Mutations in ORP1 Conferring Oxathiapiprolin Resistance Confirmed by Genome Editing using CRISPR/Cas9 in *Phytophthora capsici* and *P. sojae*

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Accepted for publication 4 July 2018.

ABSTRACT

Oxathiapiprolin is a novel fungicide that was recently registered in a number of countries to control plant-pathogenic oomycetes such as *Phytophthora capsici*. In our previous study, point mutations G770V and G839W in oxysterol binding protein-related protein 1 (ORP1) were detected in oxathiapiprolin-resistant *P. capsici* isolates (PcORP1). Here, we used the CRISPR/Cas9 system to verify the effects of these two point mutations on *P. capsici* phenotypes. Transformants containing heterozygous G770V and G839W mutations in PcORP1 showed high levels of oxathiapiprolin resistance. The G770V transformants showed otherwise similar phenotypes compared with the wild-type isolate BYA5, including sporangia and zoospore production, cyst germination, and pathogenicity.

However, two independent transformants with heterozygous G839W mutations in PcORP1 could not produce sporangia. Three transformants with an unexpected point mutation in PcORP1 (ΔN837) showed high oxathiapiprolin resistance, and either similar or significantly reduced fitness compared with BYA5. The same deletion (ΔN837) was confirmed to confer oxathiapiprolin resistance in *P. sojae* by using CRISPR/Cas9. These homozygous *P. sojae* mutants also showed either similar or strongly reduced fitness compared with the wild-type parent isolate P6497. These results improve our understanding of oxathiapiprolin resistance in *Phytophthora* spp., and will be useful for the development of novel oxysterol-binding protein homolog inhibitor fungicides.

Oomycetes are among the most problematic group of disease-causing organisms in agriculture. Well-known oomycete plant pathogens include *Phytophthora* spp., *Pythium* spp., and downy mildew agents (Blair et al. 2008; Derevnina et al. 2016; Fichtner et al. 2012; Meitz-Hopkins et al. 2014).

Generally, chemical fungicides still play an important role in the management of diseases caused by oomycetes. However, most oomycetes exhibit a tremendous ability to adapt to chemicals through the emergence of genetically resistant strains (Gisi and Sierotzki 2008; Urban and Lebeda 2006; Ziogas et al. 2006).

Oxathiapiprolin, developed by DuPont, belongs to a novel fungicide class: namely, oxysterol-binding protein homolog inhibitors (FRAC 2017; Pasteris et al. 2016). It has excellent preventive, curative, and remanence efficacy against many important plant oomycete diseases, such as Phytophthora blights and downy mildews (Bittner and Mila 2017; Cohen 2015; Ji and Csinos 2015; Ji et al. 2014). It can be applied through foliar sprays (ground, aerial, and chemigation), soil applications (in-furrow and banded), or soil drenches. Application rates are much lower than regular fungicides, ranging from 6.42 to 61.78 lb. of active ingredient per square kilometer. Currently, oxathiapiprolin has been registered in China, the United States, the European Union, New Zealand, Australia, and some other countries to control plant-pathogenic oomycetes.

Because of its single-site mode of action, oxathiapiprolin readily selected resistant mutants in *P. capsici* and *P. nicotianae* in the laboratory (Andreassi et al. 2013; Bittner et al. 2017; Miao et al. 2016). In oxathiapiprolin-resistant mutants, nine point mutations have been detected in the oxysterol-binding domain of the target protein, oxysterol binding protein-related protein 1 (ORP1). According to the proposal of Mair et al. (2016) to unify the labeling of amino acids in fungicide target proteins, the oxathiapiprolin target protein in *P. infestans* was used as the archetype protein and numerical labels on the position of the residue in it were assigned. In *P. capsici* ultraviolet mutagenesis mutants, L733W, S768I/F/K/Y, G770A/I/P/V/L, N837I/F/Y, G839W, P861H, L863W/F, and I877F/Y were detected in *P. capsici* ORP1 (PcORP1) (Andreassi et al. 2013). Andreassi et al. (2013) obtained grape downy mildew isolates from field trials where oxathiapiprolin had been continuously used for 4 years, and detected two amino acid changes (N837I and L863W) in resistant isolates. Bittner et al. (2017) also detected G770V and ΔG818/F819 mutations in *P. nicotianae*-resistant mutants produced by UV radiation. G770V and G839W were also found in *P. capsici* fungicide adaptation mutants, and G839W was confirmed by traditional overexpression transformation to confer oxathiapiprolin resistance (Miao et al. 2016). Because the mutants containing G770V showed significantly reduced fitness, we did not verify whether this point mutation confers resistance to oxathiapiprolin.

Distinct from the zinc-finger nucleases and transcription activator-like effector nucleases, the RNA-guided Cas9 endonucleases from the microbial clustered regularly interspaced short palindromic repeats (CRISPR) have emerged as a powerful tool for mediating genome alterations with high precision (Hsu et al. 2014). Recently, the CRISPR/Cas9 system was established in *P. sojae* as an effective gene-editing method in oomycetes (Fang and Tyler 2016; Fang et al. 2017). Here, we used CRISPR/Cas9 to confirm that the point mutations G770V and G839W, as well as a novel mutation, ΔN837, confer resistance to oxathiapiprolin in *P. capsici*, and to determine how they affect the biological characteristics of *P. capsici* and *P. sojae*.

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MATERIALS AND METHODS

Fungicide. Technical-grade oxathiapiprolin (96.7% active ingredient) was kindly provided by Dupont Crop Protection. It was accurately weighed and dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution at 10^4 µg/ml, which was stored in darkness at 4°C until required.

