Two *Phytophthora parasitica* cysteine protease genes, *PpCys44* and *PpCys45*, trigger cell death in various *Nicotiana* spp. and act as virulence factors

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**Funding information**
Programme of Introducing Talents of Innovative Discipline to Universities (project 111) from the State Administration of Foreign Experts Affairs, Grant/Award Number: #B18042; National Natural Science Foundation of China, Grant/Award Number: 31561143007 and 31125022; China Agriculture Research System, Grant/Award Number: CARS-09

**Abstract**
Proteases secreted by pathogens have been shown to be important virulence factors modifying plant immunity, and cysteine proteases have been demonstrated to participate in different pathosystems. However, the virulence functions of the cysteine proteases secreted by *Phytophthora parasitica* are poorly understood. Using a publicly available genome database, we identified 80 cysteine proteases in *P. parasitica*, 21 of which were shown to be secreted. Most of the secreted cysteine proteases are conserved among different *P. parasitica* strains and are induced during infection. The secreted cysteine protease proteins *PpCys44*/*45* (proteases with identical protein sequences) and *PpCys69* triggered cell death on the leaves of different *Nicotiana* spp. A truncated mutant of *PpCys44*/*45* lacking a signal peptide failed to trigger cell death, suggesting that *PpCys44*/*45* functions in the apoplastic space. Analysis of three catalytic site mutants showed that the enzyme activity of *PpCys44*/*45* is required for its ability to trigger cell death. A virus-induced gene silencing assay showed that *PpCys44*/*45* does not induce cell death on NPK1 (*Nicotiana Protein Kinase 1*)-silenced *Nicotiana benthamiana* plants, indicating that the cell death phenotype triggered by *PpCys44*/*45* is dependent on NPK1. *PpCys44* and *PpCys45*-deficient double mutants showed decreased virulence, suggesting that *PpCys44* and *PpCys45* positively promote pathogen virulence during infection. *PpCys44* and *PpCys45* are important virulence factors of *P. parasitica* and trigger NPK1-dependent cell death in various *Nicotiana* spp.

**KEYWORDS**
cell death, cysteine protease, *Phytophthora parasitica*, virulence factor
1 | INTRODUCTION

Proteolysis of plant substrates is a strategy employed by pathogens to alter plant physiology (Hotson and Mudgett, 2004). Many enzyme effectors secreted by pathogens have been shown to manipulate plant immunity, such as the glycose hydrolase 12 protein XEG1 (Ma et al., 2015, 2017) from Phytophthora sojae; small phospholipase D-like proteins (Meijer et al., 2019) and serine proteases (Paris and Lamattina, 1999) from Phytophthora infestans; and metalloproteases, subtilisin, aspartic proteases, aspartyl acid proteases, non-aspartyl acid proteases, and cysteine proteases from plant pathogenic fungi (Chandrasekaran et al., 2016) and bacteria (Hotson and Mudgett, 2004; Potempa and Pike, 2009; Niño et al., 2014).

In the MEROPS database (https://www.ebi.ac.uk) all the cysteine proteases from different organisms are classified into 15 clans and several unassigned families. Cysteine proteases from different families play different roles in plant pathosystems (Niño et al., 2014). In plants, cysteine proteases function in resistance against pathogens. In Arabidopsis thaliana, cathespin B proteins contribute to cell death and are involved in plant basal resistance against Pseudomonas syringae pv. tomato DC3000 (McLellan et al., 2009). The A. thaliana cysteine protease RD19 was shown to be relocated from vesicles to the nucleus by PopP2, a Ralstonia solanacearum effector, to form a complex that is required for RESISTANT TO RALSTONIA SOLANACEARUM 1-R (RRS1-R)-mediated resistance (Bernoux et al., 2008). The extracellular tomato cysteine protease Rcr3 is bound and inhibited by the Cladosporium fulvum effector Avr2, and the Rcr3/Avr2 complex can trigger cell death (Rooney, 2005). A Nicotiana benthamiana C14 cysteine protease interacts with the P. infestans effectors EPIC1, EPIC2B, and AVRb1b2, and contributes to resistance against this pathogen (Kaschani et al., 2010; Bozkurt et al., 2011).

In contrast to cysteine proteases in plants, those secreted by plant pathogens interfere with plant immunity. For example, AvmPphB from P. syringae is modified in the plant cell (Puri et al., 1997) and promotes P. syringae colonization by suppressing the activation of the A. thaliana R protein RPM1 (Russell et al., 2015). AvmRpt2 from P. syringae degrades RIN4 and induces RPS2-mediated resistance. In the absence of RPS2, AvmRpt2 also interferes with the AvmRpm1/Rpm1-mediated defence response (Hotson and Mudgett, 2004). As effectors share similar protease domains with host protease proteins, some effector proteases mimic those from plants to modify plant immunity. The Xanthomonas effector AvrXv4 possesses SUMO-isopeptidase activity and significantly reduces the level of SUMO-protein conjugates when it is heterologously expressed in plant cells (Roden et al., 2004). The Xanthomonas XopD effector, a cysteine protease possessing both SUMO peptidase and isopeptidase activity, also mimics plant SUMO proteases to modify downstream plant proteins (Hotson et al., 2003).

