDF)

## ■ AUTHOR INFORMATION


### Corresponding Author

**Xili Liu** − *China Agricultural University, Beijing 100193, China; State Key Laboratory of Crop Stress Biology for Arid Areas, Northwest A&F University, Yangling 712110, China*; Email: seedling@cau.edu.cn, seedling@nwafu.edu.cn; Fax: +86-010-62731013

### Authors

**Dong Lin** − *China Agricultural University, Beijing 100193, China*

**Zhaolin Xue** − *China Agricultural University, Beijing 100193, China*; orcid.org/0000-0003-2469-7968

**Jianqiang Miao** − *State Key Laboratory of Crop Stress Biology for Arid Areas, Northwest A&F University, Yangling 712110, China*; orcid.org/0000-0002-4683-6933

**Zhongqiao Huang** − *China Agricultural University, Beijing 100193, China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jafc.0c05531


### Author Contributions

[†]These first co-authors have contributed equally to this work.

### Author Contributions

X.L., D.L., and J.M. designed the experiments. D.L., Z.X., J.M., and Z.H. conducted the experiments. D.L. and Z.X. analyzed

**Table 6. Sensitivity of Point Mutation Transformants Obtained by CRISPR/Cas9 System in *Phytophthora capsici* to R034-1 and Oxathiapiprolin**

| | | R034-1 | | oxathiapiprolin | |
|---|---|---|---|---|---|
| isolate | genotype[b] | $EC_{50}$ (μg/mL) | RF[a] | $EC_{50}$ (μg/mL) | RF[a] |
| BYA5 | wild type | 0.0044 | | 0.0003 | |
| 4-T-2 | heterozygous N767I | >2 | >454.5 | 0.0234 | 78.0 |
| 4-T-X | heterozygous N767I | >2 | >454.5 | 0.0292 | 97.3 |
| T47-17 | homozygous ΔN767 | >10 | >2272.7 | 0.8701 | 2900.3 |
| T41-1 | heterozygous G700V | >10 | >2272.7 | 0.6753 | 2251.0 |
| T41-16 | heterozygous G700V | >10 | >2272.7 | 0.4330 | 1443.3 |
| T42-10 | heterozygous G700V | >10 | >2272.7 | 0.6374 | 2124.7 |

[a]RF, resistance factor, ratio of $EC_{50}$ of fungicide-resistant mutants relative to the $EC_{50}$ of the parental isolate. [b]This is consistent with amino acid change in PcORP1 protein.



https://dx.doi.org/10.1021/acs.jafc.0c05531
J. Agric. Food Chem. 2020, 68, 13651−13660

the data. Z.X. and X.L. wrote the manuscript. X.L. revised the manuscript. All authors read and approved the manuscript.

### Funding

This work was supported by the National Natural Science Foundation of China (31730075 and 31471791) and National Key Research and Development Program of China (2017YFD0200501).

### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

The authors would like to thank Beijing Deerle Agricultural High-tech R&D Center and DuPont Corporation for providing the fungicides used in this study.

## ■ REFERENCES

(1) Baldauf, S. L.; Roger, A. J.; WenkSiefert, I.; Doolittle, W. F. A kingdom level phylogeny of eukaryotes based on combined protein data. *Science* **2000**, *290*, 972−977.

(2) Cavalier-Smith, T. Nucleomorphs: enslaved algal nuclei. *Curr. Opin. Microbiol.* **2002**, *5*, 612−619.

(3) Kamoun, S.; Furzer, O.; Jones, J. D. G.; Judelson, H. S.; Ali, G. S.; Dalio, R. J. D.; Roy, S. G.; Schena, L.; Zambounis, A.; Panabières, F.; Cahill, D.; Ruocco, M.; Figueiredo, A.; Chen, X. R.; Hulvey, J.; Stam, R.; Lamour, K.; Gijzen, M.; Tyler, B. M.; Grünwald, N. J.; Mukhtar, M. S.; Tomé, D. F. A.; Tör, M.; van den Ackerveken, G.; McDowell, J.; Daayf, F.; Fry, W. E.; Lindqvist-Kreuze, H.; Meijer, H. J. G.; Petre, B.; Ristaino, J.; Yoshida, K.; Birch, P. R. J.; Govers, F. The top 10 oomycete pathogens in molecular plant pathology. *Mol. Plant Pathol.* **2015**, *16*, 413−434.

