Research Article

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Multiple point mutations in *PsORP1* gene conferring different resistance levels to oxathiapiprolin confirmed using CRISPR–Cas9 in *Phytophthora sojae*

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Abstract

BACKGROUND: Oxathiapiprolin is among the first commercial oxysterol-binding protein inhibitors (OSBPIs) developed by DuPont Corporation and shows excellent activity against plant-pathogenic oomycetes. Although more than 21 target site mutations have been identified in insensitive oomycetes, only G770V, G839W, and ΔN837 have been verified to confer oxathiapiprolin resistance in *Phytophthora capsici* or *P. sojae*. The effect of other mutations on OSBPIs sensitivity requires urgent investigation.

RESULTS: *P. sojae* transformants containing 16 mutations of *PsORP1* were recovered using the CRISPR–Cas9 system. Transformants containing L733W, S768F, S768Y, N837Y, N837F, P861H, L863W, or I877Y showed high oxathiapiprolin resistance, with resistant factors (RFs) > 3000. Point mutations S768K, S768I, G770L, G770P, G770A, ΔG818/F819, N837I, and I877F exhibited low resistance, with RFs < 80. Phenotype assays revealed that the most highly resistant transformants showed enhanced or similar pathogenicity, oospore production, and cyst gemination. However, most transformants displayed decreased sporangia and zoospore production compared with parental wild-type P6497.

CONCLUSION: This study demonstrated that L733W, S768F, S768Y, N837Y, N837F, P861H, L863W, and I877Y in PsORP1 confer high oxathiapiprolin resistance in *P. sojae*.

Supporting information may be found in the online version of this article.

Keywords: oxathiapiprolin; fungicide resistance; point mutation; CRISPR–Cas9; PsORP1; *Phytophthora sojae*

1 INTRODUCTION

Oomycetes are fungal-like eukaryotes that belong to the kingdom Chromista and are phylogenetically grouped with diatoms and brown algae.[1,2] They are among the most problematic groups of disease-causing microorganisms in both agriculture and aquaculture, and represent an important threat to food security.[3] The most prominent representative plant-pathogenic oomycetes are *Phytophthora* spp., *Pythium* spp., and *Peronospora* spp.[4] In order to avoid yield loss, plant oomycete disease control is required. Control measures consist mainly of chemical products; the most common being phenylamides, quinone outside inhibitors, carboxylic acid amides, and multisite inhibitors.[5] However, many oomycete pathogen species are now resistant to most single-site inhibitors.[5–8] The development of new oomycete fungicides with novel modes of action is therefore urgently required.

Oxathiapiprolin, developed by DuPont Corporation,[9] is the first member of a new class of piperidinyl thiazole isoxazoline fungicide that is effective against plant oomycete pathogens. It acts via inhibition of a novel molecular target—an oxysterol-binding protein (OSBP) homolog (FRAC classification F9). It exhibits excellent preventative, curative, and residual efficacy against oomycete diseases.[10–13] Oxathiapiprolin is authorized as a foliar or soil treatment at planting and for delivery via drip irrigation in North America and several countries in the Pacific, Asia, and Central and South America; authorizations are pending in Europe (Lunn D, http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/Evaluation2016/OXATHIAPIPROLIN.pdf). In China, oxathiapiprolin was registered to control plant oomycete diseases in 2015.

Because of oxathiapiprolin's excellent efficacy and novel mode of action, a Fungicide Resistance Action Committee (FRAC) OSBPI Working Group was established in 2015 to generate common resistance management recommendations for it and other OSBP inhibitors. Although oxathiapiprolin had been used for just

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3 years, field- and laboratory-resistant isolates have been detected in *Plasmopara viticola* and *Phytophthora infestans*, *P. capsici*, *P. parasitica*, and *Pseudoperonospora cubensis*[14–16] (https://www.frac.info/). In these oxathiapiprolin-insensitive isolates, 21 mutations were detected at 11 *P. infestans* homolog positions, namely, L733W, S768I/F/K/Y, G770A/I/P/V/L, ΔG818/F819, N837I/F/Y, ΔN837, G839W, P861H, L863W/F, and I877F/Y.[14–16] However, only G770V, G839W, and ΔN837 in *P. capsici* or *P. sojae* have been investigated, and shown to confer high oxathiapiprolin resistance (resistant factor > 1000) using the CRISPR–Cas9 system.[16] The impact of the other 18 mutations on fungicide sensitivity and pathogen fitness has not been reported.