Phytophthora parasitica is a typical hemibiotrophic plant pathogen with a wide host spectrum (Meng et al., 2014; Kamoun et al., 2015). At the early stages of infection, P. parasitica evades or suppresses programmed cell death; however, late in the infection process it triggers cell death, causing disease lesions, which may promote pathogen colonization. P. parasitica secretes an arsenal of “weapons” to manipulate plant immunity. The elicitin ParA1 (Kamoun et al., 1993) and the NEP1-like protein NPP1 (Fellbrich et al., 2002) from P. parasitica were shown to trigger hypersensitive cell death on plant leaves. The RXLR effector PSE1 suppresses plant immunity by modulating auxin accumulation during infection (Evangelisti et al., 2013). Another RXLR effector PpE4, which is highly expressed early in infection, elicits strong cell death and contributes to pathogen virulence (Huang et al., 2019). However, the molecular mechanism by which P. parasitica infects plants is largely unclear. There are many uncharacterized proteolytic enzymes annotated in the P. parasitica genome, and whether or not they participate in pathogen infection is still unknown. In this study, we identified 80 cysteine proteases from P. parasitica and analysed the sequence conservation and expression pattern of those predicted to be secreted. PpCys44 and PpCys45, which are up-regulated during the late stages of infection and trigger cell death in N. benthamiana leaves, were further investigated.

2 | RESULTS

2.1 | P. parasitica encodes numerous cysteine proteases

Considering that cysteine proteases have been identified as virulence factors in bacteria (Shao et al., 2002; Dowen et al., 2009) and Leishmania (Descoteaux, 1998), we aimed to investigate the distribution and function of cysteine proteases in P. parasitica. Based on the publicly available P. parasitica INRA-310 genome, 80 candidate genes encoding cysteine proteases were identified (Supporting Information S1) and divided into 12 families (Supporting Information S2). C48 is the largest family with 35 members, the C1 family has 20 members, and both the C2 and OTU-like families have 7 members. Each of the other families has fewer than three members.

Virulence factors secreted by pathogens usually act in the extracellular space or enter the plant cell, therefore we also performed in silico prediction of the signal peptides of the candidate cysteine proteases. Twenty-two proteins have a predicted N-terminal signal peptide (Figure 1), most (19) of which belong to the clan CA (C1, C2, and C12 families) and clan CD (C13 family). To confirm that the predicted signal peptides are indeed functional, we tested the ability of the signal peptides to direct secretion of invertase using a yeast secretion system. All the strains expressing fusions of the predicted cysteine protease signal peptides with invertase (except for PpCys54) could reduce triphenyltetrazolium chloride (TTC) to the insoluble red compound triphenylformazan (TPF), indicating that the signal peptides are indeed functional. The yeast strain expressing a functional Avr1b signal peptide fused with invertase was used as a positive control (Dou et al., 2008; Song et al., 2015). In contrast, no colour change was observed for the control strain expressing the empty vector (Figure 2).
FIGURE 1  Phylogenetic analysis of the predicted secreted cysteine proteases of Phytophthora parasitica and analysis of their domain architectures. Phylogenetic analysis of secreted cysteine protease proteins was performed with MEGA X using the maximum-likelihood method. The coloured dots near the nodes of the branches represent the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates). The colours of each protein in the phylogenetic tree represent the cysteine protease families they belong to: brown, C69; purple, C26; grey, C2; sea green, C1; pink, C12; orange, C13. Coloured rectangles in the schematic diagrams of protein structures represent the different domains in each protein. These domains were predicted using NCBI’s Conserved Domain Database (Marchler-Bauer et al., 2017).

FIGURE 2  Functional analysis of predicted signal peptides using a yeast secretion system. Upper row photographs show yeast strains expressing the signal peptides from cysteine proteases fused with invertase grown on a CMD-W agar plate. Lower photographs show the reactions with triphenyltetrazolium chloride (TTC) solution; a colour change indicates the conversion of TTC to triphenylformazan catalysed by secreted invertase. A yeast strain carrying a construct expressing the Avr1b signal peptide fused with invertase was used as a positive control. A yeast strain carrying the empty vector (pSUC2) was used as a negative control.
Cysteine proteases usually have a prodomain that prevents unwanted protein degradation by preventing access of the substrate to the catalytic domain (Coulombe et al., 1996). However, most secreted cysteine proteases in P. parasitica did not have the N-terminal auto-inhibitory domain and only had a predicted catalytically active peptidase domain (Figure 1).

2.2 | Most predicted secreted cysteine proteases are conserved in P. parasitica and induced during infection

To investigate the similarity between predicted secreted cysteine proteases from different P. parasitica strains, we compared the protein sequences from reference strain INRA-310 with those from the other 12 sequenced P. parasitica strains. All the proteases were found in nearly all the P. parasitica strains tested, and the sequence similarities were higher than 90%, indicating their high level of conservation among different P. parasitica strains (Figure 3). We also analysed the sequence similarities between cysteine proteases from different Phytophthora species, i.e., P. sojae and P. infestans. We identified orthologs of all 22 predicted secreted proteases in the P. infestans genome and orthologs of 16 proteins in the P. sojae genome, indicating their conservation among different Phytophthora species (Supporting Information S3).

