Identification and Characterization of *Plasmodiophora brassicae* Primary Infection Effector Candidates that Suppress or Induce Cell Death in Host and Nonhost Plants

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ABSTRACT

Clubroot caused by *Plasmodiophora brassicae* is one of the most important diseases in cruciferous crops. The recognition of *P. brassicae* by host plants is thought to occur at the primary infection stage, but the underlying mechanism remains unclear. Secretory proteins as effector candidates play critical roles in the recognition of pathogens and the interactions between pathogens and hosts. In this study, 33 *P. brassicae* secretory proteins expressed during primary infection were identified through transcriptome, secretory protein prediction, and yeast signal sequence trap analyses. Furthermore, the proteins that could suppress or induce cell death were screened through an *Agrobacterium*-mediated plant virus transient expression system and a protoplast transient

expression system. Two secretory proteins, PBCN_002550 and PBCN_005499, were found to be capable of inducing cell death associated with H₂O₂ accumulation and electrolyte leakage in *Nicotiana benthamiana*. Moreover, PBCN_002550 could also induce cell death in Chinese cabbage. In addition, 24 of the remaining 31 tested secretory proteins could suppress mouse Bcl-2-associated X protein-induced cell death, and 28 proteins could suppress PBCN_002550-induced cell death.

Keywords: plant immunity, *Plasmodiophora brassicae*, primary infection, secretory proteins

Clubroot is one of the most important diseases in cruciferous crops, including Chinese cabbage, oilseed rape, and radish, and it has been observed in >60 countries worldwide (Dixon 2009). In China, clubroot disease has been found in most provinces and affects ~4 million ha per year (Chai et al. 2014). Clubroot disease is caused by *Plasmodiophora brassicae*, which is a soilborne obligate protist that belongs to the genus *Plasmodiophora* in Rhizaria. Rhizaria shares a common ancestor with Stramenopiles and Alveolata (Schwelm et al. 2016, 2018; Sierra et al. 2016). The disease cycle of *P. brassicae* in plants consists of two phases: primary infection, which mainly occurs in root hairs, and secondary infection, which occurs in tissues adjacent to the root cortex (Kageyama and Asano 2009). Primary infection can occur in both host and nonhost plants, but secondary infection can occur only in host plants (Feng et al. 2012). The recognition of *P. brassicae* by host plants is thought to occur during primary infection (McDonald et al. 2014), but the mechanism underlying the recognition of *P. brassicae*, the interactions between *P. brassicae* and plants, and the mechanism through which *P. brassicae* overcomes plant immunity to achieve successful infection are not well understood.

The plant immune system has two branches (Jones and Dangl 2006). One is referred to as pathogen-associated molecular pattern or microbe-associated molecular pattern-triggered immunity (PTI), which can halt further colonization by both pathogens and nonpathogens. However, successful pathogens can deploy effectors to interfere with PTI, which results in effector-triggered susceptibility. The second branch of the plant immune system utilizes nucleotide-binding site leucine-rich repeat (NB-LRR) proteins to recognize pathogen effectors and consequently, activate plant immunity. This branch is referred to as effector-triggered immunity.

From the perspective of pathogens, effectors are critical for successful infection. Specifically, effectors secreted by pathogens are important molecules that can modulate plant defenses and enhance microbial fitness (Hogenhout et al. 2009). Secretory proteins as effector candidates have been widely studied in fungi, oomycetes, bacteria, and nematodes (Block et al. 2014; Erickson et al. 2018; Li et al. 2017; Lyu et al. 2016; Ma et al. 2017; Nguyen et al. 2018; Zheng et al. 2018). Many secretory proteins play a role as pathogen-associated molecular patterns or microbe-associated molecular patterns that can elicit plant immunity (Chen et al. 2013; Kamoun et al. 1998; Wei et al. 1992; Yang et al. 2018). Many other secretory proteins have been reported to suppress plant immunity (King et al. 2014; Lin et al. 2016; Liu et al. 2016).