Owing to the mature CRISPR–Cas9 system for *P. sojae*,[17] the current study aimed to investigate whether the reported point mutations in *PsORP1* could confer oxathiapiprolin resistance.

2 MATERIALS AND METHODS

2.1 Fungicides

Technical-grade oxathiapiprolin (96.7% active ingredient), provided by DuPont Corporation (Wilmington, DE, USA), was dissolved in dimethyl sulfoxide (DMSO) to produce a stock solution with a 10 mg mL^{-1} concentration of the active ingredient, which was then stored in the dark at 4 °C until required.

2.2 *P. sojae* strain and growth conditions

The reference *P. sojae* isolate P6497 and all transformants used in this study were grown routinely on a 10% V8 medium at 25 °C in the dark. The sensitivity of *P. sojae* isolates to oxathiapiprolin was determined using the mycelia growth assay described in our previous study.[18] Mycelial plugs (5 mm) were taken from the colony margin and transferred to fresh V8 agar amended with various concentrations of oxathiapiprolin (see Table S1). The final concentration of DMSO in the medium was 0.1%, and plates containing only 0.1% DMSO were used as the negative control. The diameters of the oxathiapiprolin cultures were measured when the colonies on the corresponding control plates had covered 75% of the agar surface. Percentage radial growth inhibition and median effective concentration (EC_{50}) for each strain were calculated using a formula described previously.[18] Each treatment consisted of three replicate plates.

2.3 Plasmid construction

The CRISPR–Cas9 system in *P. sojae* was used to investigate whether L733W, S768I/F/K/Y, G770A/I/P/L, ΔG818/ΔF819, N837I/F/Y, P861H, L863W/F, and I877F/Y in PsORP1 confer resistance to oxathiapiprolin. Two single-guide RNA (sgRNA) sequences (sgRNA2009-ACACGCTCCAAGTTCGTACG and sgRNA1542-GGCAACTTACATTACCGACC) were cloned separately into the all-in-one plasmid PYF515 according to the protocol used by Fang *et al.*[17] A 2262 bp fragment (1185 to downstream 476 of *PsORP1*) was amplified and infused into pBS-SK^+ using the primers listed in Table S2 and the In-Fusion HD Cloning Kit (Clontech, Mountain View, CA, USA). To prevent the sgRNA-guided Cas9 from cutting the donor plasmid, the sgRNA2009 and sgRNA1542 binding residues in the donor plasmid were mutated without changing the encoded amino acids using the primers listed in Table S2, producing the basic donor plasmid (Fig. 1). Next, the corresponding nucleotide, as shown in Table 1, was also mutated using the primers listed in Table S2 and the In-Fusion HD Cloning Kit (Clontech), respectively. All DNA fragments were amplified using TransStart® FastPfu DNA Polymerase (TransGen Biotech, Beijing, China).

2.4 Transformation of *P. sojae*

Polyethylene glycol-mediated protoplast transformation was conducted using the previously described protocol.[17] PYF515–sg1542 and PYF515–sg2009 were co-transformed with the corresponding donor plasmid. Transformants were transferred to a V8 agar medium containing 50 μg mL^{-1} G418 and incubated for 3 to 5 days at 25 °C. Next, the viable transformants were transferred to a V8 agar medium containing 0.005 μg mL^{-1} oxathiapiprolin. The hyphae of viable transformants on V8 amended with oxathiapiprolin were then collected for genomic DNA (gDNA) extraction. For verification of the transformants, the amplification products of the full-length *PsORP1* genes in all transformants were sequenced. For each target non-synonymous mutation type, one or two transformants were selected for fungicide sensitivity and phenotype analyses.

