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Gene editing with an oxathiapiprolin resistance selection marker reveals that PuLLP, a loricrin-like protein, is required for oospore development in *Pythium ultimum*

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Abstract

Oomycetes, such as *Pythium* species, contain numerous devastating plant pathogens that inflict substantial economic losses worldwide. Although CRISPR/Cas9-based genome editing is available, the selection markers available for genetic transformation in these species are limited. In this study, a mutated version of the *Phytophthora capsici* oxysterol-binding protein-related protein 1 (PcMuORP1), known to confer oxathiapiprolin resistance, was introduced into the CRISPR/Cas9 system for in situ complementation in *Pythium ultimum*. We targeted *PuLLP*, which encodes a loricrin-like protein, and showed significant downregulation when the Puf RNA-binding protein-encoding gene *PuM90* was knocked out. The *PuLLP* knockout mutants could not produce oospores, indicating a similar biological function as PuM90. The reintroduction of *PuLLP* into the knockout mutant using *PcMuORP1* as a selection marker restored oospore production. Further comparisons with the conventional selection marker *NPTII* indicated that *PcMuORP1* could be applied at a lower concentration and cost, resulting in a higher screening efficiency. Successive subculturing in the absence of selective pressure showed that PcMuORP1 had little long-term effect on the fitness of transformants. Hence, it could be reused as an alternative selection marker. This study demonstrates the successful implementation of the *PcMuORP1* gene as a selection marker in the genetic transformation of *Py. ultimum* and reveals the loricrin-like protein PuLLP as a sexual reproduction-related factor downstream of the Puf RNA-binding protein PuM90. Overall, these results will help accelerate the functional genomic investigation of oomycetes.

Keywords Oomycete, *Pythium*, Genetic transformation, Selection marker, Oxathiapiprolin

Background

Oomycetes are a class of eukaryotic microorganisms with similar life cycles and growth habits as filamentous fungi (Yin and Hulbert 2018). In general, oomycetes differ from fungi in many aspects, including genome size, vegetative hyphae ploidy, cell wall composition (e.g., cellulose rather than chitin), and the type of mating hormones that they produce (Judelson and Blanco 2005; Widyawan et al. 2015). Owing to lower transformation efficiency and homologous recombination frequency, it is more difficult to genetically manipulate oomycetes

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than most amenable true fungi (Judelson 1997). Consequently, functional genomics research on oomycetes has been limited. In 2016, the CRISPR/Cas9 system was successfully applied in *Phytophthora sojae* (Fang et al. 2017), followed by *P. palmivora* (Gumtow et al. 2017), *P. capsici* (Wang et al. 2018), *P. infestans* (Hoogen et al. 2018), and *Peronophythora litchii* (Kong et al. 2019). These results have significantly promoted the study of gene function in oomycete pathogens.

Functional genomics investigations of *Pythium* have been even more limited compared to *Phytophthora*. The species within the oomycete genus *Pythium* cause devastating losses to a wide range of crop species globally, making the genus of great economic and ecological importance (Park et al. 2016). To investigate the unique biology of *Pythium*, different genetic strategies, including gene silencing, have been adopted (Vijn and Govers 2001; Weiland 2003). Gene silencing involves transferring an endogenous gene into *Pythium* in a forward or reverse manner, resulting in gene silencing. Using this method, the tyrosine-rich protein-coding gene *PoStr* was successfully silenced in *Pythium oligandrum*, and the resulting mutants were significantly defective in oospore formation, marking the first-time gene silencing had been used to study gene function in *Pythium* (Grenville-Briggs et al. 2013). Despite these successes, this gene silencing strategy has several drawbacks, including an unpredictable degree of gene silencing and instability in some transformants. Moreover, it is also difficult to reliably silence several unrelated genes simultaneously (Bargmann 2001; Kamath et al. 2001; Alonso et al. 2003). To promote functional genomics analysis of *Pythium*, we successfully developed the CRISPR/Cas9 system-mediated gene knockout and in situ complementation methods for *Pythium ultimum* recently, providing a robust platform for further molecular studies (Feng et al. 2021).

In transformation methodologies, selection markers like antibiotic resistance genes and auxotrophic markers are essential as they help distinguish transformed cells from non-transformed ones. These markers are widely used for screening transformants of microorganisms (Dornte et al. 2020). However, the selection markers available for oomycete (including *Pythium* and *Phytophthora*) transformation is limited, with many relying on a single marker, the *NPTII* gene, which encodes the enzyme neomycin phosphotransferase II that confers resistance to kanamycin, neomycin, and geneticin (G418) (Bashir et al. 2006). A few species, such as *P. infestans*, can also use the *hpt* gene that confers resistance to hygromycin (Ah-Fong and Judelson 2011). After successful transformation, resistance screening markers will lose their resistance selection role in knockout mutants and may even have adverse effects on the mutants (Rogge and Meyhoefer 2021). Some resistance screening markers may be expressed at high levels, leading to excess protein production and abnormal growth (Qi et al. 2015). To lose resistance, mutants are typically cultured for several generations on antibiotic-free plates until they can no longer grow in the presence of antibiotics (Qiu et al. 2021). However, losing resistance screening markers can be difficult and may cause issues for subsequent experiments. Thus, the lack of selection markers in oomycetes has impeded the progress of functional gene analyses, making it crucial to develop alternative selection markers for complementation experiments and multiplex editing of genomes (Jiang 2001).

In 2007, DuPont developed oxathiapiprolin, an anti-oomycete compound that has proven to be highly effective against most oomycete plant pathogens, including *P. capsici*, *P. sojae*, *Peronophythora litchii*, and *Py. ultimum* (Ji et al. 2014; Miao et al. 2016a). The oxysterol binding protein (OSBP), a member of the OSBP-related protein (ORP) family, is the primary target protein of oxathiapiprolin in oomycetes (Pasteris et al. 2016). A recent study in *Phytophthora* revealed that point mutations in the *ORP1* gene could result in high levels of resistance to oxathiapiprolin (Miao et al. 2016b), which is concerning from a crop protection standpoint. However, this discovery also presents an opportunity to develop an alternative selection marker for *Phytophthora*. The previous studies have determined that the mutated version of the *P. capsici ORP1* sequence (*PcMuORP1*) could serve as a candidate selection marker in *P. capsici*, *P. sojae*, and *Peronophythora litchi*, and the screening efficiency was compared by calculating the number of resistant transformants obtained from the two plasmids pYF2.3G-PcMuORP1-N and pYF2.3G-NPT-N expressing *PcMuORP1* and *NPTII*, respectively (Wang et al. 2019). However, it remains unknown whether there is a difference in the efficiency of gene editing, such as gene knockout or complementation, when using the two markers and whether the use of PcMuORP1 could be extended to the *Pythium* genus.

Oospores, which are produced through sexual reproduction in oomycetes, play a crucial role in their survival, genetic variation, and long-distance transmission (Yang et al. 2013; Lava et al. 2013). The notorious oomycete pathogen *Py. ultimum* is responsible for a variety of diseases in a broad range of plant species (Uzuhashi et al. 2008). In a previous study, we revealed the mechanism through which PuM90, a stage-specific Puf family RNA-binding protein, regulates oospore formation in *Py. ultimum*. *PuM90* knockout mutants were significantly defective in oospore formation, with empty oogonia or larger oospores and thinner oospore walls compared with the wild type (Feng et al. 2021).