Five hundred fifty-three of the 9,730 genes in the *P. brassicae* isolate e3 genome are predicted to encode secretory proteins (Schwelm et al. 2015). Although clubroot and *P. brassicae* have been studied for >140 years, few studies have investigated the functions of secretory proteins and effectors in the *P. brassicae* infection process and subsequent disease progression (Pérez-López et al. 2018). Studies showed that a methyltransferase secreted by *P. brassicae* can methylate salicylic acid and alter host susceptibility to *P. brassicae* (Bulman et al. 2019; Ludwig-Müller et al. 2015). However, the identities of the other proteins involved in the *P. brassicae* infection process and subsequent disease progression remain unknown.

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In our research, five transcriptomes of hydroponic oilseed rape during primary infection with *P. brassicae* were sequenced. The *P. brassicae* genes expressed during primary infection were screened by mapping to the *P. brassicae* isolate e3 genome and analyzing the fragments per kilobase per million reads (FPKM) value. Putative secretory protein genes were then predicted using bioinformatics software, and their signal peptide (SP) functions were tested through a yeast trap assay. The capacities of these secretory proteins to induce or suppress cell death were subsequently assessed. The results of these experiments revealed the functions of *P. brassicae* secretory proteins and lay a foundation for additional studies of the mechanisms through which *P. brassicae* interferes with plant immunity.

MATERIALS AND METHODS

***P. brassicae* and plants.** The *P. brassicae* isolate used in this study was collected from an oilseed rape field located in Yichang city, Hubei Province, China. The club roots were stored at -80°C until resting spore extraction and *P. brassicae* DNA extraction. *Nicotiana benthamiana* and the hydroponic oilseed rape line ZS11 were grown in a greenhouse at $\sim 22^{\circ}\text{C}$ with a 16-h light/8-h dark cycle. Chinese cabbage (Beijing New No. 3) plants were grown on Murashige and Skoog Basal medium at $\sim 22^{\circ}\text{C}$ in total darkness for protoplast preparation.

Transcriptome analysis and prediction of putative secretory proteins. Hydroponic oilseed rape was inoculated with resting spores of *P. brassicae* as described previously (Luo et al. 2014). After seed germination, the seedlings were fastened in 1-cm-thick foam in the top of opaque tubes filled with half-strength Hoagland nutrient solution (pH 5.5). Seven days later, the seedlings were inoculated with *P. brassicae* resting spores at a final concentration of $10^7/\text{ml}$. At 3 days postinoculation (dpi), five sets of roots from *P. brassicae*-infected plants were collected for RNA extraction using the TRIzol reagent (Invitrogen) according to the manufacturer's recommended protocol. Five micrograms of total RNA from each sample was subjected to one round of poly-A selection on oligo (dT) Sera-Mag beads, and the resultant messenger RNA was fragmented to an average size of 500 base pairs according to the manufacturer's recommended protocol (Illumina). The manufacturer's (Illumina) recommended protocols were used for first-strand and second-strand complementary DNA (cDNA) synthesis and preparation of the RNA sequencing samples. All of the libraries were sequenced on a HiSeq 2500 instrument to obtain 125-base pairs paired-end reads, and five transcriptome datasets were obtained. For gene expression analysis, the reads were mapped to the *P. brassicae* e3 genome (accession no. CDSF00000000) (Schwelm et al. 2015) using SOAP, a bioinformatics software for sequence assembly and analysis (Li et al. 2008) with the default parameters. The uniquely mapped reads were then extracted, and the expression of the unigenes was normalized using the FPKM value.

The protein sequences derived from the *P. brassicae* isolate e3 genome were used for the prediction of secretory proteins. Only those proteins that contained SPs but not additional transmembrane regions and that were ≤ 300 amino acids were identified as secretory proteins. The SPs were predicted using SignalP v4.1 (Petersen et al. 2011) with sensitive D-cutoff values, and TMHMM v2.0 (Krogh et al. 2001) was then used for the prediction of transmembrane regions based on the protein sequences without SPs.