2.5 Nucleic acid extraction

Total gDNA was extracted according to a previous study,[19] with some modifications. The mycelium sample was ground after being frozen in liquid nitrogen. Then 650 μL of the 3% CTAB extraction buffer (3% CTAB, 2.8 M NaCl, 0.1 M Tris–HCl pH 8.0, 0.02 M EDTA) was added, and the mix was blended gently for 10 min and incubated at 65 °C for 60 min. Next, 650 μL of hydroxybenzene/chloroform/isoamyl alcohol (25:24:1) was added to the tubes and mixed gently for 10 min. After centrifugation (11 750 g for 10 min), 600 μL of the supernatant was transferred to a new 1.5-mL tube following the addition of 650 μL of chloroform/isoamyl alcohol (24:1) and centrifuged at 11 750 g for 10 min. The supernatant was transferred to a fresh tube with 360 μL of cold isopropanol (−20 °C) and centrifuged (11 750 g for 10 min) after being mixed gently via inversion. The liquid solution was then released, and the DNA pellet was washed with 800 μL of 70% ethanol. The pellet was dried for 1 h in a vacuum drier at 45 °C, and then resuspended in 100 μL H_2O.

For RNA extraction, mycelium was harvested from *P. sojae* strains grown on a V8 liquid medium for 2 days and frozen at −80 °C until required. Total RNA was extracted from the frozen samples using the SV Total RNA Isolation kit (Promega Corp., Madison, WI, USA), and cDNA was synthesized using the FastKing RT kit (with gDNase; Tiangen Biotech, Beijing, China), with each procedure being carried out in accordance with the manufacturer's protocol.

2.6 Phenotype analysis of *P. sojae*

Mycelial growth was determined after transferring a mycelial plug (5 mm in diameter) onto a 10% V8 agar medium and incubating the plates for 7 days in the dark at 13, 18, 25, 30, or 37 °C, respectively. Sporangium production, zoospore production, and cyst gemination of *P. sojae* isolates were assessed according to our previous study.[16] Experiments were repeated three times.

To analyze oospore production in *P. sojae*, all strains were cultured on a 10% V8 agar medium for 10 days at 25 °C in the dark. The total number of oospores formed in nine fields of view (magnification ×40) was recorded. Each experiment used three replica plates, and the experiment was repeated three times.

The first true leaves of 10-day-old cultivar Kenfeng 22 soybean seedlings were used to evaluate the pathogenicity of *P. sojae*. Leaves were placed in Petri dishes containing wet tissue to prevent dehydration, and ~ 20 μL of zoospore suspension (10^5 zoospores mL^{-1}) was placed in the middle of each soybean leaf. After inoculation, the leaves were incubated at room temperature for 4 days before lesion areas were measured using Image J 1.x.[20]

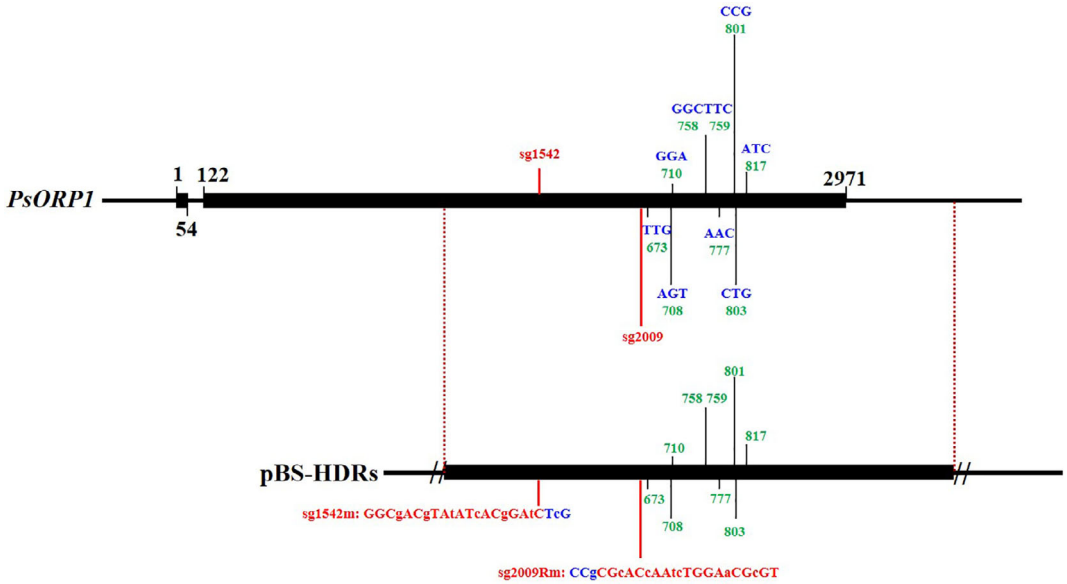

Figure 1. Schematic of homologous donor DNA plasmids. Homology arms (1185 to downstream 476 of *PsORP1*) flanking the target mutation sites were used. The sgRNA2009 and sgRNA1542 binding residues in the donor plasmid were mutated without changing the encoded amino acids and the new sequence was shown in red text. All target mutation codons are shown in blue text and codon positions are marked in green.