We next analysed the expression patterns of the genes encoding secreted cysteine proteases during infection using RNA-Seq data for N. benthamiana infected with P. parasitica Pp016 (Jia, 2017). Based on expression pattern, these genes were classified into two large clusters. The 12 genes in cluster II are highly induced during pathogen infection. Among these, PpCys44 and PpCys45 were barely detectable during the early stages of infection and highly induced at 24 and 48 hr post-inoculation (hpi). Among the genes in cluster I, four (PpCys54, PpCys43, PpCys70, and PpCys19) had barely detectable transcripts at all stages, and two (PpCys78 and PpCys39) were slightly down-regulated, and four (PpCys69, PpCys60, PpCys67, and PpCys65) were slightly induced during infection (Figure 4).

2.3 | Cysteine proteases induce cell death in various Nicotiana species

To investigate the influence of secreted cysteine proteases on plant immunity, we performed Agrobacterium tumefaciens-mediated transient expression of candidate cysteine proteases in N. benthamiana leaves to test whether they trigger cell death. PpCys44/45 (these two proteins share identical amino acid sequences, with similar nucleotide sequences except three single nucleotide polymorphism [SNPs] detected in their coding sequences and similar promoter sequences) and PpCys69 induced cell death in N. benthamiana leaves (Figure 5a), whereas no cell death occurred in the leaves expressing the other predicted secreted cysteine proteases (exclude PpCys54 and PpCys79, as these two genes were not amplified from cDNA successfully). The expression of all secreted cysteine proteases examined in N. benthamiana leaves were confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis (Supporting Information S4). We also tested the

FIGURE 3 The secreted cysteine proteases are conserved among different sequenced Phytophthora parasitica strains. P1976, P1569, PN47, 364, 149, 329, CJ01A1, H02, CJ02B3, IAC01/95, CJ05E6, and CHvinca01 are the P. parasitica strains whose genomes have been sequenced. The colours in this heatmap represent the percentage identity between genes in each strain compared with those in the reference strain INRA-310. Bright green indicates that the gene sequence is split between different contigs. Black indicates that the gene is absent in a strain.
ability of PpCys44/45 and PpCys69 to induce cell death in the leaves of other Nicotiana spp. and found that they also triggered cell death in N. tabacum, N. glutinosa, and N. nesophila leaves (Figure 5b). We also tested the influence of the secreted cysteine proteases on the pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) signalling pathway. The results showed that all secreted cysteine protease genes examined could not influence the hypersensitive response triggered by INF1 (Supporting information S5). As P. parasitica is a typical hemibiotrophic plant pathogen, and cell death in the late stage of infection may facilitate pathogen infection, we paid more attention to the function of PpCys44/45 (highly induced at 48 hpi).

Considering that virulence factors may function in different subcellular locations, we examined whether PpCys44/45 functions in the apoplastic space. A. tumefaciens cells carrying vectors harbouring the PpCys44/45 gene with or without a signal peptide were infiltrated into N. benthamiana leaves. As expected, the protein harbouring native signal peptide sequence induced typical cell death in N. benthamiana leaves; however, the protein without a signal peptide (PpCys44/45NC-3 × FLAG) did not induce cell death (Figure 5c). To confirm this result, we also replaced the native signal peptide sequence of PpCys44/45 with that of PR1a from N. benthamiana; the protein harbouring the PR1a signal peptide also triggered cell death (Figure 5c), confirming that PpCys44/45 functions in the apoplastic space. The expression of PpCys44/45NC-3 × FLAG in N. benthamiana leaves was confirmed by RT-PCR assay (Figure 5d).

2.4 The cysteine protease activity of PpCys44/45 is required for cell death activity

PpCys44/45 contains a typical cysteine protease domain, and we tested whether this domain has catalytic activity. We made three mutant versions of PpCys44/45 by replacing the three predicted catalytic residues with alanine (Ala) (Figure 6a). These three active site mutants failed to trigger cell death in N. benthamiana leaves, indicating that the predicted catalytic residues are required for the ability of PpCys44/45 to induce cell death (Figure 6b). The total protein of the infiltrated leaves was extracted at 18 hr after infiltration before the cell death was apparent (about 2 days after infiltration) and western blotting was performed to determine whether these genes were translated properly. All these five proteins accumulated in leaves and were of the expected size (Figure 6c). The failed detection of wild-type PpCys44/45 at 1.5 days post-agroinfiltration probably resulted from the poor cell functionality caused by the cell death, not by the specific degradation of PpCys44/45, though the cell death was not visible.

We next tested whether a typical cysteine protease inhibitor, E-64, could suppress the cell death triggered by PpCys44/45. We transiently expressed PpCys44/45 in N. benthamiana leaves, and 16 hr after infiltration the leaves were infiltrated with 400 μM E-64 or 4% dimethyl sulfoxide (DMSO) (vol/vol). As expected, the leaves
treated with DMSO showed a typical cell death phenotype, while the leaves treated with E-64 did not show any visible cell death (Figure 6d). This result indicates that the induction of cell death by PpCys44/45 depends on its cysteine protease activity.