***Phytophthora* strains and growth conditions.** The wild-type *P. capsici* strain BYA5, the reference *P. sojae* isolate P6497 (race 2), and all transgenic isolates used in this study were routinely grown on 10% V8 medium at 25°C in darkness. The sensitivity of wild-type isolates and all transgenic lines to oxathiapiprolin was determined using the mycelia growth assay described by Pang et al. (2013). The final concentration of DMSO in the medium was standardized at 0.1% (vol/vol). Each treatment consisted of three replicates and all experiments were repeated twice.

Plasmid construction. The sequences of the *PcORP1* and *P. sojae* ORP1 (*PsORP1*) genes were obtained by searching the *P. capsici* genome, v11.0 (https://genome.jgi.doe.gov/Phyca11/Phyca11.home.html), and the *P. sojae* genome, v3.0 (https://genome.jgi.doe.gov/Physo3/Physo3.home.html). The full-length genes of *PcORP1* from *P. capsici* BYA5 and *PsORP1* from *P. sojae* P6497 were amplified using CloneAmp HiFi PCR Premix (Takara) and sequenced. All other fragments were also amplified using CloneAmp HiFi PCR Premix (Takara).

According to our previous study (Miao et al. 2016), point mutations at nucleotides 2,173 (G→T) and 2,379 (G→T) in *PcORP1* produced the amino acid substitutions G770V and G839W, respectively, in oxathiapiprolin-resistant mutants of *P. capsici*. To use the CRISPR/Cas9 system to confirm whether these point mutations confer resistance to oxathiapiprolin, constructs encoding two single-guide RNA (sgRNA) (sg2180-GAAACCATTCAACCCGATCC and sg2358-GCTATGCTCAACACCAACAA) were cloned separately into plasmid PYF515 (Fang et al. 2017). The sgRNA were designed using the web tool sgRNA Designer (https://portals.broadinstitute.org/gpp/public/) (Doench et al. 2014). Then, the sgRNA-encoding fragments were inserted into the all-in-one plasmid pYF515, according to the protocol of Fang et al. (2017). Flanking sequences, 1,000 bp upstream and downstream of the mutation site, were amplified and infused into pBS-SK+ using the primers listed in Table 1 and the In-fusion HD Cloning Kit (Clontech), to create the basic donor plasmid.

Transformation of *P. capsici* and *P. sojae*. Polyethylene glycol-mediated protoplast transformations were conducted using previously described methods (Fang and Tyler 2016), with a minor modification. The wild-type *P. sojae* or *P. capsici* strain was cultured on a V8 agar plate. After 2 to 5 days, five to eight mycelium discs, cut from the edge of actively growing colonies, were grown into 50 ml of nutrient pea broth to produce fresh hyphae for protoplast preparation. All other steps were performed as in the previous protocol.

To avoid the sgRNA-mediated Cas9 cleavage of the homology-directed repair (HDR) donor, the sgRNA recognition site was also mutated. In order to prevent the sgRNA-guided Cas9 from cutting the donor plasmid, the sgRNA binding residues in the donor plasmid were mutated without changing the encoded amino acids, producing the two donor plasmids pBS-HDR1 and pBS-HDR2, respectively (Fig. 1). PYF515-sg2358 was cotransformed together with pBS-HDR1 and pYF515-sg2180 was cotransformed with pBS-HDR2.

Transformants were transferred to V8 liquid medium containing G418 at 50 µg/ml or oxathiapiprolin at 0.005 µg/ml and incubated for 2 to 3 days at 25°C prior to analysis. The hyphae of the transformants were then collected for genomic DNA (gDNA) extraction. For verification of the transformants, the amplification products of the full-length *PcORP1* or *PsORP1* genes in all transformants were sequenced.

Nucleic acid extraction. Total gDNA was extracted according to Edwards et al. (1991), with some modifications. The mycelium sample was ground after freezing in liquid nitrogen. Then, 400 µl of extraction buffer (200 mM Tris HCl [pH 7.5], 250 mM NaCl, 25 mM EDTA, and 0.5% sodium dodecyl sulfate) was added, and the mix was vortexed for 10 s and centrifuged at 13,000 rpm for 10 min, before 300 µl of the supernatant was transferred to a new 1.5-ml tube. Then, 300 µl of isopropanol was added into the supernatant and chilled at −20°C for 1 h. Following centrifugation (13,000 rpm for 5 min), the supernatant was removed and the pellet was vacuum dried. Then, the gDNA was suspended in 50 µl of H_2O. For RNA extraction, mycelium was harvested from *P. capsici* or *P. sojae* isolates grown on V8 liquid medium for 2 or 3 days, respectively, and frozen at −80°C until required. Total RNA was extracted from the frozen samples using the SV Total RNA Isolation kit (Promega Corp.), and cDNA was synthesized using the PrimeScript II First-Strand cDNA Synthesis Kit (Takara) according to the protocol of the manufacturers.

Phenotype analysis of *P. sojae* and *P. capsica*. Mycelial growth was determined after depositing a mycelial plug (5 mm in diameter) on 10% V8 agar medium and incubating the plates for 7 days (*P. sojae*) or 5 days (*P. capsici*) in the dark at 25°C.