Assessment of secretory activity directed by SPs from putative secretory proteins in yeast. A yeast signal sequence trap assay was adopted to assess the secretory activity directed by SPs (Jacobs et al. 1997). The DNA sequences encoding SPs were amplified by a high-fidelity DNA polymerase (Tsingke Biotech Co., LTD), and the purified DNA was then cloned into the linearized pSUC2T7M13ORI plasmid using the ClonExpress II One Step Cloning kit (Vazyme Biotech Co., LTD). The primers used in this assay are listed in Supplementary Table S1. The recombinant vectors were transformed into the invertase-negative yeast strain YTK12. Positive clones were used for the assessment of secretory activity through growth assays on YPRAA (a medium containing

1% yeast extract, 2% peptone, 2% raffinose, 2 $\mu\text{g}/\text{ml}$ antimycin, and 2% agar) plates (with raffinose as the sole carbon source) and through a 2,3,5-triphenyltetrazolium chloride (TTC) assay according to a previous study (Zhang et al. 2018). The transformed yeast can secrete invertase into the extracellular environment and grow on YPRAA plates only if the YTK12 strain is transformed with a pSUC2 vector containing a functional SP, and the secreted invertase can reduce TTC to insoluble, red-colored 1,3,5-triphenylformazan (TPF). Otherwise, the yeast is unable to grow on YPRAA plates or reduce TTC to insoluble, red-colored TPF.

***Agrobacterium*-mediated plant virus transient expression in *N. benthamiana* leaves.** Proteins with a functional SP in yeast were tested for the ability to induce or suppress plant cell death triggered by the mouse BAX protein (Lacomme and Cruz 1999) and the cell death inducer from *P. brassicae* as described previously (Wang et al. 2011). The binary potato virus X (PVX) vector pGR107 was used to express genes in *N. benthamiana*. This vector was derived from the pgRenn0000 vector. A 35S promoter promoting the transcript of the PVX genome was located between the left and right transfer DNA border sequences, and a multiple clone site was inserted into the coat protein promoter (Wang et al. 2011). The sequences encoding mature secretory proteins (without SPs) were amplified from cDNA (derived from RNAs for transcriptome sequencing) or genomic DNA of *P. brassicae* as the templates. The coding sequence (without introns) was cloned into pGR107 using the ClonExpress II One Step Cloning kit (Vazyme Biotech Co., LTD). The sequenced recombinant plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101. The positive clones were used for additional injection according to a previously described method (Wang et al. 2011).

To identify the secretory proteins that induced cell death or suppressed cell death triggered by BAX, the optical density (OD_{600}) value of the *A. tumefaciens* suspension was adjusted to 0.4, and *A. tumefaciens* carrying pGR107-BAX was injected 12 h after the injection of *A. tumefaciens* carrying secretory protein-encoding genes. For the screening of secretory proteins that suppress cell death induced by PBCN_002550, the OD_{600} value of the *A. tumefaciens* suspension was adjusted to 1.0. Equal amounts of *A. tumefaciens* carrying pGR107-PBCN_002550 and *A. tumefaciens* carrying genes encoding other secretory proteins were mixed and injected into *N. benthamiana* leaves. The necrosis of *N. benthamiana* leaves was investigated 7 dpi of the genus *Agrobacterium* carrying pGR107-BAX or the mixture of the genus *Agrobacterium* carrying pGR107-PBCN_002550 and genes encoding other secretory proteins. *N. benthamiana* leaves injected with the genus *Agrobacterium* carrying pGR107-eGFP were used as a negative control.

The expression levels of the secretory proteins and BAX were assessed by western blotting. BAX was fused with an HA tag, and thus, an anti-HA tag antibody (Sigma-Aldrich) was used for BAX detection. The secretory proteins were fused with an FLAG tag, and an anti-FLAG tag antibody (Sigma-Aldrich) was used for the detection of secretory proteins. The *N. benthamiana* leaves injected with the genus *Agrobacterium* carrying pGR107 were used as a negative control for detection of the unspecific binding of the antibody.

Detection of H_2O_2 accumulation and quantification of electrolyte leakage. The accumulation of H_2O_2 was detected by 3,3'-diaminobenzidine (DAB) staining (Thordal-Christensen et al. 1997). Whole *N. benthamiana* leaves were harvested at 3 dpi with *A. tumefaciens* and soaked in 1 mg/ml of DAB solution (pH 3.8) at room temperature. Eight hours later, the leaves were discolored by boiling in 95% ethanol for 15 min, cleared with 2.5 mg/ml of trichloroacetic aldehyde solution, and photographed. The degree of electrolyte leakage was quantified at 7 dpi. Leaf discs ~ 1 cm in diameter were harvested and soaked in 5 ml of double-distilled water for 3 h, and the electrical conductivity was then quantified and recorded as E1. The leaf discs were boiled in double-distilled water for 25 min. After cooling to room temperature, the electrical conductivity was quantified and recorded as E2. The degree of

electrolyte leakage was calculated as follows: electrolyte leakage = $E1/E2 \times 100\%$ (Mittler et al. 1999).