Table 1. Point mutation type and resistant factor of *Phytophthora sojae* transformants

Isolate	Point mutation type				Ho/He[b]	EC_{50} (μg mL^{-1})	RF[a]
	Amino acid position in PsORP1	Amino acid position in PiORP1	Codon change in *PsORP1*				
			Before[c]	After[d]			
P6497	—	—	—		—	5.0×10^{-4}	-
T673-W1	L673W	L733W	TTG	TGG	He	>2	>4000
T673-W2	L673W	L733W	TTG	TGG	Ho	>2	>4000
T708-K3	S708K	S768K	AGT	AAG	He	1.4×10^{-3}	3
T708-K5	S708K	S768K	AGT	AAG	Ho	4.2×10^{-3}	8
T708-F1	S708F	S768F	AGT	TTC	He	>2	>4000
T708-I2	S708I	S768I	AGT	ATT	He	6.9×10^{-3}	14
T708-Y1	S708Y	S768Y	AGT	TAC	He	>2	>4000
T710-L3	G710L	G770L	GGA	CTG	He	3.6×10^{-2}	72
T710-P2	G710P	G770P	GGA	CCG	He	2.6×10^{-3}	5
T710-P3	G710P	G770P	GGA	CCG	Ho	2.2×10^{-2}	44
T710-A3	G710A	G770A	GGA	GCA	He	1.9×10^{-3}	4
T758/759–10	ΔG758/F759	ΔG818/F819	GGCTTC	—	Ho	3.3×10^{-2}	66
T777-I2	N777I	N837I	AAC	ATC	He	8.9×10^{-3}	18
T777-Y2	N777Y	N837Y	AAC	TAC	Ho	>2	>4000
T777-F1	N777F	N837F	AAC	TTT	He	>2	>4000
T801-H1	P801H	P861H	CCG	CAC	Ho	1.9	3800
T803-W1	L803W	L863W	CTG	TGG	He	2.0	4000
T803-W6	L803W	L863W	CTG	TGG	Ho	>2	>4000
T817-F5	I817F	I877F	ATC	TTC	He	2.8×10^{-2}	56
T817-Y7	I817Y	I877Y	ATC	TAT	He	>2	>4000

[a] RF, resistance factor; ratio of EC_{50} of transformants relative to the EC_{50} of the parental isolate P6497.
[b] Ho/He, homozygous or heterozygous in the target non-synonymous point mutation position.
[c] 'Before' indicates the corresponding codon of wild-type *PsORP1*.
[d] 'After' indicates the corresponding non-synonymous point mutation introduced from pBS-HDRs.

Table 2. Mycelial growth of *Phytophthora sojae* transformants and their parental isolate P6497 on V8 agar at various temperatures