To analyze sporangia production of *P. sojae*, 10 hyphal tip plugs (5 mm in diameter) cut from the edge of an actively growing culture of each strain were deposited in a plastic plate containing 20 ml of

TABLE 1. Primers used in the study

Primer	Sequence (5′–3′)	Application[z]
P1	GCTTGATATCGAATTCAAGTGGAAGCTTTGCCCAG	Amplification of upstream and downstream regions around codon 770 of *PcORP1*
P2	TAGAACTAGTGGATCCTCCGACTTCAACCACGAGATG	…
P3	GCTTGATATCGAATTCTCTTCGTCATGTGCGTGAGTCT	Amplification of upstream and downstream regions around codon 839 of *PcORP1*
P4	TAGAACTAGTGGATCCCAGGCTGAAGGTCTGATGATGTAC	…
P5	GCGATGCTGAATACGAATAAAGGGGCCTGTGCGTGTTAC	Synonymous mutation of sg2358 target sequence in pBS-HDR1
P6	TTTATTCGTATTCAGCATCGCGTTGGACTTGACGCTCAT	…
P7	GAAGCCGTTTAATCCCATTCTCTGGGCGAAACATACCAGT	Synonymous mutation of sg2180 target sequence in pBS-HDR2
P8	GAGAATGGGATTAAACGGCTTCAGCTGTCCGATACTGTGG	…
P9	GCTTGATATCGAATTCGGTGCTGCTGCGTCACGT	Amplification of upstream and downstream regions around codon 837 of *PsORP1*
P10	TAGAACTAGTGGATCCGATTTACAAATCGATCGATCTAAAT	…
P11	GCCATGCTGAATACGAAAGGTCCTGTGCGCGTGACGTT	Synonymous mutation of sg2381 target sequence in pBS-HDR3
P12	CCTTTCGTATTCAGCATGGCGTTGGACTTGACGCTCAT	…
P13	CACCACAGTATCGGACAGC	Amplify *PcORP1* transcripts for quantitative real-time PCR
P14	CAAACCCAGCAATGGAGTA	…
P15	ACTGCACGTTCCAGACGATC	Amplify *PcActin* transcripts for quantitative real-time PCR
P16	CCACCACCTTGATCTTCATG	…
P17	TGCAATTCCCGCAGTCGT	Amplify *PsORP1* transcripts for quantitative real-time PCR
P18	GCGTACACCATCGCAGTCAGT	…
P19	ACTGCACCTTCCAGACCATC	Amplify *PsActin* transcripts for quantitative real-time PCR
P20	CCACCACCTTGATCTTCATG	…

[z] PCR = polymerase chain reaction.

liquid 10% V8 medium. These plates were incubated in darkness at 25°C for 2 days. After washing the mycelia seven times in 30 ml of sterile tap water, another 20 ml of sterile water was added and the resulting preparation was incubated in darkness at 25°C for 24 h to induce sporangia production. For *P. capsici*, 10 hyphal tip plugs (5 mm in diameter) of each strain were placed into a Petri dish containing 20 ml of sterile deionized water and incubated at 25°C under continuous light for 48 h. The number of all sporangia formed on 30 plugs was counted.

To analyze zoospore production in *P. sojae*, all strains were cultured on 10% V8 agar medium for 10 days in darkness at 25°C. Then, the plates containing mycelium were washed seven times using 30 ml of sterile tap water for each washing. Finally, to induce sporangia production and zoospore release, another 15 ml of sterile water was added, and the plates were incubated in darkness 25°C for 8 h. For *P. capsici*, all of the isolates were cultured on 10% V8 agar medium for 3 days in darkness at 25°C. Next, all of the plates were incubated at room temperature under continuous light for another 5 days. Then, 20 ml of sterile distilled water was added to the container and the plates were placed at 4°C for 30 min, followed by room temperature for 30 min. Finally, the zoospore suspension from one Petri dish was poured into a sterile beaker and the zoospores were counted using a hemocytometer.

Cyst germination was assessed by plating approximately 500 zoospores on 10% V8 agar medium. After 4 h of incubation in the dark at 25°C, the percent germination of each isolate was observed by counting 100 cysts. Cysts were considered to have germinated if the length of their germ tube was greater than the diameter of the cyst. Each experiment contained three replica plates and all experiments were repeated three times.

To evaluate the pathogenicity of *P. sojae*, the first true leaves of 9- to 10-day-old soybean seedlings, cultivar Williams (rps), were used. Leaves were placed in Petri dishes containing wet tissue to prevent dehydration, and a mycelial plug was placed in the middle of each soybean leaf. For the pathogenicity assay of *P. capsici*, detached leaves of *Nicotiana benthamiana* were used. For each isolate, there were six replicate leaves. After inoculation, leaves were incubated at room temperature for 4 days before measuring the lesion area using Image J 1.x (Schneider et al. 2012). The experiment was repeated three times.

Quantitative real time polymerase chain reaction analysis of transcript levels. Quantitative real-time polymerase chain reaction (PCR) analysis of transcript levels was performed using the ABI7500fast sequence detection system (Applied Biosystems) and the Fast SYBR Green Master Mix (Thermo Fisher Scientific) with the primers listed in Table 1 and the protocol of the manufacturer. The relative quantities of the PCR products were calculated using the $2^{-\Delta\Delta Ct}$ method and the *PcACTIN* and *PsACTIN* genes were used as a reference (Yan and Liou 2006) to normalize the quantification of *PcORP1* and *PsORP1* transcript levels, respectively. The entire experiment was conducted twice, and each experiment included three replicates for each treatment.

RESULTS

CRISPR/Cas9 system for the point mutation in *P. capsici* and validation of transformants. A total of eight colonies representing at least seven independent transformants showed oxathiapiprolin resistance (Figs. 2 and 3). T41-1 and T41-16 were obtained from the same transformation reaction and might not represent independent transformation events. The remaining six transformants were all obtained independently from one another. Sequencing results showed that T65-1 and T65-2 were both heterozygous at nucleotide 2,379. One allele was the wild type and the other allele contained the point mutation G→T at this position. The synonymous point mutations at the sg2180 target sequence introduced from pBS-HDR2 were also heterozygous (Fig. 2). Similarly, T41-1, T41-16, and T42-10 were heterozygous at nucleotide 2,173 (G and T). However, they were all homozygous for the synonymous point mutations at the sg2381 target sequence. Interestingly, in T47-1, T47-17, and T47-19, three nucleotides (2,379, 2,380, and 2,381) were deleted at the Cas9 cleavage site and no other mutations were found. This 3-bp deletion resulted in a deletion of one amino acid (N837) (Fig. 2). This mutation was presumably produced by nonhomologous end joining (NHEJ), triggered by CRISPR/Cas9.