Loricrin is a major component of the cornified cell envelope in animals and a significant component of plant cell walls (Cassab 1998). Loricrin-like protein (LLP), a glycine-rich protein and a major component of the plant cell wall, plays important role in the development of vascular tissues, nodules, and flowers (Keller 1992). In *Phytophthora infestans*, the loricrin-like protein PiLLP plays a key role in oospore formation. Gene-silenced transformants of PiLLP were defective in oospore formation and blocked at the stage of oospore wall formation compared to the wild type (Guo et al. 2017). RNA sequencing (RNA-seq) analysis of *Py. ultimum* revealed that the expression of the *PuLLP* gene was downregulated in the *PuM90* knockout mutants, indicating it may play an important role in oospore formation. We investigated the biological roles of *PuLLP* in *Py. ultimum* using *PcMuORP1*, an oxathiapiprolin resistance gene, as an alternative selection marker for CRISPR/Cas9-mediated genome editing. Our study provides novel insights into LLP functions and M90-regulated oospore formation, as well as an alternative selection marker for *Pythium* transformation.

Results

CRISPR/Cas9-mediated gene knockout of *PuLLP*

RNA-seq analysis unraveled 623 significantly downregulated genes in the *PuM90* knockout mutants compared to the wild-type strain. One of these genes, *PYU1_G001136*, was identified as encoding a loricrin-like protein (LLP) and was named *PuLLP*. At 96 h, when mature thick-walled oospores were visible, the transcript level of *PuLLP* showed a significant increase, similar to *PuM90* (Fig. 1a).

To determine the biological function of *PuLLP* in *Py. ultimum*, the CRISPR/Cas9 system was used to knock it out using *NPTII* as a selection marker. Two sgRNAs (sgRNA1 and sgRNA2; Additional file 1: Table S1) targeting *PuLLP* were designed using online tools (Fang and Tyler 2015). Two plasmids, the 'all-in-one' plasmid pYF515 and the donor DNA plasmid containing the hygromycin B phosphotransferase gene (*hph*) flanked by 1 kb arms of homology on either side of the *PuLLP* gene, were used to cotransform *Py. ultimum* (Fig. 1b). Individual G418-resistant transformants were isolated and subjected to genomic DNA extraction and PCR analyses. We obtained 100 primary transformants and identified 12 candidate mutants based on genomic DNA (gDNA) PCR and sequencing (Fig. 1c). One of these candidate mutants was randomly selected for further purification using the single mycelial fragment isolation method and subsequent phenotypic analysis.

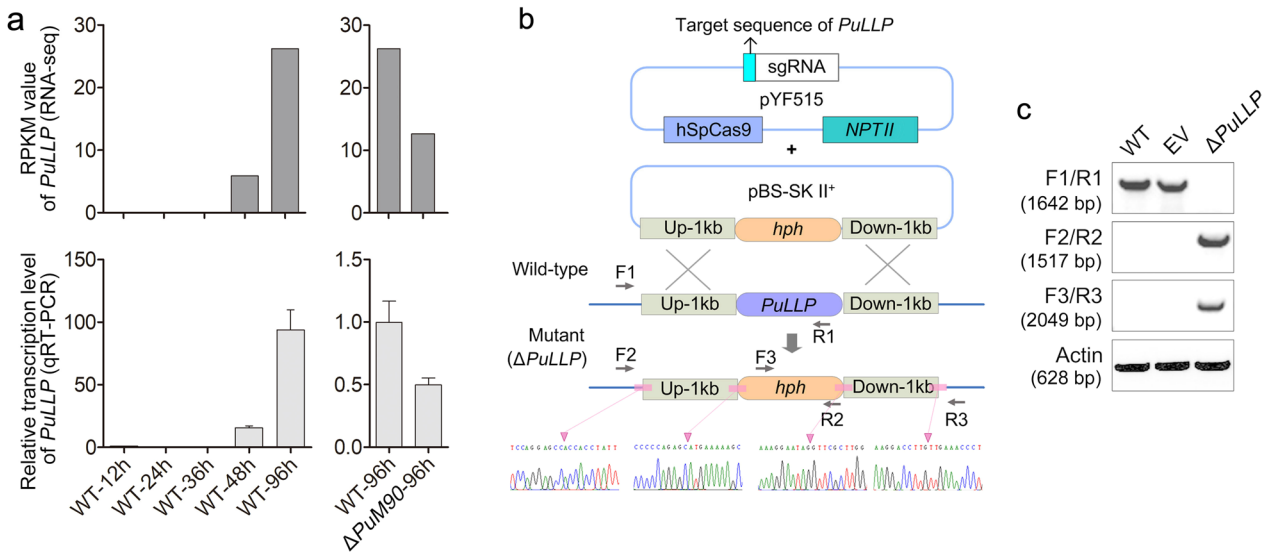

Fig. 1 Transcript levels and CRISPR/Cas9-mediated gene knockout of *PuLLP*. **a** Transcript levels of *PuLLP* in the *Py. ultimum* wild type and *PuM90* knockout mutant strains detected using RNA-seq (upper) and qRT-PCR (bottom) when mycelia were inoculated in V8 liquid medium for 12, 24, 36, 48, or 96 h. **b** Schematic diagram of homology-directed repair (HDR)-mediated modification of the *PuLLP* gene. Top: an 'all-in-one' plasmid (pYF515) harboring both Cas9 and sgRNA cassettes was co-transformed with a plasmid (pBS-SK II+) containing homologous donor DNA, *hph* with *PuLLP* flanking sequences. Locations of the primers used to screen the HDR mutants and Sanger sequencing traces of junction regions confirming that the *PuLLP* ORF was precisely replaced are shown. **c** Analysis of genomic DNA from the wild-type (WT), an empty-vector control line (EV), and *PuLLP* knockout mutants (Δ*PuLLP*) using the primers shown above and actin primers as a positive control

Determination of oxathiapiprolin concentration for *Pythium* transformation

The potential of *PcMuORP1* as a selection marker in *Pythium* was initially evaluated through gene complementation. To determine a concentration for transformation, the sensitivity of *Py. ultimum* to oxathiapiprolin was tested. Oxathiapiprolin exhibited a strong inhibitory effect on the mycelial growth of *Py. ultimum*, with EC_{50} and EC_{90} values of 1 and 2 μg/mL, respectively (Fig. 2). A two-step screening process was utilized to select oxathiapiprolin-resistant transformants, using increasing concentrations of oxathiapiprolin; 1 and 2 μg/mL were selected as first and second cover concentrations for transformation (Table 1). The regenerated protoplasts were initially plated on KPYG2 agar, with 1 μg/mL oxathiapiprolin used. After 2 or 3 days, mycelia emerged from the KPYG2 agar, and the medium was overlaid with molten V8 agar supplemented with 2 μg/mL oxathiapiprolin. After 2–3 days of further incubation, several discrete colonies appeared. These colonies were transferred to fresh V8 agar supplemented with 2 μg/mL oxathiapiprolin, and all of them continued to exhibit normal growth with minimal inhibition.