	Colony diameter (mm) [a]				
Isolate	13 °C	18 °C	25 °C	30 °C	37 °C
P6497	20.3 ef	32.2 a–d	43.8 hi	38.8 h	6.0 a
T673-W1	21.7 cd	31.0 de	47.8 d–f	49.0 ab	6.0 a
T673-W2	21.2 c–e	30.2 e–g	50.7 b	43.7 f	6.0 a
T708-K3	21.8 cd	31.3 b–e	39.7 j	35.7 i	6.0 a
T708-K5	24.3 ab	32.1 b–d	43.8 hi	41.3 g	6.0 a
T708-F1	21.7 cd	29.0 g	50.2 bc	44.7 d–f	6.0 a
T708-I2	23.7 b	31.5 b–e	46.8 e–g	46.0 cd	6.0 a
T708-Y1	23.8 b	31.2 c–e	48.3 c–e	45.3 de	6.0 a
T710-L3	20.0 fg	30.7 d–f	47.0 e–g	43.8 ef	6.0 a
T710-P2	25.3 a	32.7 a–c	45.8 f–h	41.2 g	6.0 a
T710-P3	22.2 c	30.0 e–g	47.7 d–f	43.7 f	6.0 a
T710-A3	25.2 a	33.7 a	49.5 b–d	49.3 a	6.0 a
T758/759–10	20.8 d–f	31.0 de	53.2 a	49.3 a	6.0 a
T777-I2	21.8 cd	32.7 a–c	48.5 c–e	47.3 c	6.0 a
T777-Y2	20.0 fg	31.2 c–e	48.3 c–e	45.3 de	6.0 a
T777-F1	24.3 ab	31.0 de	48.0 de	47.5 bc	6.0 a
T801-H1	15.2 i	23.3 i	42.5 i	36.3 i	6.0 a
T803-W1	22.2 c	32.8 ab	47.3 ef	40.0 gh	6.0 a
T803-W6	19.2 g	27.0 h	36.7 k	36.3 i	6.0 a
T817-F5	20.8 d–f	31.2 c–e	51.3 ab	40.5 g	6.0 a
T817-Y7	17.3 h	29.2 fg	45.2 gh	41.2 g	6.0 a

[a] Colony diameters were measured 5 days post inoculation. Values followed by the same letter with in a column do not differ significantly ($P < 0.05$).

Table 3. Fitness of *Phytophthora sojae* transformants compared with wild-type parental isolate P6497[a]

Isolate	Sporangia[b] (no./field of view)	Zoospores (10^4 mL^{-1})	Cyst germination (%)	Oospores[b] (no./field of view)	Lesion area (cm^2)
P6497	27 ± 3 b	15 ± 7 a	92 ± 4 a	20 ± 3 a–d	2.4 ± 0.4 e–g
T673-W1	19 ± 13 b–e	5 ± 4 de	90 ± 5 ab	18 ± 4 a–d	3.2 ± 0.8 b
T673-W2	14 ± 3 c–e	9 ± 7 a–e	84 ± 6 b–d	18 ± 6 b–d	3.2 ± 0.8 ab
T708-K3	15 ± 4 c–e	7 ± 5 c–e	78 ± 18 de	18 ± 4 b–d	2.1 ± 1.1 fg
T708-K5	16 ± 5 c–e	11 ± 7 a–d	84 ± 13 b–d	18 ± 2 b–d	3.1 ± 0.7 bc
T708-F1	23 ± 8 b–d	12 ± 6 a–c	93 ± 2 a	24 ± 5 a	3.0 ± 0.7 b–d
T708-I2	14 ± 2 de	7 ± 6 c–e	80 ± 5 de	18 ± 4 b–d	3.2 ± 1.1 b
T708-Y1	25 ± 8 bc	15 ± 4 ab	93 ± 4 a	19 ± 3 a–d	3.9 ± 0.6 a
T710-L3	21 ± 12 b–e	9 ± 4 a–e	92 ± 2 a	24 ± 6 a–c	2.8 ± 0.6 b–f
T710-P2	14 ± 13 c–e	8 ± 7 c–e	66 ± 10 f	18 ± 8 b–d	2.8 ± 0.8 b–e
T710-P3	15 ± 6 c–e	5 ± 3 de	77 ± 14 de	19 ± 4 a–d	2.8 ± 0.5 b–e
T710-A3	15 ± 9 c–e	7 ± 7 c–e	94 ± 2 a	15 ± 1 d	3.0 ± 0.2 b–e
T758/759–10	14 ± 2 c–e	7 ± 3 c–e	92 ± 1 a	17 ± 2 cd	2.9 ± 0.6 b–e
T777-I2	15 ± 4 c–e	5 ± 4 de	89 ± 4 a–c	18 ± 3 b–d	2.6 ± 0.6 b–f
T777-Y2	15 ± 4 c–e	4 ± 1 de	93 ± 4 a	18 ± 6 a–d	1.9 ± 0.7 gh
T777-F1	13 ± 1 de	4 ± 2 de	94 ± 2 a	22 ± 1 a–c	2.4 ± 0.5 d–g
T801-H1	16 ± 9 c–e	5 ± 3 de	79 ± 13 de	14 ± 1 d	1.2 ± 0.6 i
T803-W1	16 ± 7 c–e	8 ± 6 b–e	74 ± 5 e	21 ± 5 a–d	2.6 ± 0.6 c–f
T803-W6	38 ± 14 a	9 ± 5 a–d	82 ± 12 cd	24 ± 2 ab	1.4 ± 0.7 hi
T817-F5	12 ± 7 de	6 ± 1 c–e	79 ± 9 de	14 ± 2 d	3.0 ± 0.5 b–d
T817-Y7	10 ± 5 e	2 ± 2 e	88 ± 2 a–c	19 ± 3 a–d	2.6 ± 1.0 b–f