Phenotype of *P. capsici* transformants. All eight transformants grew on V8 plates containing various concentrations of oxathiapiprolin, up to 0.5 µg/ml (Fig. 3A), whereas the wild-type
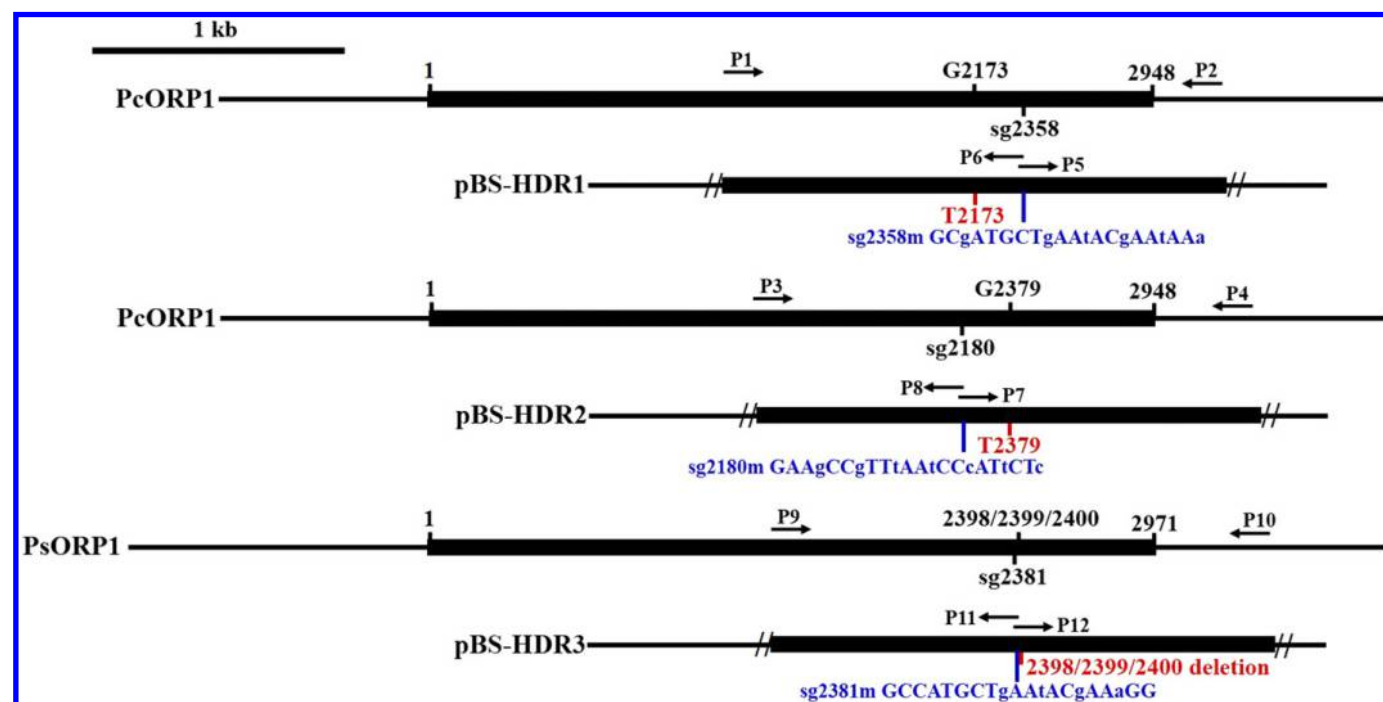

Fig. 1. Schematic of homologous donor DNA plasmids. Primer binding sites are indicated by arrows. Homology arms (1 kb) flanking the target mutation site were used. The subgenomic RNA recognition sites in pBS-HDR1, pBS-HDR2, and pBS-HDR3 were mutated and the new sequence was shown in red text.

isolate BYA5 could not grow on V8 plates containing oxathiapiprolin at 0.005 μg/ml. This confirms that individual heterozygous mutations of G770V or G839W in PcORP1 could produce high oxathiapiprolin resistance. The sensitivity results for T47-1, T47-17, and T47-19 suggested that the homozygous ΔN837 mutation in PcORP1 also could confer high oxathiapiprolin resistance (Fig. 3A).