CRISPR/Cas9-mediated in situ gene complementation using PcMuORP1 as a selection marker

The resistance of the *PuLLP*-knockout mutant (Δ*PuLLP*) to G418 did not affect the further use of *PcMuORP1* as a selection marker. For gene complementation, three plasmids, pYF2.3G-PcMuORP1-N (Wang et al. 2019) containing *PcMuORP1* and sgRNA targeting the *hph* gene, pYF-Cas9-EI expressing the Cas9 protein and the open reading frame (ORF) of *PuLLP* with two mutated sgRNA sites (*PuLLP*-m), and the exploited donor, were simultaneously transformed into Δ*PuLLP* (Fig. 3a). After transformation, putative complementation transformants were selected using oxathiapiprolin antibiotic plates, which yielded 42 resistant colonies. gDNA PCR using specific primers was performed to identify oxathiapiprolin-resistant transformants (Additional file 1: Table S1); 29 transformants produced positive bands, indicating that the *PuLLP*-m ORF was reintroduced (Fig. 3b and Table 1).

Table 1 Relative screening efficiency and cost of the selection markers *PcMuORP1* and *NPTII*

Selection marker	Concentration of first use (μg/mL)	Concentration of second use (μg/mL)	Efficiency of gene complementation	
			PuM90	***PuLLP***
NPTII	30	50	12% (10/86)	67% (23/34)
PcMuORP1	1	2	58% (54/93)	69% (29/42)

Gene complementation of *PuLLP* using *NPTII* as a selection marker was also performed to compare the screening efficiency. After culturing the mutants for at least ten generations in antibiotic-free plates for more than 2 weeks, the mutants could no longer grow on G418 antibiotic plates. The ORF of *PuLLP*-m, exploited as donor DNA along with pYF515 containing *NPTII*, Cas9, and new RNA templates targeting the *hph* gene, was co-transformed into Δ*PuLLP* that had lost G418 resistance. Following transformation, putative complementation transformants were selected using G418 antibiotic plates, which yielded 34 resistant colonies. After gDNA PCR and amplicon sequencing, 23 independent *PuLLP*-m complemented strains were identified from 34 primary transformants (Table 1).

PcMuORP1 was also used as a selection marker to complement *PuM90* in our previously obtained *PuM90* knockout mutant. Using *PcMuORP1* as a selection

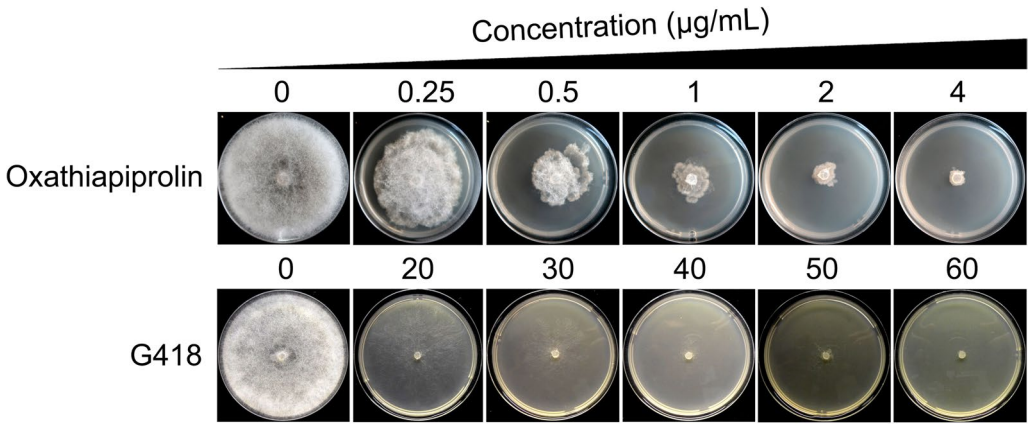

Fig. 2 In vitro effects of oxathiapiprolin (upper) and G418 (bottom) on *Py. ultimum* growth

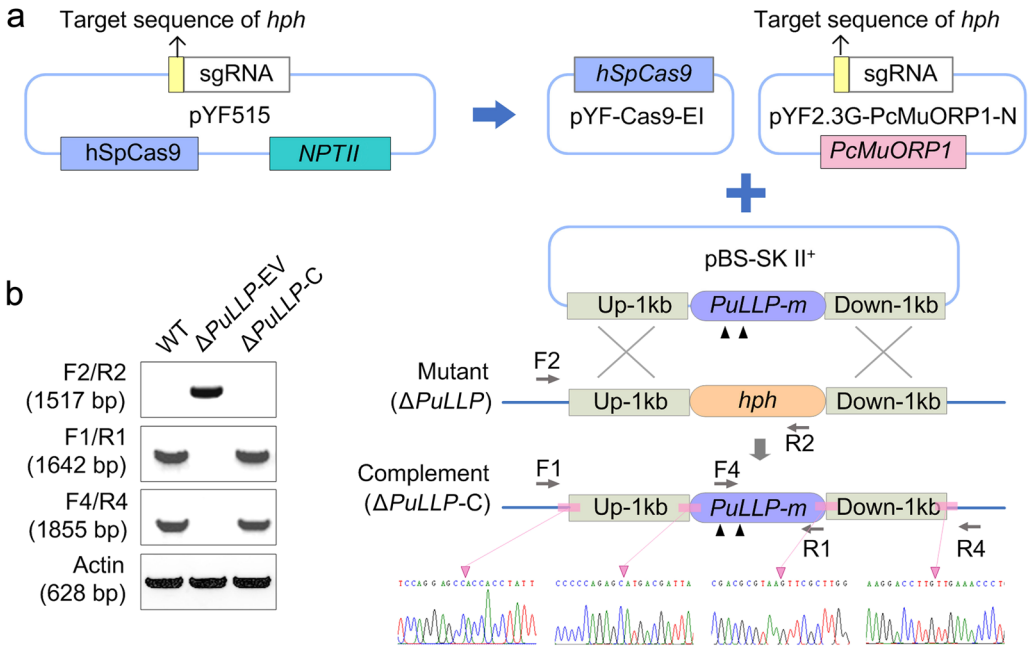

Fig. 3 CRISPR/Cas9-mediated in situ *PuLLP* gene complementation using *PcMuORP1* as a selection marker. **a** Schematic representation of plasmid modification and homologous recombination for *PuLLP* gene complementation. Cas9 and sgRNA were divided into two plasmids, and the *NPTII* gene was replaced with *PcMuORP1*. The location of the primers used to validate complementation and Sanger sequencing traces of junction regions confirming complementation of the *PuLLP* ORF are shown. **b** Analysis of genomic DNA from the wild type (WT), Δ*PuLLP*-EV (in which *PuLLP*-m was not successfully complemented), and *PuLLP*-complemented mutants (Δ*PuLLP*-C) using the primers shown above and actin primers as a positive control

marker, *hph* was replaced with a mutated *PuM90* ORF, and 54 independent *PuM90*-m complemented strains from 93 primary transformants were identified (Table 1). Taken together, these results validate the use of the oxathiapiprolin-resistance gene *PcMuORP1* as an alternative selection marker for *Py. ultimum* transformation.

Complementation of *PuLLP* restores oospore formation

To verify whether the complemented strains obtained using *PcMuORP1* as a selection marker had recovered some of the phenotypes described below, we analyzed the phenotypes of the Δ*PuLLP* and *PuLLP*-complemented strains (Δ*PuLLP*-C) using *PcMuORP1* as a selection marker. The WT strain and empty vector line (EV, in which *PuLLP* knockout was not successful, for comparison with the WT; and Δ*PuLLP*-EV, in which *PuLLP*-m was not successfully complemented, for comparison with Δ*PuLLP*) were included as controls. After 7 days of growth on V8 liquid medium, WT and EV produced oospores, while Δ*PuLLP* and Δ*PuLLP*-EV were unable to form oospores and instead produced a lemon-like structure (Fig. 4a).