Protoplast preparation and transformation. The vectors used for protoplast transformation were derived from pGL3 control (Groskreutz et al. 1995). The SV40 promoter was replaced by the 35S promoter from the pBI121 vector (Chen et al. 2003). DNA fragments containing the 35S gene Tnos sequence were inserted between the *Sall* and *Bam*HI restriction enzyme sites using the ClonExpress II One Step cloning kit (Vazyme Biotech Co., LTD). The recombinant plasmids were then extracted using an EndoFree Maxi Plasmid kit (TIANGEN Biotech Co., LTD) and adjusted to 2 mg/μl for transformation.

The hypocotyls of Chinese cabbage were collected for protoplast preparation according to the method described previously (Cheng et al. 1994b) with several modifications. Seven-day-old hypocotyls were harvested. The hypocotyls were cut in half along the longitudinal axis, and then, they were cut into 1-cm segments. The segments were plasmolyzed in cell and protoplast washing (CPW) solution (27.2 mg/liter of KH_2PO_4 , 101.0 mg/liter of KNO_3 , 1,480.0 mg/liter of $CaCl_2 \cdot 2H_2O$, 246.0 mg/liter of $MgSO_4 \cdot 7H_2O$, 0.025 mg/liter of $CuSO_4 \cdot 5H_2O$, and 0.16 mg/liter of KI, pH 5.6) supplemented with 13% mannitol for ~1 h; transferred into CPW solution supplemented with 9% mannitol solution containing 2% (wt/vol) Rhozyme, 4% Meicelase, and 0.3% Macerozyme; and incubated overnight at 25°C. The impurities were filtered by passing the solution through a cell strainer, and the protoplasts were centrifuged at $100 \times g$ for 5 min and further cleaned by suspension in CPW solution supplemented with 20% sucrose. Approximately 20 μg of plasmid was subsequently transformed into clean protoplasts through polyethylene glycol-mediated transformation according to the method described previously (Cheng et al. 1994a). After incubation for 24 h at 26°C, the transformed protoplasts were harvested for the quantification of luciferase (LUC) activity using an LUC reporter kit (Beyotime Biotech Co., LTD) according to the technical manual.

In this system, the gene was coexpressed with *LUC* in Chinese cabbage hypocotyl cells. If the protein can induce cell death, low LUC expression will be obtained, and sufficiently low chemiluminescence cannot be detected (Yoshida et al. 2009). Therefore, the capacity to induce cell death of a gene can be quantified by measuring the LUC activity and subsequent chemiluminescence intensity.

RESULTS

Fifty-three genes encoding putative secretory proteins of *P. brassicae* were expressed during primary infection. The transcriptome analysis revealed different FPKM values (Supplementary Table S2) in the five transcriptome datasets (all from the infected roots at 3 days after inoculation with *P. brassicae*; Sequence Read Archive accession no. PRJNA527625). The genes with FPKM values greater than zero in more than three transcriptomes were considered to be expressed during primary infection. After the prediction of secretory proteins, 53 genes encoding putative secretory proteins were considered to be expressed during primary infection. Among these 53 genes encoding putative secretory proteins, 6 (*PBCN_000422*, *PBCN_000804*, *PBCN_004558*, *PBCN_006805*, *PBCN_0008074*, and *PBCN_008892*) were partial genes, and 2 (*PBCN_005498* and *PBCN_009221*) could not be cloned. Ultimately, 45 genes were obtained and used for subsequent experiments.