Surprisingly, neither T65-1 nor T65-2, containing the heterozygous point mutation G839W in PcORP1, could produce sporangia or zoospores, although they maintained pathogenicity (Table 2). By
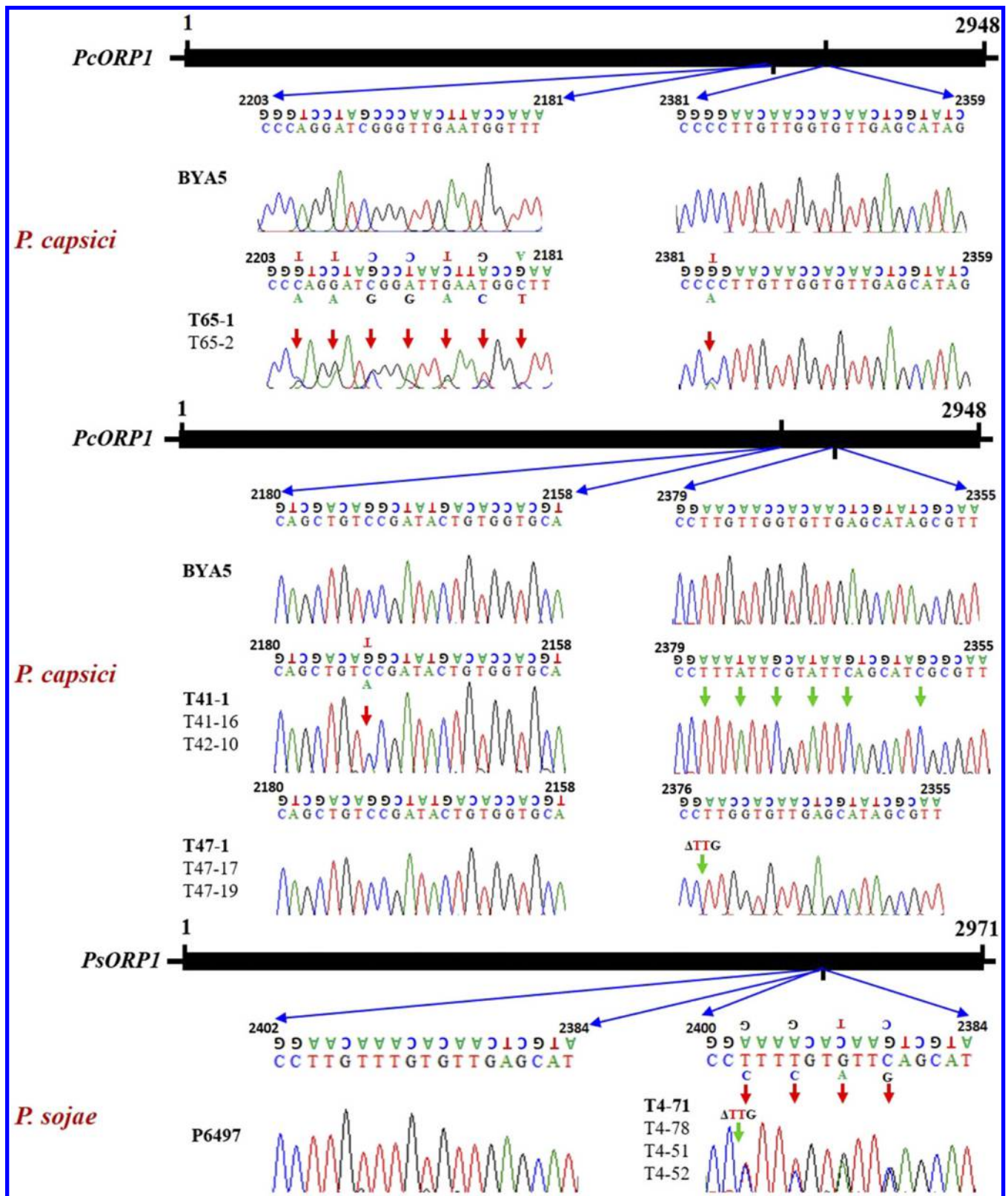

Fig. 2. Sanger sequencing traces of mutation regions of transformants. Mutations are marked using short dark arrows (heterozygous mutation) or short light arrows (homozygous mutation). Long arrows show the locations of the mutation sites in the full-length *Phytophthora capsici* or *P. sojae* oxysterol binding protein-related protein 1 genes (*PcORP1* or *PsORP1*, respectively). Upper sequences represent the coding strand and the lower sequence is the complement. Numbers indicate the nucleotide positions within the gene of the sequences shown.

contrast, sporangia production, zoospore production, cyst germination, and pathogenicity in T41-1, T41-16 and T42-10 that contain the heterozygous mutation G770V were similar to that of the parental isolate BYA5, except for T41-1, which produced more sporangia than BYA5 (Table 2). T47-1, T47-17, and T47-19, carrying the homozygous mutation ΔN837, showed normal cyst germination but somewhat lower sporangia and zoospore production and pathogenicity (Table 2) relative to the wild-type parent.

Phenotypes conferred by the N837 deletion in *P. sojae*. Four independent oxathiapiprolin-resistant colonies (T4-71, T4-78, T4-51, and T4-52) with the ΔN837 in PcORP1 were obtained using the CRISPR/Cas9 system and PYF515-sg2381 (sg2381: GCCA TGCTCAACACAAACAA) and pBS-HDR3 (Fig. 1) in *P. sojae* P6497. Interestingly, all four colonies were homozygous for ΔN837, even though the synonymous mutations in the sgRNA target sequence were heterozygous (Fig. 2). One ΔN837 mutation might have been produced by NHEJ and one by HDR. All four strains showed high oxathiapiprolin resistance (Fig. 3B): they grew very well on V8 plates containing oxathiapiprolin concentrations up to 0.5 µg/ml (Fig. 3B), when the wild-type isolate P6497 could not grow on V8 plates containing oxathiapiprolin at 0.005 µg/ml.

Compared with the wild-type P6497, all four *P. sojae* transformants containing the ΔN837 mutation in PsORP1 showed normal cyst germination and a somewhat lower mycelial growth rate.

Sporangia and zoospore production were reduced slightly in T4-71 but were dramatically reduced in T4-78, T4-51, and T4-52. All four strains showed greatly reduced pathogenicity on soybean (Table 3).

Gene expression level analysis. Expression of *PcORP1* was lower in all of the *P. capsici* transformants than in BYA5 (Fig. 4A). In addition, the expression of *PcORP1* differed between these eight transformants. Expression was nearly normal in T65-2 and T47-16 but very low in T65-1 and T47-1. Among the *P. sojae* transformants, expression of *PsORP1* in T4-71 and T4-51 did not differ from the wild-type P6497. However, the expression of *PsORP1* appeared higher in T4-78 and lower in T4-52 (Fig. 4B).