2.5 | The cell death triggered by PpCys44/45 is dependent on NPK1

Hypersensitive cell death is also associated with the reactive oxygen species (ROS) burst. To test whether PpCys44/45 could induce the ROS burst, we performed a 3,3′-diaminobenzidine (DAB) staining assay on N. benthamiana leaves expressing PpCys44/45 or FLAG-green fluorescent protein (GFP). The leaves expressing PpCys44/45 were stained brown at 36 hr after infiltration, which indicates that PpCys44/45 induced a strong ROS burst. The control leaves expressing FLAG-GFP did not change colour (Figure 7a). We also tested whether PpCys44/45 induces the expression of the ROS marker genes RbohA and RbohB. The results showed that PpCys44/45 could significantly induce the expression of RbohB, but could not induce the expression of RbohA (Figure 7b, Supporting Information S6).

To investigate which pathway PpCys44/45 may elicit, we tested whether PpCys44/45 induces cell death in the plants with silenced expression of genes involved in plant immune signalling. We found that PpCys44/45 failed to trigger cell death in NPK1-silenced plants (Figure 7c,d), which indicates that the cell death triggered by PpCys44/45 is dependent on NPK1. The expression level of NPK1 in TRV-GFP and TRV:NPK1 plants was confirmed by quantitative reverse transcription PCR (RT-qPCR) (Figure 7e) and the expression of PpCys44/45 in NPK1-silenced plants was confirmed by RT-PCR (Figure 7f). The PpCys44/45-triggered cell death is not compromised in MEK1-, MEK2-, RAR1-, SIPK-, WRKY3-, SGT1-, and MYB1-silenced plants (Supporting Information S7).
To investigate the function of \( PpCys44 \) and \( PpCys45 \) in pathogen virulence, we performed CRISPR/Cas9-mediated genome modification to knock out \( PpCys44 \) and \( PpCys45 \). As these genes share similar nucleotide sequences (only three SNPs) and have similar expression patterns, we knocked out these two genes simultaneously. Based on PCR analysis, we identified three knockout transformants (3C-1B-3, 5B-5, and a2sg1+2-2) (Figure 8a), which were obtained from three independent transformations. The sequences of \( PpCys44 \) and \( PpCys45 \) in the genome of transformants were confirmed by sequencing. Both the \( PpCys44 \) and \( PpCys45 \) gene sequences are partially deleted in the three transformants (Supporting Information S8), which led to mutations in the cysteine protease domain. To test the influence of mutation of \( PpCys44 \) and \( PpCys45 \) on pathogen virulence, we inoculated detached \( N. \) benthamiana leaves with wild-type \( P. \) parasitica, knockout transformants and control (3C-2-3, a non-edited transformant recovered from the transformation experiment). These three knockout transformants produced lesions similar to those caused by the wild-type or control (Figure 8b,c). However, the biomass of \( P. \) parasitica on the leaves infected by knockout transformants was lower than that on leaves infected by the wild-type or control (Figure 8d). This result indicates that \( PpCys44/45 \) contributes to the virulence of \( P. \) parasitica.

### 3 | DISCUSSION

Cysteine proteases have been implicated as important virulence factors in bacteria (Hotson and Mudgett, 2004) and fungi (Ball et al., 1991). Zuluaga et al. (2016) found that cathepsin B and papain-like cysteine proteases were abundant during the \( P. \) infestans–tomato compatible interaction, peaking at 96 hr after inoculation, indicating that cysteine proteases may play a role in Phytophthora–plant interactions. In this study, we evaluated the expression patterns, protein sequence similarities among different strains, and cell-death-triggering abilities of the cysteine proteases secreted by \( P. \) parasitica. We then systematically investigated the functions of two cysteine proteases, \( PpCys44 \) and \( PpCys45 \) during the compatibility interaction.
and PpCys45, which are highly expressed during the late stage of infection and trigger cell death in different Nicotiana species.

Apart from a few unconventionally secreted effectors (Liu et al., 2014), effectors usually possess an N-terminal signal peptide. In this study, we found that more than a quarter of P. parasitica cysteine protease proteins contained a predicted signal peptide, and the secretion of most of these proteins was verified using a yeast secretion system (Figure 2), indicating that these cysteine proteases may be secreted by the pathogen and play a role in P. parasitica pathogenesis. As reported previously, functionally important effectors are usually induced during infection (Wang et al., 2011a). In this study, 16 out of 21 secreted cysteine proteases were induced during P. parasitica infection (Figure 4). A similar result was also reported in another Phytophthora pathosystem, in which 12 cysteine proteases from P. infestans were found to accumulate at three different stages during infection of tomato plants (Zuluaga et al., 2016), four of which are orthologs of the cysteine proteases from P. parasitica (PpCys10, PpCys17, PpCys39, and PpCys53). We found that all the secreted cysteine proteases are highly conserved among different P. parasitica strains (Figure 3) and most are conserved among three different Phytophthora species. Taken together, these characteristics of cysteine proteases indicate that they may contribute to the pathogenesis of P. parasitica.