SPs from 33 genes directed secretory activity in yeast. The secretory activity directed by the SPs in the 45 candidate genes was tested individually. Thirty-three of these SP sequences showed secretory activity in yeast as demonstrated by the growth assay on YPRAA plates and the TTC assay. Two typical SPs (*PBCN_007868* and *PBCN_009214*) with secretory activity in yeast are shown in Figure 1. Similar to the positive control (containing the SP of *PS87*) (Gu et al. 2011), the transformed yeast grew normally on YPRAA

plates and produced insoluble, red-colored TPF in the TTC assay. The SP sequences of the remaining 12 genes were considered to not direct secretory activity in the yeast assay. Two typical results (the SPs from *PBCN_004660* and *PBCN_007663*) are shown in Figure 1. The transformed yeast exhibited weak growth on YPRAA plates but could not convert the colorless TTC solution to the insoluble, red-colored TPF, which was similar to the results obtained for the negative control (containing the SP of *Mg87*) (Gu et al. 2011). Therefore, only the 33 genes containing active secretory SPs in yeast (Supplementary Fig. S1) were considered secretory proteins and used in subsequent studies.

Two secretory proteins induced cell death in *N. benthamiana*. To identify whether these 33 proteins could induce cell death, each gene was transiently expressed in the leaves of *N. benthamiana* using the *Agrobacterium*-mediated plant virus

Fig. 1. Functional validation of signal peptides from putative secretory proteins of *Plasmodiophora brassicae*. Two active signal peptides (SPs) from putative secretory proteins (*PBCN_007868* and *PBCN_009214*) and two inactive SPs from putative secretory proteins (*PBCN_004660* and *PBCN_007663*) are listed. SPs from *Phytophthora sojae* Avr1b were used as the positive control, and *Magnaporthe oryzae* Mg87 served as the negative control. CMD-W: 0.67% YNB, 0.075% tryptophan dropout supplement, 2% sucrose, 0.1% glucose, and 2% agar. YPRAA: 1% yeast extract, 2% peptone, 2% raffinose, 2 μg/ml antimycin A, and 2% agar. TTC, triphenyltetrazolium chloride.

transient expression system, and leaf necrosis was investigated at 7 dpi. Similar to the positive control (leaves expressing *BAX*), necrosis was found on the leaves expressing the secretory proteins encoded by the *PBCN_002550* and *PBCN_005499* genes, which indicated that these two secretory proteins could induce cell death in *N. benthamiana* leaves (Fig. 2A). Although no necrosis was found in the negative control (leaves expressing *eGFP*), only a little yellowing was found. The leaves expressing other secretory genes are shown in Supplementary Figure S2. In addition, *N. benthamiana* leaves expressing *eGFP*, *BAX*, *PBCN_002550*, and *PBCN_005499* were photographed at 3, 5, and 7 dpi. Severe necrosis emerged at 3 dpi on *N. benthamiana* leaves expressing *BAX*. Although necrosis was observed at 5 dpi on *N. benthamiana* leaves expressing *PBCN_002550*, only chlorosis was observed on *N. benthamiana* leaves expressing *PBCN_005499* (Fig. 2A). Compared with the effects of *BAX* expression, the *N. benthamiana* leaves expressing

Fig. 2. Secretory proteins of *Plasmodiophora brassicae* induced programmed cell death, H_2O_2 accumulation, and electrolyte leakage in *Nicotiana benthamiana*. **A**, Necrosis and H_2O_2 accumulation in *N. benthamiana* leaves expressing *eGFP*, *PBCN_002550*, *PBCN_005499*, and *BAX*. **B**, Electrolyte leakage at 3, 5, and 7 days postinoculation (dpi) from *N. benthamiana* leaves expressing *eGFP*, *PBCN_002550*, *PBCN_005499*, and *BAX*. DAB, 3,3'-diaminobenzidine. ** indicates $P < 0.01$.

PBCN_002550 and *PBCN_005499* showed decreased cell death and delayed necrosis, which indicated that the proteins encoded by these two genes induced cell death less effectively than *BAX* in *N. benthamiana*.