DISCUSSION

Oxathiapiprolin has been used in China for approximately 2 years, and no resistant isolates of any oomycete pathogens have yet been reported in the field. However, resistant mutants could readily be obtained in the lab, and mutations G770V and G839W in the target in PcORP1 have been found in *P. capsici* oxathiapiprolin-resistant mutants (Miao et al. 2016). Therefore, validating the genetic basis for resistance can provide the ground knowledge needed for rapid resistance monitoring and establishment of a resistance management strategy.
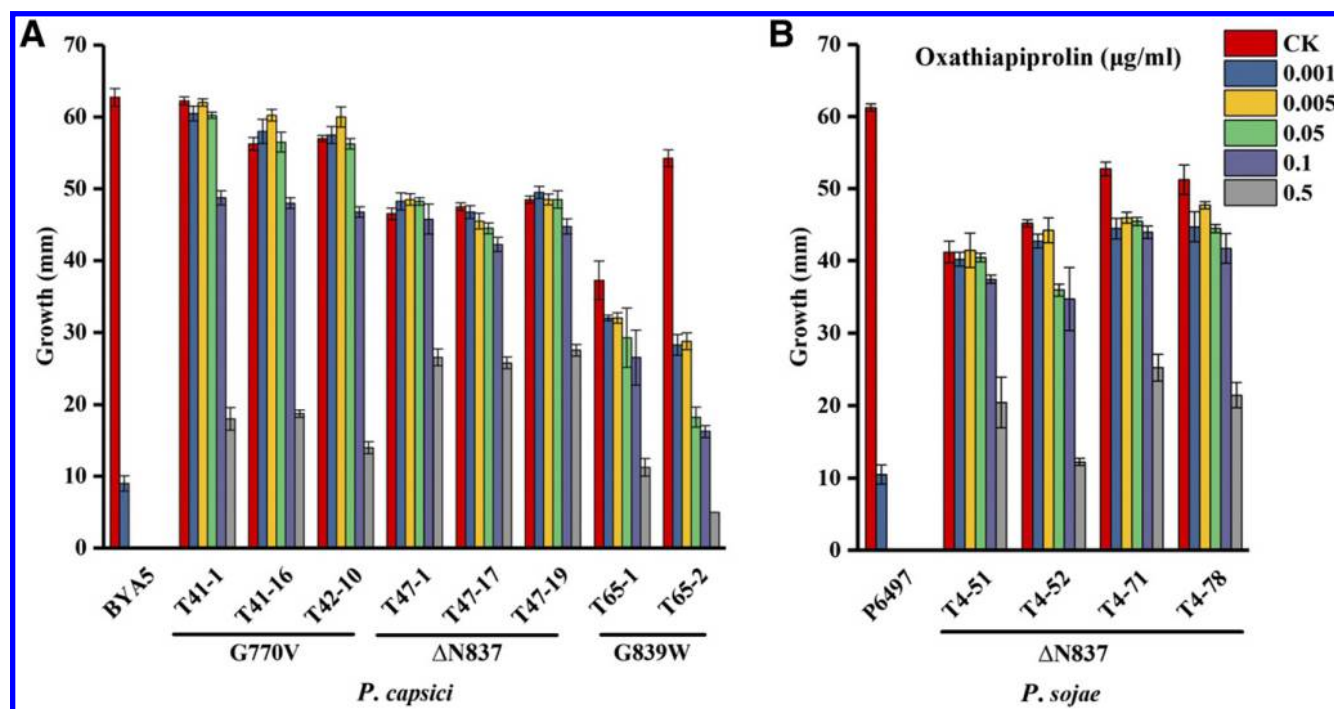

Fig. 3. Oxathiapiprolin sensitivity of CRISPR/Cas9 transformants (CK = control) for **A,** *Phytophthora capsici* and **B,** *P. sojae*.

TABLE 2. Characteristics of *Phytophthora capsici* transformants and their parental isolates[y]

Isolate	Gene type	Mycelia growth (mm)	Sporangia (n/field of view)[z]	Zoospores (10^4/ml)	Cyst germination (%)	Lesion area (cm^2)
BYA5	Wild type	68 ± 2 a	148 ± 42 bcd	97 ± 21 ab	89 ± 4 a	3.4 ± 0.5 ab
T65-1	Heterozygous G839W	41 ± 4 f	0 ± 0 f	0 ± 0 e	…	2.1 ± 1.4 abc
T65-2	Heterozygous G839W	59 ± 2 d	0 ± 0 f	0 ± 0 e	…	2.6 ± 0.4 abc
T41-1	Heterozygous G770V	65 ± 2 ab	193 ± 28 a	101 ± 18 ab	90 ± 2 a	3.9 ± 0.1 a
T41-16	Heterozygous G770V	61 ± 2 cd	150 ± 30 bc	90 ± 14 bc	89 ± 6 a	2.4 ± 1.8 abc
T42-10	Heterozygous G770V	63 ± 2 bc	163 ± 40 ab	105 ± 9 ab	85 ± 3 a	2.8 ± 0.7 abc
T47-1	Homozygous ΔN837	51 ± 1 e	113 ± 16 de	75 ± 7 cd	90 ± 3 a	1.8 ± 0.9 bc
T47-17	Homozygous ΔN837	53 ± 1 e	106 ± 13 e	69 ± 9 d	86 ± 4 a	1.2 ± 0.7 c
T47-19	Homozygous ΔN837	53 ± 2 e	126 ± 13 cde	111 ± 3 a	84 ± 8 a	1.6 ± 1.9 bc

[y] Data are mean ± standard deviation. Means within a column followed with the same letter are not significantly different by Fisher's protected least significant difference test ($P < 0.05$).

[z] Sporangia were counted using light microscope (magnification ×40). The number (*n*) was the mean of 30 fields of view.

Here, we successfully used the CRISPR/Cas9 system to obtain transformants heterozygous for the PcORP1 point mutation G839W and transformants heterozygous for the PcORP1point mutation G770V. This technology avoids the potentially undesirable effects of overexpression of target genes, frequent in the more traditional transformation methods. In addition, the point mutation G839W in PcORP1 was verified using traditional ectopic overexpression transformation. All five transformants showed high oxathiapiprolin resistance, confirming that G770V or G839W in PcORP1 do confer oxathiapiprolin resistance in *P. capsici*.