As cell death can limit the development of some pathogens, it has long been considered as a resistance phenotype. However, in certain pathosystems, cell death is often uncoupled with resistance (Coll et al., 2010). Some necrotrophic pathogens, such as Cochliobolus victoriae, secrete toxins to hijack the plant cell death mechanism to kill plant cells and feed on the cell remnants (Lorang et al., 2012). P. parasitica is a typical hemibiotrophic plant pathogen and induces plant cell death during compatible interactions, which may promote infection. In this
study, we identified two papain-like cysteine proteases with identical protein sequences, PpCys44 and PpCys45, which induced cell death in the leaves of different Nicotiana spp. (Figure 5). PpCys44 and PpCys45 contain a conserved cysteine protease C1 domain and the ability of PpCys44/45 to trigger cell death depends on its cysteine protease activity. There are two possible explanations for how PpCys44/45 induces cell death. First, as an active enzyme, the mature PpCys44/45 protein may cleave plant substrates, and the modified substrates may trigger a downstream cell death pathway. A similar mechanism was reported previously: AvrPphB cleaves PBS1 and modified PBS1 is recognized by an R protein, leading to hypersensitive cell death (Shao et al., 2003). Second, as reported before, pathogen effectors may promote infection by mimicking the activity of plant proteins (Hotson et al., 2003).

Plant papain-like cysteine proteases have been shown to participate in hypersensitive cell death (McLellan et al., 2009). PpCys44/45 shares similar enzyme domains with plant proteins, and may mimic plant papain-like proteins and take part in the cell death pathway.

In this research, we determined that the cell death triggered by PpCys44/45 is dependent on NPK1 (Figure 7). NPK1 is a tobacco MAP kinase kinase kinase (MAPKKK) homolog of human MEKK1, and the silencing of NPK1 could interfere with the function of R genes N, Bs2, and Rx (Jin et al., 2002) and the ROS burst elicited by INF1 (Asai et al., 2008). The involvement of NPK1 in PTI and R gene-mediated plant immune signalling indicates that PpCys44/45-triggered cell death is possibly a consequence of plant recognition. However, PpCys44/45-triggered cell death is not dependent on the

FIGURE 8 PpCys44- and PpCys45-deficient double mutants of Phytophthora parasitica showed reduced pathogenicity. (a) PCR detection of the mutations in PpCys44 and PpCys45 in four transformants. PpCys44 and PpCys45 were knocked out in 3C-1B-3, a2sg1+2-2, and 5B-5. 3C-2-3 is a non-edited transformant recovered from the transformation experiment that was included as a control. (b) Trypan blue staining of Nicotiana benthamiana leaves infected with P. parasitica. There were no differences in lesion size between the true transformants and the wild-type strain Pp016 or the control transformant 3C-2-3. (c) Statistical analysis of the diameters of lesions on N. benthamiana leaves infected with P. parasitica. Student’s t test; ns, p > .05. (d) Biomass of P. parasitica on the infected N. benthamiana leaves determined using quantitative PCR. Pp/Nb, the level of WS041 from P. parasitica (Zhang, 2012) relative to the level of NbActin from N. benthamiana. Student’s t test; ** p < .01; * p < .05.
MAPK cascade genes MEK1, MEK2, and SIPK, the transcription factors WRKY3 and MYB1, and the R gene function-related genes RAR1 and SGT1. To illustrate the exact immune pathway PpCys44/45 elicited, identification of the host target of PpCys44/45 is helpful and should be performed in the future.

The CRISPR/Cas9 system has been widely used for genome modification in different species. As for Phytophthora spp., CRISPR/Cas9-mediated genome modification has been established in P. sojae (Fang and Tyler, 2016; Fang et al., 2017) and P. capsici (Wang et al., 2018). In this study, we found that with minor modification the protocol reported by Fang et al. (2017) also works for P. parasitica. The successful modification of the P. parasitica genome using the CRISPR/Cas9 method will advance the functional analysis of P. parasitica effectors and other genes. In this study, we tried to knock out both PpCys44 and PpCys45 in the P. parasitica genome simultaneously, and the mutation efficiency was not very high. From about 400 transformants, we only obtained three independent homozygous knockout transformants. The results of PCR product sequencing confirmed that all alleles of PpCys44 and PpCys45 were edited at two different sites, which lead to partial deletion of these alleles. In the future, gene replacement and gene complementation may be performed to investigate the functions of candidate genes in P. parasitica using this CRISPR/Cas9 system.

We found that these successful transformants showed decreased virulence. This result indicates that PpCys44 and PpCys45 contribute to pathogenicity. Expression pattern analysis showed that PpCys44 and PpCys45 are very lowly expressed during the early stages of infection and then highly induced during the late stages of infection. Taken together, we speculate that these two genes do not participate in early stage lesion expansion but function at the late stages of infection, during which the pathogen kills the plant cell and feeds on cell remnants.

In summary, we show that most genes encoding the highly conserved secreted cysteine proteases from P. parasitica were up-regulated during infection. Two of them, PpCys44 and PpCys45, contribute to the pathogen virulence by inducing NPK1-dependent cell death in different Nicotiana spp. leaves, which is dependent on its enzymatic activity.