(4) Derevnina, L.; Petre, B.; Kellner, R.; Dagdas, Y. F.; Sarowar, M. N.; Giannakopoulou, A.; De la Concepcion, J. C.; Chaparro-Garcia, A.; Pennington, H. G.; van West, P.; Kamoun, S. Emerging oomycete threats to plants and animals. *Phil. Trans. R. Soc. B* **2016**, *371*, 20150459.

(5) Granke, L. L.; Quesada-Ocampo, L.; Lamour, K.; Hausbeck, M. K. Advances in research on *Phytophthora capsici* on vegetable crops in the United States. *Plant Dis.* **2012**, *96*, 1588−1600.

(6) Lamour, K. H.; Stam, R.; Jupe, J.; Huitema, E. The oomycete broad-host-range pathogen *Phytophthora capsici*. *Mol. Plant Pathol.* **2012**, *13*, 329−337.

(7) Hausbeck, M. K.; Lamour, K. H. *Phytophthora capsici* on vegetablecrops: research progress and management challenges. *Plant Dis.* **2004**, *88*, 1292−1303.

(8) Hwang, B. K.; Kim, C. H. *Phytophthora* blight of pepper and its control in Korea. *Plant Dis.* **1995**, *79*, 221−228.

(9) Lamour, K. H.; Hausbeck, M. K. Mefenoxamin sensitivity and the sexual stage of *Phytophthora capsici* in Michigan cucurbit fields. *Phytopathology* **2000**, *90*, 396−400.

(10) Lee, J. Y.; Sherman, D. H.; Hwang, B. K. *In vitro* antimicrobial and *in vivo* antioomycete activities of the novel antibiotic thiobutacin. *Pest Manag. Sci.* **2008**, *64*, 172−177.

(11) Lu, X. H.; Zhu, S. S.; Bi, Y.; Liu, X. L.; Hao, J. J. Baseline sensitivity and resistance-risk assessment of *Phytophthora capsici* to iprovalicarb. *Phytopathology* **2010**, *100*, 1162−1168.

(12) Randall, E.; Young, V.; Sierotzki, H.; Scalliet, G.; Birch, P. R. J.; Cooke, D. E. L.; Csukai, M.; Whisson, S. C. Sequence diversity in the large subunit of RNA polymerase I contributes to mefenoxamin sensitivity in *Phytophthora infestans*. *Mol. Plant Pathol.* **2014**, *15*, 664−676.

(13) Miao, J.; Cai, M.; Dong, X.; Liu, L.; Lin, D.; Zhang, C.; Pang, Z.; Liu, X. Resistance assessment for oxathiapiprolin in *Phytophthora capsici* and the detection of a point mutation (G769W) in PcORP1 that confers resistance. *Front. Microbiol.* **2016**, *7*, 615.

(14) Wu, J.; Xue, Z.; Miao, J.; Zhang, F.; Gao, X.; Liu, X. Sensitivity of different developmental stages and resistance risk assessment of *Phytophthora capsici* to fluopicolide in China. *Front. Microbiol.* **2020**, *11*, 185.

(15) Grimmer, M. K.; van den Bosch, F.; Powers, S. J.; Paveley, N. D. Evaluation of a matrix to calculate fungicide resistance risk. *Pest Manag. Sci.* **2014**, *70*, 1008−1016.

(16) Pasteris, R. J.; Hanagan, M. A.; Shapiro, R. Fungicidal azocyclic amides. *Int Patent*: WO08/013622. 2008.

(17) Pasteris, R. J.; Hanagan, M. A.; Bisaha, J. J.; Finkelstein, B. L.; Hoffman, L. E.; Gregory, V.; Andreassi, J. L.; Sweigard, J. A.; Klyashchitsky, B. A.; Henry, Y. T.; Berger, R. A. Discovery of oxathiapiprolin, a new oomycete fungicide that targets an oxysterol binding protein. *Bioorgan. Med. Chem.* **2016**, *24*, 354−361.