Because he CRISPR/Cas9 system can directly target a gene of interest for editing (Hsu et al. 2014), it usually generates transformants heterozygous at the edited locus. It is interesting that both the adaptation mutants and the CRISPR/Cas9 transformants were indeed heterozygotes for the G839W and G770V point mutations.

For *P. capsici* transformants T65-1 and T65-2, one allele was fully wild type and the other one was replaced via HDR, as expected. For T41-1, T41-16, and T42-10, the two alleles actually were replaced via HDR but the nonsynonymous point mutation was heterozygous. This might mean that only part of the donor template was used during HDR, because the distance from the sg2180 site and T2379 is approximately 200 bp. Paquet et al. (2016) also reported that homozygous mutations introduced using CRISPR/Cas9 require a guide RNA targeting close to the intended mutation. Therefore, when the CRISPR/Cas9 system is used to directly introduce a point mutation, the interval between the targeted mutation point and the guide RNA should also be considered during design of the constructs, especially for gene editing of recessive alleles such as CESA3 in oomycetes (Gisi et al. 2007). In T4-71, T4-78, T4-51, and T4-52 (which were all independent *P. sojae* transformants), the 3-bp

TABLE 3. Characteristics of *Phytophthora sojae* transformants and their parental isolates[y]

Isolate	Gene type	Mycelia growth (mm)	Sporangia (*n*/field of view)[z]	Zoospores (10^4/ml)	Cyst germination (%)	Lesion area (cm^2)
P6497	Wild type	66 ± 1 a	26 ± 3 a	7.3 ± 1.7 a	97 ± 2 a	6.5 ± 2.5 a
T4-71	Homozygous ΔN837	57 ± 3 b	17 ± 6 b	5.8 ± 5.8 a	94 ± 1 a	0.15 ± 0.14 b
T4-78	Homozygous ΔN837	56 ± 2 b	4.3 ± 4 c	0.06 ± 0.03 b	95 ± 3 a	0.24 ± 0.09 b
T4-51	Homozygous ΔN837	46 ± 2 d	2.3 ± 2 c	0.14 ± 0.01 b	96 ± 2 a	0.13 ± 0.12 b
T4-52	Homozygous ΔN837	50 ± 1 c	4.0 ± 4 c	0.06 ± 0.02 b	94 ± 4 a	0.13 ± 0.02 b

[y] Data are mean ± standard deviation. Means within a column followed with the same letter are not significantly different by Fisher's protected least significant difference test ($P < 0.05$).

[z] Sporangia were counted using light microscope (magnification ×40). The number (*n*) was the mean of 30 fields of view.
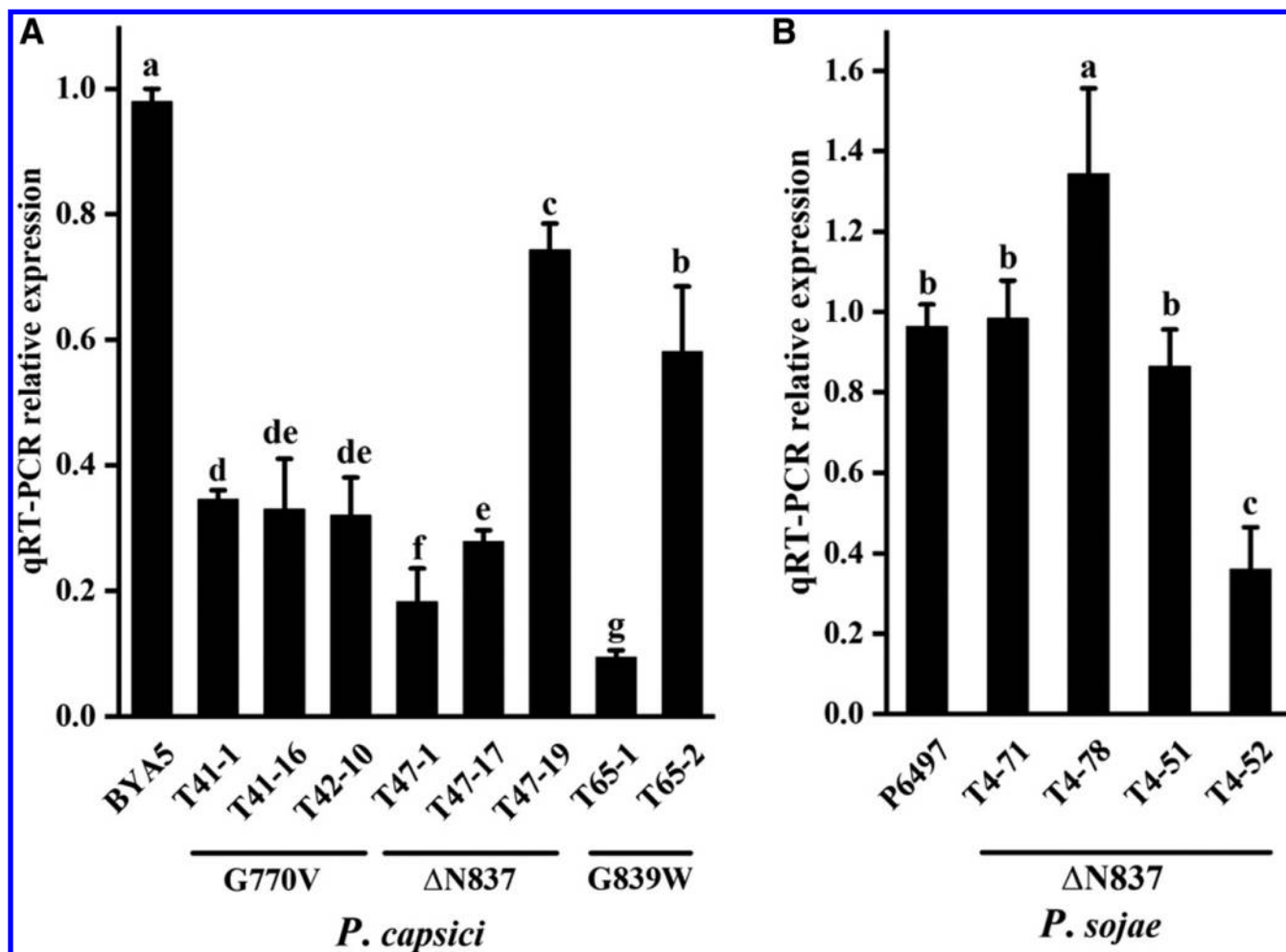