4  |  EXPERIMENTAL PROCEDURES

4.1  |  Plant and P. parasitica cultivation and inoculation assays

N. benthamiana seeds were sown on soil in a matrix and cultured in a Phytootron with a 13 hr light:11 hr dark photoperiod for about 30–40 days. P. parasitica strains were routinely maintained on 5% carrot agar (CA) plates (Zhang et al., 2019). Incubation conditions were as follows: 23 °C, darkness. The P. parasitica strain used in this study is Pp016, which was obtained in Australia (Wang et al., 2011b).

For P. parasitica inoculation, P. parasitica strains were grown on a 5% CA plate for 3 days, then 5-mm diameter mycelia plugs were taken around the colony edge. Detached N. benthamiana leaves were placed in a plastic tray covered with moist filter paper. Slight wounds were made by poking both sides of a vein with a toothpick. Mycelia plugs from transformants and the control were inoculated on opposite sides of one leaf and incubated at 23 °C for 48 hr. Ten leaves were used for one experiment and the experiment was performed three times.

4.2  |  Bioinformatic analysis of the cysteine protease family

Cysteine protease members in the publicly available P. parasitica INRA-310 v. 2 genome (GenBank assembly accession: GCA_000247585.2) were identified by aligning the predicted proteins against NCBI’s Conserved Domain Database (Marchler-Bauer et al., 2017). The orthologs of the secreted cysteine proteases in the sequenced oomycete genomes, P. infestans (GenBank assembly accession: GCA_000142945.1) and P. sojae (GenBank assembly accession: GCA_000149755.2), were identified using the BLASTP algorithm. Multiple sequence alignments were performed with ClustalW2 (Larkin et al., 2007) using the default parameters. Phylogenetic dendrograms were generated in MEGA X (Kumar et al., 2018) using the maximum-likelihood method with 1,000 bootstrap replications. Signal peptide prediction was performed using SignalP v. 4.0 (Petersen et al., 2011). The heatmap of protein sequence similarity was generated with TBtools (Chen et al., 2018). The expression pattern of secreted cysteine proteases was analysed based on the RNA-Seq data for N. benthamiana plants infected with P. parasitica Pp016 (Jia, 2017). Jia (2017) collected the infected leaves for RNA sequencing at 3, 6, 12, 24, and 48 hpi depending on the life cycle of P. parasitica. The fragments per kilobase of transcript per million mapped reads (FPKM) values of all the genes were calculated with TopHat and Cufflinks (Trapnell et al., 2012) with the default parameter. The FPKM values of secreted cysteine protease genes were sorted out and the heatmap was generated with TBtools.

4.3  |  Cloning procedures and plasmid constructs

To generate 3 × FLAG-tagged versions of the candidate proteins, we first constructed a vector carrying the 3 × FLAG sequence. The 3 × FLAG sequence was synthesized by annealing long primers and extending them with the high-fidelity DNA polymerase FastPfu (TransGen Biotech) to form double-stranded DNA (dsDNA). Then this short dsDNA fragment was inserted into the XhoI/XbaI restriction sites of pKannibal (Wesley et al., 2001) using a ClonExpress II One Step Cloning Kit (Vazyme). The vector was digested with NotI restriction enzyme (NEB), and the smaller fragment was inserted into the binary vector pART27 (Gleave, 1992) at the NotI restriction site. The resulting plasmid was named P27:3 × FLAG. The open reading frame sequence of each gene was amplified from P. parasitica Pp016 cDNA and inserted into P27:3 × FLAG at the XbaI restriction site using a ClonExpress II One Step Cloning Kit (Vazyme).
To create the vectors used for signal peptide functional analysis, the signal peptide sequences of the secreted cysteine proteases were cloned from *P. parasitica* Pp016 genomic DNA and inserted into the EcoRI/Xhol restriction sites of pSUC2 (Jacobs et al., 1997; Yin et al., 2018) using a ClonExpress II One Step Cloning Kit (Vazyme).

To generate vectors for expressing the truncated version of PpCys44/45, the truncated fragment was amplified from vector P27:PPpCys44/45-3 × FLAG and inserted into P27:3 × FLAG at the XhoI/Xhol restriction sites using a ClonExpress II One Step Cloning Kit (Vazyme). The signal peptide sequence of PR1a was amplified from *N. benthamiana* cDNA and cloned in frame with the N-terminus of truncated PpCys44/45. The three catalytic site mutants of PpCys44/45 were created by introducing a point mutation with traditional overlap PCR. All primers used in this study are listed in Supporting Information S9.

### 4.4 Functional evaluation of the signal peptide sequences of secreted proteins

Functional evaluation of the predicted signal peptide sequences was performed according to a previously published protocol (Yin et al., 2018). Briefly, the sequence-verified pSUC2 constructs were transformed into yeast YTK12 competent cells. The transformants that grew on a selective CMD–W (complete minimal media lacking tryptophan) agar plate were inoculated into 3 ml of liquid CMD–W broth and incubated at 30 °C with shaking for 36 hr. The cells were harvested by centrifugation and then washed with sterile distilled water of the solution.