[a] Mean ± standard deviation. Means within a column followed with the same letter are not significantly different by Fisher's protected LSD test ($P < 0.05$).
[b] Sporangia and oospores were counted using light microscope (magnification × 40). The number was the mean of 30 fields of view.

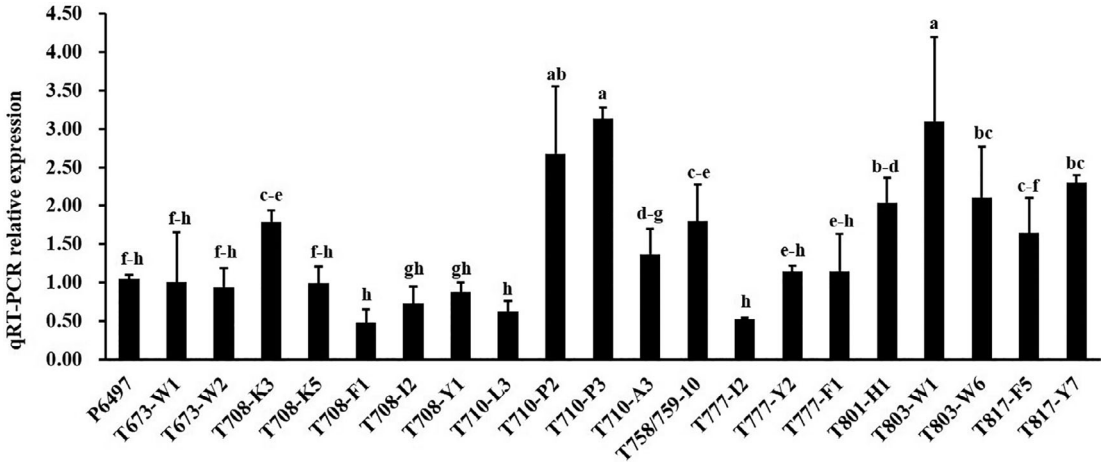

Figure 2. Transcript levels of *PsORP1* in *Phytophthora sojae* transformants. Columns and bars indicate means ± standard deviation. Columns marked with the same letter are not significantly different by Fisher's protected least significant difference test ($P < 0.05$).

Eight leaves were used in each experiment and this experiment was repeated three times.

2.7 Quantitative real-time polymerase chain reaction analysis of transcript levels

Quantitative real-time polymerase chain reaction (PCR) analysis of the transcript levels was performed using the CFX Connect™ Real-Time system (Bio-Rad, Hercules, CA, USA) and SuperReal PreMix Color (SYBR Green; Tiangen Biotech) with the primers listed in Table S2 and following the manufacturers' protocol. The relative quantities of PCR products were calculated using the $2^{-\Delta\Delta Ct}$ method, and the *PsActin* gene was used as a reference[21] to normalize quantification of the *PsORP1* transcript levels. The entire experiment was conducted twice, and each experiment included three replicates for each treatment.

3 RESULTS

3.1 CRISPR–Cas9 system for point mutations in *P. sojae* and validation of transformants

For G770I and L863F, no positive transformant was recovered in this study. However, for the other point mutations, at least one independent transformant was recovered. According to the sequencing results, the positive transformants could be classified into five types, depending on whether they were homozygous or heterozygous for the synonymous mutations introduced at the two sgRNA sites and whether they were homozygous or heterozygous at the site of the intended amino acid substitution (summarized in Table 1 and detailed in Fig. S1). Five transformants were homozygous at all sites, T673W-2, T708K-5, T710P-3, T777Y-1, and T801H-1. The remaining transformants were all heterozygous at the site of the intended amino acid substitution, but contained various combinations of homozygous or heterozygous states at the two sgRNA sites. T708-I2 was unusual in that one allele at the sg2009 site was a mixture of wild-type and mutant nucleotides.