This lemon-like structure was identified as a chlamydospore, rather than mycelium enlargement or intersporangium, because the cell wall of the lemon-like structure was thick and had a septum at both ends, while the mycelia of oomycetes have no septum and the cell wall is thin. In addition, several methods were used to induce this lemon-like structure to produce zoospores, but internal substances and zoospores were not released, indicating that this lemon-like structure was not likely a sporangium. The average width and length of the chlamydospores were 19.2 and 22.3 μm, respectively, in Δ*PuLLP*. The average oospore and oogonia diameter of the WT strain were 16.0 and 19.7 μm, respectively (Fig. 4b, c).

Δ*PuLLP*-C restored the ability to produce oospores and showed no significant differences compared with the WT, EV, Δ*PuLLP*-C, Δ*PuLLP*, and Δ*PuLLP*-EV showed no significant differences in the rate of mycelial growth on nutrient-rich V8 medium (Additional file 2: Figure S1a, b) or nutrient-poor Plich medium (Additional file 2: Figure S1a, c), indicating that knocking out of *PuLLP* had no effect on vegetative growth of *Py. ultimum*. All hypocotyls of etiolated soybean seedlings inoculated with mycelia of Δ*PuLLP* or Δ*PuLLP*-EV developed disease symptoms, with lesion sizes comparable to WT, EV, and Δ*PuLLP*-C at 24 h after infection (Additional file 2: Figure S1a, d), suggesting that the deletion of *PuLLP* did not affect virulence. Taken together, the loss of *PuLLP* leads

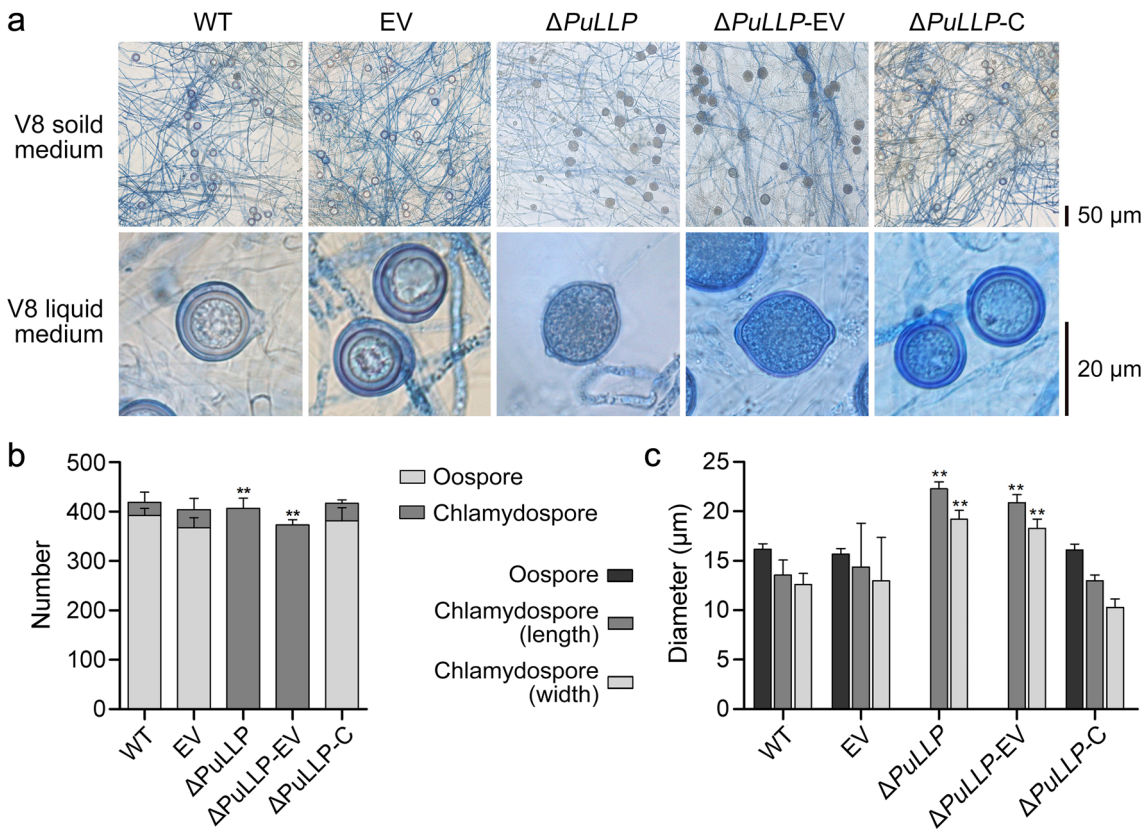

Fig. 4 The deletion of *PuLLP* disrupts oospore formation. **a** Morphology of oospores and chlamydospores produced in 7-day-old cultures of WT, EV, *PuLLP* knockout transformants (Δ*PuLLP*), Δ*PuLLP*-EV (in which *PuLLP*-m was not successfully complemented), and complemented transformants (Δ*PuLLP*-C). **b, c** Statistical analysis of oospore and chlamydospore number from 7-day-old cultures on V8 solid medium (**b**), and diameter from 7-day-old cultures grown in V8 liquid medium (**c**). Asterisks indicate significant differences compared to WT at $P < 0.01$

to severe defects in oospore formation, and subsequent complementation can compensate for these deficiencies.

Comparison of screening efficiency and cost between *PcMuORP1* and *NPTII*

After confirming the successful use of *PcMuORP1* as a selection marker for the transformation of *Pythium*, further experiments were conducted to compare the screening efficiency of *PcMuORP1* and *NPTII*. Each transformation experiment introduced equivalent quantities of the plasmids to 4.5×10^6 protoplasts/mL of *Py. ultimum*. The screening efficiency was determined by calculating the number of complemented transformants obtained using the two selection markers. Using *NPTII* as a selection marker, ten independent *PuM90*-m complemented strains were identified from 86 primary transformants (screening rate of 12%) (Feng et al. 2021). On the other hand, using *PcMuORP1* as a selection marker, 54 independent *PuM90*-m complemented strains were obtained from 93 primary transformants (screening rate of 58%). Moreover, using *NPTII* as a selection marker, twenty-three independent *PuLLP*-m complemented strains were identified from 34 primary transformants (screening rate of 67%). When *PcMuORP1* was used as a selection marker, 29 independent *PuLLP*-m complemented strains were obtained from 42 primary transformants (screening rate of 69%). The complementation efficiency of *PuLLP* and *PuM90* genes using *PcMuORP1* as a screening marker was higher than that using *NPTII* as a screening marker (Table 1). Overall, the results indicate that *PcMuORP1* can increase screening efficiency relative to *NPTII*, producing a higher screening rate of complemented transformants in *Py. ultimum*.

The concentrations of G418 used in the first and second cover were 30 and 50 μg/mL, respectively, while, for oxathiapiprolin, they were 1 and 2 μg/mL, respectively. Thus, the dosage of oxathiapiprolin was lower than that of G418. The price of Dupont™ Zorvec™ 10% Oxathiapiprolin was 16 ¥/g, while the price of G418 (Solarbio) was 1000 ¥/g. Considering the dose and unit price of antibiotics, using *PcMuORP1* as a marker had significantly lower transformation costs than *NPTII*.