After the addition of 900 μl of sterile ddH₂O, and 250 μl of 10 mM NaOAc (pH 4.7) and 500 μl of 10% sucrose (wt/vol) were added into the tube. After incubation in a 37 °C water bath for 10 min, the tubes were spun at 12,000 × g for 1 min and 100 μl of supernatant was transferred into a new tube. After the addition of 900 μl of 0.1% TTC solution, the tubes were incubated at room temperature for 5 min. Those strains carrying a functional signal peptide can secrete invertase and reduce TTC to the insoluble red compound TPF, which leads to a change in the colour of the solution.

### 4.5 Agrobacterium-mediated transient expression and virus-induced gene silencing assay

*A. tumefaciens* GV3101 carrying different constructs were cultured in Luria Bertani (LB) liquid medium supplemented with the appropriate antibiotics. After incubation with shaking at 28 °C for about 36 hr, the bacterial cells were harvested, resuspended in infiltration buffer (Huang et al., 2019), and adjusted to the appropriate concentration. For transient expression, the bacterial concentration was adjusted to OD₆₀₀ = 0.4. The biggest leaves of 30- to 40-day-old *N. benthamiana* seedlings were infiltrated with a needleless syringe. An *A. tumefaciens* strain harbouring a plasmid expressing FLAG-GFP was used as a control. To test whether the secreted cysteine protease genes influence the cell death triggered by INF1, the *A. tumefaciens* strains carrying vectors with a construct of secreted cysteine protease gene or GFP were infiltrated into the *N. benthamiana* leaves (OD₆₀₀ = 0.4). Twenty-four hours later, the *A. tumefaciens* strains carrying INF1 (OD₆₀₀ = 0.05) were infiltrated into the same area of the leaves. The cell death phenotype was photographed at 5 days after infiltration. Statistical significance was determined by the Wilcoxon rank-sum test. For the virus-induced gene silencing (VIGS) assay, *A. tumefaciens* strains harbouring different pTRV2 constructs were co-infiltrated in equal ratios with those harbouring pTRV1 vectors with a final OD₆₀₀ of 0.3. pTRV2:GFP was used as a control and pTRV2:PDS (phytoene desaturase) (Liu et al., 2002a) was used to monitor the silencing process. The lowest two leaves of four-leaf stage seedlings were infiltrated as previously described (Ratcliff et al., 2001; Liu et al., 2002b).

Eight plant genes involved in the plant immune signalling (Huang et al., 2019), SG1, NPK1 (*Nicotiana Protein Kinase* I), MEK1, MEK2, SIPK, MYB1, RAR1, and WRKY3 were silenced to test which pathway PpCy44/45 may elicit. The pTRV2 constructs for silencing these genes and primers used for the gene expression level detection are the same as described previously (Huang et al., 2019).

### 4.6 DAB staining

DAB staining was performed according to a previously published protocol (Daudi and O’Brien, 2012) with minor modifications. Briefly, *N. benthamiana* leaves were stained with DAB solution (1 mg/ml) at 1.5 days after infiltration before the cell death phenotype was apparent. The leaves were incubated in DAB solution with shaking (100 rpm) for 4 hr. Then the DAB staining solution was replaced with bleaching buffer (ethanol:acetic acid:glycerol, 3:1:1 vol/vol/vol) and the leaves were extensively cleared by incubating them in a boiling water bath for about 15 min.

### 4.7 Single-guide RNA design and CRISPR/Cas9 plasmid preparation

The protocols for single-guide RNA (sgRNA) design and CRISPR/Cas9 plasmid preparation were modified from previously published protocols (Fang et al., 2017). The sgRNA for PpCys44/45 was designed with EuPaGDT (http://grna.ctegd.uga.edu/), and sgRNAs with a total score larger than 0.5 were chosen for RNA secondary structure prediction and off-target analysis. Three candidate sgRNAs (sg1, sg2, and sg3) without strong RNA secondary structure and off-target sites were chosen for primer synthesis. Synthesized primer pairs were annealed to each other and extended using the high-fidelity DNA polymerase FastPfu (TransGen Biotech) to make the dsDNA fragment. The dsDNA fragment was inserted into the *NheI/Bsal* restriction sites of pyFS15 (Fang et al., 2017) using a ClonExpress II One Step Cloning Kit (Vazyme). The sequence-verified plasmid was purified from *Escherichia coli* DH5α cells with a Maxi Plasmid Kit (Omega Bio-Tek). The concentration of prepared plasmid was...
measured using a NanoDrop spectrophotometer, and the DNA solution was diluted to 0.5 μg/μl with ddH₂O.