3.2 Phenotype of *P. sojae* transformants

3.2.1 Oxathiapiprolin sensitivity

The resistant factors (RFs) of T673-W1, T673-W2, T708-F1, T708-Y1, T777-Y2, T777-F1, T801-H1, T803-W1, T803-W6, and T817-Y7 were all > 3000. The RFs of T708-I2, T710-L3, T710-P3, T758/759–10, T777-I2, and T817-F5 ranged from 14 to 72 (Table 1). However, T708-K3, T708-K5, T710-P2, and T710-A3 showed low resistance to oxathiapiprolin, and all had RFs < 10 (Table 1).

3.2.2 Effect of temperature on mycelial growth

The optimal temperature for mycelial growth was 25 °C for all *P. sojae* isolates tested (Table 2). Interestingly, the transformants grew at the same rate or faster than the wild-type isolate P6497 at 13, 18, 25, and 30 °C (with the exceptions of T801-H1 and T817-Y7 at 13 °C; T673-W2, T708-F1, T710-P3, T801-H1, T803-W6, and T817-Y7 at 18 °C; T708-K3 and T803-W6 at 25 °C; and T708-K3, T801-H1, and T803-W6 at 30 °C). At 37 °C, all *P. sojae* isolates tested could not grow.

3.2.3 Sporangium, zoospore, and oospore production and cystospore germination

The sporangium production of transformants was similar to or less than that of P6497, except for T803-W6, which produced significantly more sporangia than the wild-type P6497 *in vitro* (Table 3). Zoospore and oospore production and cystospore germination of all transformants were similar to or less than those of P6497 (Table 3).

3.2.4 Virulence on detached soybean leaves

Interestingly, most of transformants exhibited similar or enhanced virulence on detached soybean leaves, but the T801-H1 and T803-W6 showed greatly reduced virulence (Table 3).

3.3 Gene expression level analysis

Among the *P. sojae* transformants, expression of *PsORP1* in T673-W1, T673-W2, T708-K5, T708-F1, T708-I2, T708-Y1, T710-L3, T777-I2, T777-Y2, T777-F1, and T817-F5 did not differ from that in wild-type P6497. However, expression of *PsORP1* appeared higher in T708-K3, T710-P2, T710-P3, T710-A3, T758/759-10, T801-H1, T803-W1, T803-W6, and T817-Y7 than that in wild-type P6497 (Fig. 2).

4 DISCUSSION

In our previous study, only one sgRNA expression plasmid was used in the *P. capsici* and *P. sojae* genetic transformations, and no transformants for which both the non-synonymous point

mutation and sgRNA target sequence were homozygous were recovered.[16,22] To increase the efficiency of homology-directed repair (HDR) for CRISPR–Cas9 editing in *P. sojae*, two sgRNA sequences were designed and used simultaneously in this study.

Interestingly, five transformants were obtained for which both the sgRNA target sequence and non-synonymous point mutation were homozygous, meaning that after the sgRNA-guided Cas9 cut the genome, two alleles were repaired via HDR. For the other 15 transformants, either the sgRNA target sequence or the non-synonymous point mutation was heterozygous. This result was also observed in our previous study[16] and might mean that only part of the donor template was used during HDR. However, non-homologous end joining (NHEJ) was not triggered by CRISPR–Cas9 in the current study. We speculate that two sgRNA sequences in the CRISPR–Cas9 system might introduce two targeted, double-stranded breaks and that they could be repaired by HDR using a DNA repair template with greater efficiency than only one sgRNA system. Han *et al.*[23] found that, in mice, the deletion efficiency was doubled through the use of multiple sgRNAs in the *Rian* locus. However, the rates of modification on multiple target sites affecting the reading frame were much lower in *Populus tomentosa*, even if four sgRNAs were used for one locus at the same time.[24] Therefore, the relationship between the number of sgRNA sequences and NHEJ or HDR in the oomycetes should be verified in more species and genes.