Table 2 Stability of the selection markers *PcMuORP1* and *NPTII* in regenerated transformants

Selection marker	Number of mutants losing resistance in every three generations*							Number of mutants maintaining resistance
	3	6	9	12	15	18	21	
PcMuORP1	0	0	8	3	0	0	0	4
NPTII	0	0	3	0	4	2	0	6

* In each case, 15 *Py. ultimum* transformants were inoculated on V8 plates without selection pressure. After three generations, the transformants were transferred back to the plates amended with oxathiapiprolin or G418 to determine whether the transformants lost resistance

Stability of *PcMuORP1* and *NPTII* in regenerated transformants

To compare the stability of selection markers in transformants, we selected 15 strains of *Py. ultimum* that were resistant to oxathiapiprolin or G418 and had been complemented with *PuLLP*. These strains were subjected to multiple rounds of vegetative subculturing on V8 plates without oxathiapiprolin or G418. We found that eight transformants that underwent 9 rounds of subculturing and three transformants that underwent 12 rounds were unable to grow on oxathiapiprolin plates. Additionally, four transformants remained oxathiapiprolin-resistant even after 21 rounds of vegetative subculturing on V8 plates in the absence of oxathiapiprolin (Table 2). In addition, three, four, and two transformants that underwent, respectively, 9, 15, and 18 rounds of vegetative subculturing on V8 plates in the absence of G418 were unable to grow in the presence of G418, and six transformants remained G418-resistant even after 21 rounds of vegetative subculturing on V8 plates in the absence of G418 (Table 2). These results suggest that the selection marker *PcMuORP1* is more likely to be lost from transformants in the absence of selective pressure and, thus, would have minimal long-term effects on the fitness of *Py. ultimum* transformants compared to *NPTII*.

Discussion

The CRISPR/Cas9-mediated gene-editing system has recently been used in *Pythium* (Feng et al. 2021). The development of alternative selection makers for the CRISPR/Cas9 system is crucial for complementation experiments and multiplex editing of the genome. However, selection markers available for *Pythium* transformation are limited (Kennett 2003). In this study, we established a CRISPR/Cas9 system with *PcMuORP1* as a screening marker in *Pythium*. Furthermore, we also complemented the *PuLLP* gene in the knockout mutant, revealing that *PuLLP* plays an important role in the oospore formation of *Pythium*.

Although the ORP family has been reported in many organisms, such as *Homo sapiens* and *Saccharomyces cerevisiae* (Vesa 2013; Mark et al. 2018), their functions are still unknown in oomycetes. The ultra-high activity of oxathiapiprolin in many oomycetes suggests that the target protein of this fungicide, ORP1, is vital to the survival of these species (Lacey et al. 2021). Recently, point mutations in the ORP1 protein have been found to cause resistance to oxathiapiprolin in *Phytophthora* (Miao et al. 2018). This discovery has provided an opportunity to develop an alternative selection marker for *Phytophthora* transformation. While a previous study evaluated the potential of oxathiapiprolin as a means of selection during *Phytophthora* transformation, it found that *PcMuORP1* could produce a higher number of transformants than *NPTII* (Wang et al. 2019). However, the efficiency in gene editing, such as gene knockout or complementation, has not yet been compared. Our results have extended the application of *PcMuORP1* in the *Pythium* genus and revealed it to be a reliable selection marker in gene editing. The present study demonstrated that, when compared to *NPTII*, *PcMuORP1* not only increased the number of transformants but also improved complementation efficiency (*PuM90*: 58% and 12%; *PuLLP*: 69% and 67%). Therefore, using different gene combinations as selection markers in *Pythium* will allow for multiple gene deletions.

Although selection markers play an important role in transformation, they may also impair the fitness of transformants and even pose a risk to the environment or human health (Scutt et al. 2002; Vicentin et al. 2022). For example, high expression levels of selection markers can result in an excess of redundant proteins in the cell, thereby increasing the metabolic burden of the transformants (Khan and Maliga 1999). Selection markers can also lead to difficulties in subsequent experiments (Charles et al. 2002; Marcel et al. 2020). The present study found that the *PcMuORP1* gene is easily lost when selective pressure is absent. The loss of the selection marker could be useful because it allows the reuse of the same marker in subsequent transformations, facilitating gene complementation or multiplex editing (Wang et al. 2019). In addition, the pure product of oxathiapiprolin is unnecessary and the price of the active ingredient oxathiapiprolin in DuPont's Zorvec™ was 16 ¥/g, that is much cheaper than the G418 powder from Solarbio (1000 ¥/g).

Considering the dosage and unit price of antibiotics, the transformation cost of *PcMuORP1* as a screening marker is lower than that of *NPTII*.

Compared to G418, the mycelia of *Py. ultimum* were denser on medium containing oxathiapiprolin; the mycelial growth rate was also reduced, and the expansion area was smaller. This might be due to the higher sensitivity of *Py. ultimum* to oxathiapiprolin. The morphology of the mycelium is conducive to the selection of single colonies, which reduces the number of steps required for single mycelium fragment separation and the time and labor needed for transformation. Only *PcMuORP1* was used for gene complementation in this study. Further investigations are required to determine the use of *PcMuORP1* for other genetic manipulations, such as gene silencing and overexpression, as well as the subcellular localization of proteins.

In the present study, *PuLLP*-knockout mutants were unable to produce oospores, whereas in *P. infestans*, *PiLLP*-silenced transformants formed many immature oogonia, but some of these oogonia were unable to form oospores. Gene silencing results in reduced expression of the target gene; thus, low expression of *PiLLP* may cause *P. infestans* to produce abnormal oospores. *PiLLP* knockout was not performed in *P. infestans*, and the phenotype of gene deletion will require further exploration (Guo et al. 2017). In this study, the knockout of *PuLLP* had no effect on the vegetative growth and pathogenicity of *Py. ultimum*, and similar results were observed with *PuM90* knockout mutants. These findings suggest that *PuM90* and *PuLLP* may function specifically in oospore formation in *Py. ultimum*. The transcriptional level of *PuLLP* was decreased in the *PuM90* knockout mutant, but how *PuM90* regulates *PuLLP* expression remains unclear.

Many *Pythium* pathogens have a great economic and ecological impact. However, many aspects of *Pythium* biology, particularly plant–pathogen interactions, are largely unknown. Although the genome sequences of many oomycete species are available (Tyler et al. 2006; Lévesque et al. 2010; Tian et al. 2011), the lack of available selection markers hinders genome editing. A broad range of selection markers, categorized according to their modes of action, including antibiotic resistance, herbicide resistance, and auxotroph-saving genes, are available for the transformation of a variety of different organisms (Miki and Mchugh 2004; He et al. 2004; Kanda et al. 2014). Fungicide resistance genes are rarely utilized, despite the detailed characterization of resistance mechanisms for many fungicides, including carbendazim, tebuconazole, boscalid, phenamacril, zoxamide, mandipropamid, and oxathiapiprolin, have been characterized in detail (Fujimura et al. 1994; Blum et al. 2010; Meng et al. 2016). The development of these fungicides and their related resistance genes as selection markers could greatly expand the options available for the transformation and functional genomics of oomycetes and true fungi.

Conclusions

In this study, we have created an alternative selection marker for *Pythium* transformation using a fungicide-resistance gene. This alternative marker was compatible with the CRISPR/Cas9 system and was used to confirm the significance of a loricrin-like protein in oomycete sexual reproduction. Furthermore, the use of *PcMuORP1* resulted in significantly higher screening efficiency compared to *NPTII*. The stability of the marker was assessed, and it was found that it could be easily lost from transformants without selective pressure. This suggests that the marker is unlikely to have a long-term impact on the fitness of transformants and could be used as a selection marker in subsequent experiments. Overall, this study describes a new selection marker and data for *Pythium* functional gene research, advancing our understanding of sexual reproduction and development in organisms within this genus.