(18) Pasteris, R. J.; Hoffman, L. E.; Sweigard, J. A.; Andreassi, J. L.; Ngugi, H. K.; Perotin, B.; Shepherd, C. P. Oxysterol-binding protein inhibitors: oxathiapiprolin - a new oomycete fungicide that targets an oxysterol-binding protein. In *Modern Crop Protection Compounds;* Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, Germany, 2019, *2*, pp. 979−987.

(19) Ji, P.; Csinos, A. S.; Hickman, L. L.; Hargett, U. Efficacy and application methods of oxathiapiprolin for management of black shank on tobacco. *Plant Dis.* **2014**, *98*, 1551−1554.

(20) Cohen, Y. The novel oomycide oxathiapiprolin inhibits all stages in the asexual life cycle of *Pseudoperonospora cubensis* - causal agent of cucurbit downy mildew. *PLoS One* **2015**, *10*, No. e0140015.

(21) Ji, P.; Csinos, A. S. Effect of oxathiapiprolin on asexual life stages of *Phytophthora capsici* and disease development on vegetables. *Ann. Appl. Biol.* **2015**, *166*, 229−235.

(22) Miao, J.; Dong, X.; Lin, D.; Wang, Q.; Liu, P.; Chen, F.; Du, Y.; Liu, X. Activity of the novel fungicide oxathiapiprolin against plant-pathogenic oomycetes. *Pest Manag. Sci.* **2016**, *72*, 1572−1577.

(23) Andreassi, J. L.; Gutteridge, S.; Pember, S. O.; Sweigard, J. A. Detection and screening method and materials useful in performance thereof. *Int Patent*: WO13/009971. 2013.

(24) Ma, Z.; Michailides, T. J. Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. *Crop Prot.* **2005**, *24*, 853−863.

(25) Gisi, U.; Sierotzki, H. Fungicide modes of action and resistance in downy mildews. *Eur. J. Plant Pathol.* **2008**, *122*, 157−167.

(26) Avenot, H. F.; Michailides, T. J. Progress in understanding molecular mechanisms and evolution of resistance to succinate dehydrogenase inhibiting (SDHI) fungicides in phytopathogenic fungi. *Crop Prot.* **2010**, *29*, 643−651.

(27) Leroux, P.; Gredt, M.; Leroch, M.; Walker, A. S. Exploring mechanisms of resistance to respiratory inhibitors in field strains of *Botrytis cinerea*, the causal agent of gray mold. *Appl. Environ. Microbiol.* **2010**, *76*, 6615−6630.

(28) Miao, J.; Chi, Y.; Lin, D.; Tyler, B. M.; Liu, X. Mutations in ORP1 conferring oxathiapiprolin resistance confirmed by genome editing using CRISPR/Cas9 in *Phytophthora capsici* and *P. sojae*. *Phytopathology* **2018**, *108*, 1412−1419.

(29) Pang, Z.; Shao, J.; Chen, L.; Lu, X.; Hu, J.; Qin, Z.; Liu, X. Resistance to the novel fungicide pyrimorph in *Phytophthora capsici*: risk assessment and detection of point mutations in CesA3 that confer resistance. *PLoS One* **2013**, *8*, No. e56513.

(30) Brasier, C. M.; Griffin, M. J. Taxonomy of *Phytophthora palmivora* on cocoa. *Trans. Br. Mycol. Soc.* **1979**, *72*, 111−143.

(31) Miller, P. M. V-8 juice agar as a general-purpose medium for fungi and bacteria. *Phytopathology* **1955**, *45*, 461−462.

(32) Glosier, B. R.; Ogundiwin, E. A.; Sidhu, G. S.; Sischo, D. R.; Prince, J. P. A differential series of pepper (*Capsicum annuum*) lines delineates fourteen physiological races of *Phytophthora capsici*. *Euphytica* **2008**, *162*, 23−30.