This study aimed to verify the effects of all 18 point mutations in PsORP1 on oxathiapiprolin sensitivity. According to the present results, not all point mutations in PsORP1 confer high oxathiapiprolin resistance. Combining the results in this study with our previous study results, we are able to confirm that L733W, S768F, S768Y, G770V, N837Y, N837F, ΔN837, G839W, P861H, L863W, and I877Y in the target protein confer high oxathiapiprolin resistance (all with RF > 1000). Unfortunately, the three-dimensional structure of any ORP1 of oomycetes remains unknown and all the ORPs (oxysterol-binding protein-related proteins) with structure data showed low homology (< 30%) with oomycete ORP1.[11,25] This prohibits prediction of the influence of amino acid changes at key positions on the structural integrity of the protein. Thus, it can only be speculated that the three-dimensional structure of PsORP1 with any one of these 11 point mutations contributes to the oxathiapiprolin resistance in *P. sojae*.

The excellent fitness of transformants with high oxathiapiprolin resistance will allow the isolates with any of these 11 amino acid substitutions to survive, develop and spread in field. All these 11 amino acid substitutions should therefore be the focus of future work on resistance monitoring. To the best of our knowledge, fast molecular detection methods were established only for G839W.[22] However, it may be impossible for only one point mutation to appear under fungicide selection pressure in the field. Therefore, methods such as suspension array technology,[26] which could detect all point mutations simultaneously, should be developed. However, it should be noted that, in this study, the codon change that led to the amino acid substitutions was artificial, according to the codon usage bias in *P. sojae*.[27] Whether all these point mutations will appear in the field is therefore uncertain, meaning that more monitoring should be conducted to extend the effective lifespan of the fungicide.

Transformants containing S768K, S768I, G770L, G770P, G770A, ΔG818/F819, N837I, or I877F in PsORP1 showed low oxathiapiprolin resistance, with RF < 80. The same low oxathiapiprolin resistance was obtained for ΔG818/F819 in *Ph. nicotianae* mutants.[15] However, whether oxathiapiprolin will always have an excellent control effect over these mutants, which showed low resistance *in vitro*, should be studied further.

It is interesting to note that, for L733W, S768K, G770P, and L863W, both heterozygous and homozygous transformants were obtained. However, both heterozygous and homozygous transformants showed similar RFs, consistent with our speculation. In our previous study, mutants obtained via fungicide adaption and CRISPR–Cas9 at G839W and G770V were all heterozygous. However, all those mutants showed high oxathiapiprolin resistance. We speculated then that the resistance might be controlled by single dominant genes. The results of this study have provided more evidence for this.

We also found that different amino acid substitutions at the same position could confer different oxathiapiprolin RFs. For example, S768F, S768Y, G770V, N837Y, N837F, and I877Y in *PsORP1* conferred high oxathiapiprolin resistance, but transformants containing S768I, G770L, G770P, G770A, N837I, and I877F exhibited low oxathiapiprolin resistance. We speculate that different amino acid substitutions at the same position could cause different changes in the protein structure and the combination of fungicide and target protein. The same phenomenon was observed in *Botrytis cinerea* with succinate dehydrogenase inhibitor (SDHI) fungicides. Isolates possessing the H272L mutation in SdhB were highly resistant to boscalid, but H272R and H272Y showed moderate levels of resistance. Li *et al.*[28] reported that A577T in myosin5 led to low resistance to phenamacril in *Fusarium asiaticum*, but A577G was responsible for moderate phenamacril resistance.[29]

Although T708-K3, T710-P2, T710-P3, T710-A3, T758/759-10, T801-H1, T803-W1, T803-W6, and T817-Y7 showed significantly increased *PsORP1* expression levels, no other transformants exhibited significant differences in these levels. Even though these nine transformants had increased levels of *PsORP1*, we speculate that the *PsORP1* expression level has no direct relevance to oxathiapiprolin resistance because, in T710-P2, T710-P3, and T803-W1, *PsORP1* showed no significant difference in expression level, whereas T710-P2 and T710-P3 showed low oxathiapiprolin resistance, and T803-W1 displayed high oxathiapiprolin resistance. Point mutations in *PsORP1* are therefore mainly responsible for oxathiapiprolin resistance.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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