Methods

Strain source and culturing

The *Pythium ultimum* var. *ultimum* strain F18-6, used as the wild type (WT) strain, was isolated from soil from a soybean field in Shandong Province, China (Feng et al. 2020). All strains employed in this study were routinely grown on 10% V8 agar medium at 25°C in the dark. For growth rate analysis, strains were cultured on V8 and Plich media at 25°C in the dark. Plich medium is a minimal medium with low nutritional content containing 0.5 g KH_2PO_4, 0.25 g $MgSO_4{\cdot}7H_2O$, 1 g asparagine, 1 mg thiamine, 0.5 g yeast extract, 10 mg β-sitosterol, 25 g glucose, and 15 g agar per liter (Latijnhouwers et al. 2004; Kliegman et al. 2013). The diameter of each colony was measured at 24 h post-inoculation, and the average diameter was determined from two measurements taken at right angles to each other. To allow for explicit observation of the oospores, five 5 mm × 5 mm hyphal plugs were cultivated in 8 mL of V8 broth in 90 mm Petri dishes for 7 days at 25°C in the dark. Oospores stained with lactophenol-trypan blue (10 mL lactic acid, 10 mL glycerol, 10 g phenol, and 10 mg trypan blue dissolved in 10 mL distilled water) were randomly selected for examination under an inverted microscope (Zeiss). Experiments were performed in triplicate for each assay and repeated

independently three times. Results were compared using a *t*-test in Microsoft Excel.

Fungicide and antibiotics

Dupont™ Zorvec™ (10% oxathiapiprolin dispersible oil suspension), which is used for crop oomycete disease control, was purchased from a pesticide store. Ultra-pure grade G418 sulfate and ampicillin sodium salt were purchased from Beijing Solarbio Science & Technology Co., Ltd. To determine the sensitivity of *Py. ultimum* to oxathiapiprolin, Zorvec™ was dissolved in dimethyl sulfoxide and added to V8 medium at 0, 0.25, 0.5, 1, 2, and 4 μg/mL. Three replicates were generated for each treatment. After incubation in the dark for 2 days at 25°C, mycelial growth was assessed. G418 and ampicillin were dissolved or diluted in ultra-pure grade water to prepare stock solutions (50 mg/mL) that were stored in the dark at −20°C.

Targeted gene deletion and complementation

Gene deletion mutants were generated using the CRISPR-mediated gene replacement strategy (Fang and Tyler 2015). sgRNAs were designed using the web tool at http://grna.ctegd.uga.edu. Two plasmids, including the 'all-in-one' plasmid pYF515, containing Cas9, sgRNA, and selection marker *NPTII*, and the donor DNA plasmid pBS-SK II$^+$, which contained the entire *hph* gene, flanked by 1 kb of homology arms flanking the target gene, were used for PEG-mediated protoplast transformation (Fig. 1). For complementation, the knockout mutants were transformed using *NPT II* or *PcMuORP1* as a selection marker. The entire gene coding region with a mutated single guide RNA (sgRNA) site, inserted into two 1.0 kb fragments flanking the target gene, was used as donor DNA. Polyethylene glycol (PEG)-mediated protoplast transformation was used to transform *Py. ultimum* (Li 2013). Putative transformants were selected by growth on 10% (v/v) V8 medium containing G418 or oxathiapiprolin and screened via polymerase chain reaction (PCR) (Additional file 1: Table S1) and analyzed by Sanger sequencing. The primer set F2/R2 was used to screen for the deletion of *hph* from the genome of resistant transformants. The primer sets F1/R1 and F4/R4 were used to detect HDR events.

Quantitative PCR

Total RNA of *Py. ultimum* was extracted using the EZNA Total RNA Kit I (Omega). cDNA was synthesized from 1–5 μg total RNA with the PrimeScript First Strand cDNA Synthesis Kit (TaKaRa Bio Inc.), following the manufacturer's instructions. Quantitative PCR was performed in 20 μL reactions containing 20 ng DNA/cDNA, 0.2 mM primers for the target or reference gene, 10 μL SYBR Premix ExTaq (TaKaRa Bio Inc.), and 6.8 μL double-distilled water. PCR was performed using an ABI Prism 7500 Fast Real-Time PCR System (Applied Biosystems Inc.) with the following conditions: 95°C for 30 s; followed by 40 cycles of 95°C for 5 s and 60°C for 34 s; and finally, 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. For the gene expression assay, the actin gene (*PuACTA* = *PYU1_T009609*) of *Py. ultimum* was used as a constitutively expressed endogenous control. To quantify relative *PuLLP* expression, the *Py. ultimum* genes *PuACTA* and *PuLLP* were quantified via quantitative PCR, and expression levels were calculated using the $2^{-[Ct(Ct(PuLLP)-PuACTA]}$ method (Lin et al. 2018). The primers used in this study are provided in Additional file 1: Table S1. Means and standard deviations were calculated using data from three replicates.

Pathogenicity assay

Pathogenicity assays were performed after hyphal plug inoculation of hypocotyls of etiolated soybean seedlings of the Williams cultivar, as this cultivar is compatible with *Py. ultimum*. Soybeans grown in a greenhouse at 25°C with a light/dark cycle of 16 h/8 h for 4 days were used for hypocotyl infection. Hyphal plugs (5 mm in diameter) were inoculated onto hypocotyls and incubated at 25°C in the dark for 24 h before imaging and sampling. Each strain was tested on at least five plants, and the length of the disease spot was used to quantify virulence. All assays were repeated independently at least three times.

Abbreviations

PcMuORP1	*Phytophthora capsici* Oxysterol-binding protein-related protein 1
OSBP	Oxysterol binding protein
ORP	OSBP-related protein
LLP	Loricrin-like protein
hph	Hygromycin B phosphotransferase gene
ORF	Open reading frame
PuLLP-m	ORF of *PuLLP* with two mutated sgRNA sites

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-023-00189-7.

Additional file 1 Table S1. Primers and sgRNAs used in this study.

Additional file 2 Figure S1. *PuLLP* is not involved in the mycelial growth or virulence of *Py. ultimum*. **a** Growth characteristics 24 h after inoculation on V8 and Plich media, and virulence on soybean hypocotyls of WT, EV, *PuLLP*-knockout transformants (Δ*PuLLP*), complemented transformants (Δ*PuLLP*-C), and the empty control line of Δ*PuLLP* (Δ*PuLLP*-EV). **b, c** Growth rates on V8 medium (**b**) and Plich medium (**c**). **d** Lesion length of soybean hypocotyls 24 h post hypocotyl infection.

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Author contributions
WY, XZ, YW, and HF conceived and designed the experiments. HF, TL, JL, CW, and FD carried out the experiments. HF and WY analyzed the data. HF and WY wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
Not applicable.

Declarations

Ethical approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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References

Ah-Fong AM, Judelson HS. Vectors for fluorescent protein tagging in *Phytophthora*: tools for functional genomics and cell biology. Fungal Biol. 2011;115:882–90. https://doi.org/10.1016/j.funbio.2011.07.001.

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Cheuk R. Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science. 2003;301:653–7. https://doi.org/10.1126/science.1086391.

Bargmann CI. High-throughput reverse genetics: RNAi screens in *Caenorhabditis elegans*. Genome Biol. 2001;2:1005. https://doi.org/10.1186/GB-2001-2-2-REVIEWS1005.