Fig. 4. Transcript levels of **A,** *Phytophthora capsici* or **B,** *P. sojae* oxysterol binding protein-related protein 1 genes (*PcORP1* or *PsORP1*, respectively) in transformants. Columns and bars indicate means ± standard deviation. Columns marked with the same letter are not significantly different by Fisher's protected least significant difference test ($P < 0.05$).

deletion was homozygous but the sg2381 target sequence emerged as heterozygous. We speculate that, after the sg2381-guided Cas9 cut the genome, one allele was repaired via NHEJ and the other one was repaired via HDR. If so, this would mean that the ΔN837 mutation was produced seven times independently by NHEJ in the *P. capsici* and *P. sojae* transformants. We speculate that the tandem duplication of the codon CAA adjacent to the Cas9 cleavage site predisposed one of those codons to be lost during NHEJ.

The observation that the CRISPR/Cas9 transformants at G839W and G770V were all heterozygous, even when the synonymous mutations in the sgRNA target sequence had become homozygous, suggests that G839W and G770V might have severe fitness costs, or may be lethal, when they are homozygous. In a previous study (Miao et al. 2016), heterozygous G839W mutants in PcORP1 displayed high levels of fitness, and they could produce similar numbers of sporangia and zoospores compared with their parental isolate LP3. However, T65-1 and T65-2 with G839W in PcORP1, obtained independently from the wild-type isolate BYA5, could not produce sporangia. We speculate that the CRISPR/Cas9 treatment, or the transformation itself, might have caused some off-target deleterious mutations in these two lines. In a controversial study, Schaefer et al. (2017) suggested that CRISPR-Cas9 could induce more than 1,000 unexpected mutations in mice. However, this result has been questioned (Wilson et al. 2017), and deep sequencing of CRISPR/Cas9-modified *Arabidopsis* genomes did not detect any off-target mutations (Feng et al. 2014). An alternative hypothesis is that the original G839W mutants of *P. capsici* may have benefited from polymorphisms in their parent strain LP3 that compensated for the negative effects of G839W on sporangiogenesis, or that compensating mutations may have arisen during the fungicide adaptation process. More independent transformants carrying G839W are needed to resolve whether and, if yes, how this mutation truly interferes with sporangiogenesis.

Preliminary studies also had indicated that six independent *P. capsici* heterozygous mutants carrying G770V in PcORP1 had lower fitness and produced much less sporangia compared with their parental isolate HNJZ10. However, here, transformants T41-1, T41-16, and T42-10, all heterozygous at the G770V locus, showed similar fitness compared with parental isolate BYA5. The different effects of G770V in the two studies could possibly be related to differences in the genetic backgrounds of the parent isolates, HNJZ10 and BYA5. Nonetheless, our results indicate that the point mutation G770V in PcORP1 also has survival potential in the field. In future, this type of mutation should also be monitored.

Our results have shown for the first time that the mutation ΔN837 in PcORP1 or PsORP1 could confer oxathiapiprolin resistance in *P. capsici* or *P. sojae,* respectively. Andreassi et al. (2013) previously observed that amino-acid substitutions (I, F, or Y) at N837 could confer oxathiapiprolin resistance in *P. capsici* but deletions were not previously observed. Amino-acid substitutions are the most common resistance mechanism against fungicides with a single site of action (Ma and Michailides 2005). However, amino-acid deletions in target proteins also can cause fungicide resistance in plant pathogens by altering the molecular target of the active ingredient. For example, ΔY459/G460 was found in Cyp51 from *Mycosphaerella graminicola* field isolates resistant to sterol synthesis inhibitors (Leroux and Walker 2011), and the mutation ΔL497 in the transcription factor mrr1 was detected in all *Botrytis cinerea* multidrug-resistant isolates analyzed by Leroch et al. (2012). Bittner et al. (2017) also detected the deletion ΔG818/F819 in PnORP1 in *P. nicotianae* oxathiapiprolin-resistant mutants produced by UV mutagenesis, although that mutation was not verified to cause resistance. We speculate that ΔN837 in PcORP1 or PsORP1 causes a structural change in the ORD domain of these two proteins. This change may reduce the binding affinity of oxathiapiprolin to its target protein. The fitness results for *P. capsici* CRISPR/Cas9 transformants T47-1, T47-17, and T47-19 and *P. sojae* CRISPR/Cas9

transformants T4-71, T4-78, T4-51, and T4-52 suggested that the mutants with ΔN837 may have survival potential in the field. Therefore, the ΔN837 oxathiapiprolin resistance mutation should also be monitored in the future.

In conclusion, our results using CRISPR/Cas9 confirmed that the point mutations G770V and G839W in PcORP1 do confer resistance to oxathiapiprolin in *P. capsici*. Notably, we also found a new point mutation, ΔN837, in PcORP1 that could produce oxathiapiprolin resistance in *P. capsici* and *P. sojae*. Mutants with any of these three point mutations may have survival potential in the field. This information will increase our understanding of oxathiapiprolin resistance in plant-pathogenic oomycetes and should be useful for the development of new fungicides.

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