(33) Kim, E. S.; Hwang, B. K. Virulence to Korean pepper cultivars of isolates of *Phytophthora capsici* from different geographic areas. *Plant Dis.* **1992**, *76*, 486−489.

(34) Hartman, G.; Huang, Y. Pathogenicity and virulence of *Phytophthora capsici* isolates from Taiwan on tomatoes and other selected hosts. *Plant Dis.* **1993**, *77*, 588−591.



https://dx.doi.org/10.1021/acs.jafc.0c05531
J. Agric. Food Chem. 2020, 68, 13651−13660

(35) Ristaino, J. B.; Madritch, M.; Trout, C. L.; Parra, G. PCR amplification of ribosomal DNA for species identification in the plant pathogen genus *Phytophthora*. *Appl. Environ. Microbiol.* **1998**, *64*, 948−954.
(36) Fang, Y.; Tyler, B. M. Efficient disruption and replacement of an effector gene in the oomycete *Phytophthora sojae* using CRISPR/Cas9. *Mol. Plant Pathol.* **2016**, *17*, 127−139.
(37) Doench, J. G.; Hartenian, E.; Graham, D. B.; Tothova, Z.; Hegde, M.; Smith, I.; Sullender, M.; Ebert, B. L.; Xavier, R. J.; Root, D. E. Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation. *Nat. Biotechnol.* **2014**, *32*, 1262−1267.
(38) Fang, Y.; Cui, L.; Gu, B.; Arredondo, F.; Tyler, B. M. Efficient genome editing in the oomycete *Phytophthora sojae* using CRISPR/Cas9. *Curr. Protoc. Microbiol.* **2017**, *44*, 21A.1.1−21A.1.26.
(39) Hu, J.; Pang, Z.; Bi, Y.; Shao, J.; Diao, Y.; Guo, J.; Liu, Y.; Lv, H.; Lamour, K.; Liu, X. Genetically diverse long-lived clonal lineages of *Phytophthora capsici* from pepper in Gansu, China. *Phytopathology* **2013**, *103*, 920−926.
(40) Lu, X. H.; Hausbeck, M. K.; Liu, X. L.; Hao, J. J. Wild type sensitivity and mutation analysis for resistance risk to fluopicolide in Phytophthora capsici. *Plant Dis.* **2011**, *95*, 1535−1541.
(41) Miao, J.; Liu, X.; Li, G.; Du, X.; Liu, X. Multiple point mutations in *PsORP1* gene conferring different resistance levels to oxathiapiprolin confirmed using CRISPR-Cas9 in *Phytophthora sojae*. *Pest Manag. Sci.* **2020**, *76*, 2434−2440.
(42) Mair, W.; Lopez-Ruiz, F.; Stammler, G.; Clark, W.; Burnett, F.; Hollomon, D.; Ishii, H.; Thind, T. S.; Brown, J. K. M.; Fraaije, B.; Cools, H.; Shaw, M.; Fillinger, S.; Walker, A.; Mellado, E.; Schnabel, G.; Mehl, A.; Oliver, R. P. Proposal for a unified nomenclature for target-site mutations associated with resistance to fungicides. *Pest Manag. Sci.* **2016**, *72*, 1449−1459.
(43) Veloukas, T.; Markoglou, A. N.; Karaoglanidis, G. S. Differential effect of *SdhB* gene mutations on the sensitivity to SDHI fungicides in *Botrytis cinerea*. *Plant Dis.* **2013**, *97*, 118−122.
(44) Li, B.; Zheng, Z.; Liu, X.; Cai, Y.; Mao, X.; Zhou, M. Genotypes and characteristics of phenamacril-resistant mutants in *Fusarium asiaticum*. *Plant Dis.* **2016**, *100*, 1754−1761.
(45) Brent, K. J.; Hollomon, D. W. *Fungicide resistance in crop pathogens-how can it be managed?*. FRAC Press: Brussels, 2007.


https://dx.doi.org/10.1021/acs.jafc.0c05531
*J. Agric. Food Chem.* 2020, 68, 13651−13660