Bashir KM, Kim MS, Stahl U, Cho MJ. Microalgae engineering toolbox: selection and screenable markers. Biotechnol Bioproc E. 2006;21:224–35. https://doi.org/10.1007/s12257-015-0386-4.

Blum M, Waldner M, Gisi U. A single point mutation in the novel *PvCesA3* gene confers resistance to the carboxylic acid amide fungicide mandipropamid in *Plasmopara viticola*. Fungal Genet Biol. 2010;47:499–510. https://doi.org/10.1016/j.fgb.2010.02.009.

Cassab GI. Plant cell wall proteins. Annu Rev Plant Biol. 1998;49:281–309. https://doi.org/10.1146/annurev.arplant.49.1.281.

Charles PS, Elena Z, Peter M. Techniques for the removal of marker genes from transgenic plants. Biochimie. 2002;84:1119–26. https://doi.org/10.1016/S0300-9084(02)00021-4.

Dornte B, Peng C, Fang Z, Kamran A, Yulvizar C, Kues U. Selection markers for transformation of the sequenced reference monokaryon Okayama 7/#130 and homokaryon AmutBmut of Coprinopsis cinerea. Fungal Biol Biotechnol. 2020;7:15. https://doi.org/10.1186/s40694-020-00105-0.

Fang Y, Tyler BM. Efficient disruption and replacement of an effector gene in the oomycete *Phytophthora sojae* using CRISPR/Cas9. Mol Plant Pathol. 2015;17:127–39. https://doi.org/10.1101/025023.

Fang YF, Cui LK, Gu B, Arredondo F, Tyler BM. Efficient genome editing in the oomycete *Phytophthora sojae* using CRISPR/Cas9. Curr Microbiol. 2017;6:21A11–126. https://doi.org/10.1002/cpmc.25.

Feng H, Chen J, Yu Z, Li K, Li Z, Li Y, et al. Pathogenicity and fungicide sensitivity of *Pythium* and *Phytopythium* spp associated with soybean in the Huang-Huai region of China. Plant Pathol. 2020. https://doi.org/10.1111/ppa.13187.

Feng H, Wan C, Zhang Z, Chen LZ, Jiang H, et al. Specific interaction of an RNA-binding protein with the 3'-UTR of its target mRNA is critical to oomycete sexual reproduction. PLoS Pathog. 2021;17:e1010001. https://doi.org/10.1371/journal.ppat.1010001.

Fujimura M, Kamakura T, Inoue H, Yamaguchi I. Amino-acid alterations in the -tubulin gene of Neurospora crassa that confer resistance to carbendazim and diethofencarb. Curr Genet. 1994;25:418. https://doi.org/10.1007/BF00351780.

Grenville-Briggs LJ, Horner NR, Phillips AJ, Beakes GW, van West P. A family of small tyrosine rich proteins is essential for oogonial and oospore cell wall development of the mycoparasitic oomycete *Pythium oligandrum*. Fungal Biol. 2013;117:163–72. https://doi.org/10.1016/j.funbio.2013.01.001.

Gumtow R, Wu D, Uchida J, Tian M. A *Phytophthora palmivora* extracellular cystatin-like protease inhibitor targets papain to contribute to virulence on papaya. Mol Plant Microbe Interact. 2017;31:363–73. https://doi.org/10.1094/MPMI-06-17-0131-FI.

Guo T, Wang XW, Shan K, Sun W, Guo LY. The Loricrin-Like Protein (*LLP*) of *Phytophthora infestans* is required for oospore formation and plant infection. Front Plant Sci. 2017;8:142. https://doi.org/10.3389/fpls.2017.00142.

He Z, Fu Y, Si H, Hu G, Zhang S, Yu Y, et al. Phosphomannose-isomerase (pmi) gene as a selectable marker for rice transformation via Agrobacterium. Plant Sci. 2004;166:17–22. https://doi.org/10.1016/S0168-9452(03)00338-8.

Hoogen VD, Govers J, Francine G. Attempts to implement CRISPR/Cas9 for genome editing in the oomycete *Phytophthora infestans*. *bioRxiv*. 2018. https://doi.org/10.1101/274829.

Ji PS, Alexander SC, Lara LH, Unessee H. Efficacy and application methods of oxathiapiprolin for management of black shank on Tobacco. Plant Dis. 2014. https://doi.org/10.1094/PDIS-02-14-0172-RE.

Jiang W. Advances in gene therapy of liver cirrhosis: a review. World J Gastroenterol. 2001;7:1–8. https://doi.org/10.3748/wjg.v7.i1.1.

Judelson HS. The genetics and biology of *Phytophthora infestans*: modern approaches to a historical challenge. Fungal Genet Biol. 1997;22:65–76. https://doi.org/10.1006/fgbi.1997.1006.

Judelson HS, Blanco FA. The spores of *Phytophthora*: weapons of the plant destroyer. Nat Rev Microbiol. 2005;3:47–58. https://doi.org/10.1038/nrmicro1064.

Kamath RS, Martinez-Campos M, Zipperlen P, Fraser AG, Ahringer J. Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans*. Genome Biol. 2001. https://doi.org/10.1186/gb-2000-2-1-research0002.

Kanda K, Ishida T, Hirota R, Ono S, Motomura K, Ikeda T, et al. Application of a phosphite dehydrogenase gene as a novel dominant selection marker for yeasts. J Biotechnol. 2014;182–183:68–73. https://doi.org/10.1016/j.jbiotec.2014.04.012.

Keller RB. Ultrastructural localization of a bean Glycine-rich protein in unlignified primary walls of protoxylem cells. Plant Cell. 1992;4:773–83. https://doi.org/10.2307/3869393.

Kennett RM. Transformation of *Pythium aphanidermatum* to geneticin resistance. Curr Genet. 2003;42:344–52. https://doi.org/10.1007/s00294-002-0359-y.

Khan MS, Maliga P. Fluorescent antibiotic resistance marker for tracking plastid transformation in higher plants. Nat Biotechnol. 1999;17:910–5. https://doi.org/10.1038/12907.

Kliegman JI, Fiedler D, Ryan CJ, Xu Y, Su X, Thomas D, et al. Chemical genetics of rapamycin insensitive TORC2 in *S. cerevisiae*. Cell Rep. 2013;5:1725–36. https://doi.org/10.1016/j.celrep.2013.11.040.

Kong GH, Wan L, Deng YZ, Yang WS, Wen Y. Pectin acetylesterase PAE5 is associated with the virulence of plant pathogenic oomycete *Peronophythora litchii*. Physiol Mol Plant Pathol. 2019;106:16–22. https://doi.org/10.1016/j.pmpp.2018.11.006.

Lacey RF, Fairhurst MJ, Daley KJ, Amohaere N, Patterson HR, Patrick WM, et al. Assessing the effectiveness of oxathiapiprolin toward *Phytophthora agathidicida* the causal agent of kauri dieback disease. FEMS Microbes. 2021. https://doi.org/10.1093/femsmc/xtab016.

Latijnhouwers M, Ligterink W, Vleeshouwers VG, West PV, Govers F. A Galpha subunit controls zoospore motility and virulence in the potato late blight pathogen *Phytophthora infestans*. Mol Microbiol. 2004;51:925–36. https://doi.org/10.1073/pnas.191384198.

Lava SS, Heller A, Spring O. Oospores of pustula helianthicolain sunflower seeds and their role in the epidemiology of white blister rust. IMA Fungus. 2013;4:251–8. https://doi.org/10.5598/imafungus.2013.04.02.10.