4.8 | Genome editing of *P. parasitica* using CRISPR/Cas9

Genome editing of *P. parasitica* using CRISPR/Cas9 was performed following a previously published protocol for *P. sojae* (Fang et al., 2017) with minor modifications. *P. parasitica* strain Pp016 was inoculated onto a 5% CA plate and incubated in the dark at 23 °C for 3 days. Ten fresh *P. parasitica* discs taken from the colony edge were transferred into a 250 ml flask containing 50 ml of nutrient pea broth and incubated at 23 °C in the dark without shaking for 2 days. Four flasks of *P. parasitica* culture were sufficient for four transformations. The entire culture was poured into a sterilized 1-L beaker covered with a layer of Miracloth culture were sufficient for four transformations. The entire culture was poured into a sterilized 1-L beaker covered with a layer of Miracloth and three layers of cheesecloth. The mycelial culture was washed with sterile ddH₂O, then washed in 0.8 M mannitol for 10 min. The hyphal mats were poured into another beaker covered with Miracloth and cheesecloth to remove the mycelium. Then the hyphae were uniformly dispersed in 40 ml of fresh enzyme solution (0.4 M mannitol, 20 mM KCl, 20 mM MES [pH 5.7], 10 mM CaCl₂, 2% [wt/vol] Lysing Enzyme from *Trichoderma harzianum* [cat. no. L1412, Sigma] and 2% [wt/vol] cellulose from *Trichoderma viride* [cat. no. 219466, Sigma]) and digested for about 1.5 hr with gentle shaking (40 rpm). The digestion products were filtered through a 70 μm nylon mesh to remove the hyphal debris and collected by centrifugation for 5 min at 500 × g. The protoplasts were then washed twice with W5 solution, followed by incubation on ice for 30 min. The protoplasts were collected again by centrifuging as described above, resuspended in 4 ml of MMG solution (4 mM MES, 0.4 M mannitol, 15 mM MgCl₂, pH 5.7), then placed at room temperature for 10 min. The CRISPR/Cas9 plasmid (three different combinations: 60 μg of sg1 and sg2 [1:1] or 60 μg of sg1 and sg3 [1:1:1] or 90 μg of sg1, sg2, and sg3 [1:1:1]) and 1 ml of protoplasts were gently added into a 50 ml tube. The mixture was placed on ice for 20 min. The protoplasts were mixed gently with 1.7 ml of 40% polyethylene glycol solution until well mixed and placed on ice for 20 min. Then, 10 ml of pea broth/0.5 M mannitol was added to each tube in two batches and the tube was placed on ice for 2 min after each addition. The solution was poured into a deep 90-mm Petri dish containing 10 ml of pea broth/0.5 M mannitol supplemented with 100 μg/ml ampicillin and 10 μg/ml rifampicin and mixed well. The transformed protoplasts were incubated at 23 °C without shaking for 22 hr. The regenerated protoplasts were collected by centrifuging at 2,000 × g and resuspended in 4 ml ddH₂O. Then the resuspended protoplasts were diluted with 150 ml of c.40 °C liquid 5% CA medium containing 0.8% agar supplemented with 200 μg/ml ampicillin, 20 μg/ml rifampicin, 20 μg/ml nystatin, and 10 μg/ml G418 and poured into nine 90-mm Petri dishes. Then the Petri dishes were kept at 23 °C for 5 days or until mycelial growth appeared. The candidate transformants were transferred to a new 5% CA plate containing 200 μg/ml ampicillin, 20 μg/ml rifampicin, 20 μg/ml nystatin, and 10 μg/ml G418. The nutrient pea broth and pea broth/0.5 mannitol were prepared according to Fang et al. (2017).

4.9 | DNA extraction and screening for genome-edited transformants

After incubation in the dark at 23 °C for 3 days, *P. parasitica* discs taken from the edges of the colony of candidate transformants were inoculated into a 12-well plate containing 5 ml of 5% CA broth. Mycelia were harvested after incubation at 23 °C in the dark for 4 days and ground to fine powder in liquid nitrogen. The mycellal powder was resuspended with 500 μl of DNA extraction buffer (200 mM Tris-HCl [pH 8.0], 25 mM EDTA [pH 8.0], 200 mM NaCl, 2% SDS [wt/vol], and 0.1 mg/ml RNase A) (Fang et al., 2017) and extracted with 250 μl Tris-saturated phenol (pH 8.0) and 250 μl chloroform. The DNA was precipitated with 800 μl of a mixed solution of 7.5 M NH₄OAC and isopropanol (1:3 vol/vol) and washed with 70% ethanol. The air-dried DNA pellet was dissolved with 30 μl ddH₂O. Primers PpCys44-F/PpCys44-R and PpCys45-F/PpCys45-R were used to amplify the full-length coding sequences of PpCys44 and PpCys45, respectively, and the PCR products were assessed with agarose electrophoresis. We could distinguish the wild-type and heterozygous and homozygous transformants based on the PCR product size. The PCR products from candidate genome-edited strains were reconfirmed by sequencing.

ACKNOWLEDGEMENTS

This work was supported by the China Agriculture Research System (CARS-09), National Natural Science Foundation of China (31125022 and 31561143007), and the Programme of Introducing Talents of Innovative Discipline to Universities (project 111) from the State Administration of Foreign Experts Affairs (#B18042).

AUTHOR CONTRIBUTIONS

W.S., Y.M., and Q.Z. designed the experiments. Q.Z., W.L., J.Y., Y.M., and J.X. performed the experiments. Q.Z., W.L., Y.M., and W.S. analysed the data. Q.Z., Y.M., and W.S. wrote the manuscript.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Zhang Q, Li W, Yang J, Xu J, Meng Y, Shan W. Two Phytophthora parasitica cysteine protease genes, PpCys44 and PpCys45, trigger cell death in various Nicotiana spp. and act as virulence factors. *Molecular Plant Pathology*. 2020;21:541–554. [https://doi.org/10.1111/mpp.12915](https://doi.org/10.1111/mpp.12915)