Lévesque A, Brouwer H, Cano L, Hamilton J. Genome sequence of the necrotrophic plant pathogen *Pythium ultimum* reveals original pathogenicity mechanisms and effector repertoire. Genome Biol. 2010;11:R73. https://doi.org/10.1186/gb-2010-11-7-r73.

Li S. Establishment of a PEG-mediated genetic transformation system and expression of green fluorescence protein in colletotrichum gloeosporioides. Sci Silvae Sin. 2013;49:121–7.

Lin L, Ye W, Wu J, Xuan M, Li Y, Gao J, et al. The MADS-box transcription factor *PsMAD1* is involved in zoosporogenesis and pathogenesis of *Phytophthora sojae*. Front Microbiol. 2018;9:2259. https://doi.org/10.3389/fmicb.2018.02259.

Marcel S, Sascha B, Bernhard H, Lydia K. A TRP1-marker-based system for gene complementation, overexpression, reporter gene expression, and gene modification in Candida glabrata. FEMS Yeast Res. 2020;20:8. https://doi.org/10.1093/femsyr/foaa066.

Mark C, Asako G, Neale D. Oxysterol-binding protein recruitment and activity at the endoplasmic reticulum-golgi interface are independent of Sac1. Traffic. 2018;1848:139–50. https://doi.org/10.1111/tra.12491.

Miao J, Dong X, Lin D, Wang Q, Liu P. Activity of the novel fungicide oxathiapiprolin against plant-pathogenic oomycetes. Pest Manag Sci. 2016a;72:1572–7. https://doi.org/10.1002/ps.4189.

Miao JQ, Cai M, Dong X, Liu L, Lin D, Zhang C, et al. Resistance assessment for oxathiapiprolin in *Phytophthora capsici* and the detection of a point mutation (G769W) in *PcORP1* that confers resistance. Front Microbio. 2016b;7:615. https://doi.org/10.3389/fmicb.2016.00615.

Miao JQ, Chi YD, Lin D, Tyler BM, Liu XL. Mutations in ORP1 conferring oxathiapiprolin resistance confirmed by genome editing using CRISPR/Cas9 in *Phytophthora capsici* and *P. sojae*. Phytopathology. 2018;108:1412–9. https://doi.org/10.1094/PHYTO-01-18-0010-R.

Miki B, Mchugh S. Selectable marker genes in transgenic plants: applications, alternatives and biosafety. J Biotechnol. 2004;107:193–232. https://doi.org/10.1016/j.jbiotec.2003.10.011.

Park M, Back C, Han K, Park J. Occurrence of damping-off caused by *Pythium spinosum* on cucumis melo in Korea. Res Plant Dis. 2016;22:190–3. https://doi.org/10.5423/RPD.2016.22.3.190.

Pasteris RJ, Hanagan MA, Bisaha JJ, Finkelstein BL, Hoffman LE, Gregory V, et al. Discovery of oxathiapiprolin, a new oomycete fungicide that targets an oxysterol binding protein. Bioorg Med Chem. 2016. https://doi.org/10.1016/j.bmc.2015.07.064.

Qi W, Tinnenbroek-Capel IE, Salentijn EM, Schaart JG, Cheng J, Denneboom C, et al. Screening for recombinants of Crambe abyssynica after transformation by the pMF1 marker-free vector based on chemical selection and meristematic regeneration. Sci Rep. 2015;5:14033. https://doi.org/10.1038/srep14033.

Qiu M, Li Y, Ye W, Zheng X, Wang Y. A CRISPR/Cas9-mediated in situ complementation method for *Phytophthora sojae* mutants. Mol Plant Pathol. 2021;22:373–81. https://doi.org/10.1111/mpp.13028.

Rogge SA, Meyhoefer R. Leaf age is important for assessment of resistance in chrysanthemum against frankliniella occidentalis. J Plant Dis Prot. 2021;128:511–6. https://doi.org/10.1007/S41348-021-00542-Y.

Scutt C, Zubko E, Meyer P. Techniques for the removal of marker genes from transgenic plants. Biochimie. 2002;84:1119–26. https://doi.org/10.1016/s0300-9084(02)00021-4.

Tian M, Win J, Savory E, Burkhardt A, Held M, Brandizzi F, et al. 454 Genome sequencing of *Pseudoperonospora cubensis* reveals effector proteins with a QXLR translocation motif. Mol Plant-Microbe Interact. 2011;24:543–53. https://doi.org/10.1094/MPMI-08-10-0185.

Tyler B, Tripathy S, Zhang X, Dehal P, Jiang R, Aerts A, et al. *Phytophthora* genome sequences uncover evolutionary origins and mechanisms of pathogenesis. Science. 2006;313:1261–6. https://doi.org/10.1126/SCIENCE.1128796.

Uzuhashi S, Imazu M, Kakishima M. Structure and organization of the rDNA IGS region in *Pythium ultimum*. Ann Phytopathol Soc Japan. 2008;74:224–32. https://doi.org/10.1007/s10267-008-0473-x.

Vesa O. OSBP-related proteins: liganding by glycerophospholipids opens new insight into their function. Molecules. 2013;18:13666–79. https://doi.org/10.3390/molecules181113666.

Vicentin IG, Ghione CE, Cuatrín A, Gilli JR, Bernardi CN, Darío D, et al. Identification of molecular markers and candidate genes associated with time to flowering and length of reproductive period in soybean through association mapping. Plant Mol Biol Rep. 2022;41(2):229–41. https://doi.org/10.1007/s11105-022-01361-7.

Vijn I, Govers F. Gene silencing and *Agrobacterium tumefaciens* mediated transformation of the oomycete *Phytophthora infestans*. Mol Plant-Microbe Interact. 2001; pp. 10–14.

Wang ZW, Tyler B, Liu XL. Protocol of *Phytophthora capsici* transformation using the CRISPR-Cas9 system. Methods Mol Biol. 2018;1848:265–74. https://doi.org/10.1007/978-1-4939-8724-5_17.

Wang W, Xue Z, Miao J, Cai M, Zhang C, Li T, et al. *PcMuORP1*, an oxathiapiprolin-resistance gene, functions as a novel selection marker for *Phytophthora* transformation and CRISPR/Cas9 mediated genome editing. Front Microbiol. 2019;10:2402. https://doi.org/10.3389/fmicb.2019.02402.

Weiland JJ. Transformation of *Pythium aphanidermatum* to geneticin resistance. Curr Genet. 2003;42:344. https://doi.org/10.1007/s00294-002-0359-y.

Widyawan A, Damiri BV, Santosa AI, Setyaningrum H. A Survey of Phytopathogenic fungi and oomycetes in Riyadh, Saudi Arabia. Gontor Agrotech Sci J. 2015;1:1–19. https://doi.org/10.21111/agrotech.v1i2.261.

Yang X, Zhao W, Hua C, Zheng X, Wang Y. Chemotaxis and oospore formation in *Phytophthora sojae* are controlled by G-protein-coupled receptors with a phosphatidylinositol phosphate kinase domain. Mol Microbiol. 2013;88:382–94. https://doi.org/10.1111/mmi.12191.

Yin CY, Hulbert SH. Host-induced gene silencing (HIGS) for elucidating puccinia gene function in wheat. Methods Mol Biol. 2018;1848:139–50. https://doi.org/10.1007/978-1-4939-8724-5_12.