Cell death is often associated with H_2O_2 accumulation, electrolyte leakage, and other metabolic abnormalities (Bai et al. 2012; Wojtaszek 1997). *N. benthamiana* leaves expressing *PBCN_002550*, *PBCN_005499*, *BAX*, and *eGFP* were harvested at 3 dpi for DAB staining. Brown substances showing H_2O_2 accumulation were detected in leaves expressing *PBCN_002550*, *PBCN_005499*, and *BAX* but were not detected in leaves expressing *eGFP* (Fig. 2A). Moreover, leaves expressing *BAX* were stained a deeper color. The degree of electrolyte leakage from *N. benthamiana* leaves expressing these genes was quantified at 3, 5, and 7 dpi. None of the *P. brassicae* secretory proteins induced significant leakage at 3 dpi. However, leaves expressing *PBCN_002550* and *PBCN_005499* showed significant leakage at 5 and 7 dpi compared with leaves expressing *eGFP*, and leaves expressing *PBCN_005499* showed significantly reduced leakage at 5 dpi compared with leaves expressing *PBCN_002550* and *BAX* (Fig. 2B). These results were consistent with the results described above and indicated that both *PBCN_002550* and *PBCN_005499* were weak inducers of cell death and that *PBCN_005499* was a weaker inducer than *PBCN_002550*.

PBCN_002550 induced cell death in Chinese cabbage hypocotyls. The whole lifecycle of *P. brassicae* can occur only in cruciferous plants and not in other plants, such as *N. benthamiana*.

Fig. 3. *PBCN_002550* induces cell death in Chinese cabbage hypocotyls. **A**, Scheme of the experimental protoplast transformation of Chinese cabbage hypocotyls. The lethal gene causes rapid cell death, resulting in little luciferase (LUC) activity and low chemiluminescence intensity. **B**, Chemiluminescence intensity of protoplasts coexpressing *PBCN_002550*, *PBCN_005499*, or *BAX* with *LUC*. ** indicates $P < 0.01$.

A firefly LUC coexpression system was adopted to further assess whether PBCN_002550 and PBCN_0005499 can induce cell death in host plant cells. Compared with protoplasts expressing only *LUC* (control), protoplasts coexpressing *PBCN_002550* and *LUC* exhibited significantly decreased ($P < 0.01$) chemiluminescence intensity (Fig. 3B). However, the chemiluminescence intensity of protoplasts coexpressing *PBCN_005499* and *LUC* was not significantly different from that of the control protoplasts, which indicated that only *PBCN_002550* could induce cell death in Chinese cabbage hypocotyls.

Twenty-four secretory proteins suppressed BAX-induced cell death. Among the 31 tested secretory protein genes, *N. benthamiana* leaves expressing 7 secretory protein-encoding genes (*PBCN_001856*, *PBCN_003620*, *PBCN_005440*, *PBCN_007081*, *PBCN_008608*, *PBCN_009251*, and *PBCN_009622*) exhibited a similar level of necrosis as leaves expressing *eGFP* and *BAX* (negative control), which indicated that these 7 secretory protein-encoding genes could not suppress BAX-induced cell death. In contrast to the results obtained for these 7 genes, the levels

of necrosis were noticeably reduced on *N. benthamiana* leaves expressing the other 24 secretory proteins (Fig. 4A). *PBCN_001987* showed a typical suppression of BAX-triggered cell death as illustrated in Figure 4B. The suppression effect of these proteins on BAX-triggered cell death was further confirmed by the coinfiltration of the genus *Agrobacterium* carrying putative cell death inhibitors and diluted (32-fold, $OD_{600} = 0.0125$) genus *Agrobacterium* carrying BAX.

To quantify the level of cell death suppression by these secretory proteins, the electrolyte leakage from *N. benthamiana* leaves at 7 dpi was quantified. Compared with *N. benthamiana* leaves expressing *eGFP*, *N. benthamiana* leaves expressing eight genes (*PBCN_001856*, *PBCN_003620*, *PBCN_005440*, *PBCN_007081*, *PBCN_008608*, *PBCN_009251*, *PBCN_009331*, and *PBCN_009622*) exhibited no significant reduction in electrolyte leakage. Among *N. benthamiana* leaves expressing these eight genes, *N. benthamiana* expressing *PBCN_009331* did not exhibit obvious cell death. Unlike these eight genes, however, the other 23 genes significantly suppressed electrolyte leakage induced by BAX

Fig. 4. Secretory proteins of *Plasmodiophora brassicae* suppressed BAX-induced cell death. **A**, Several typical effector candidates suppressed cell death to different degrees. *eGFP* was used as the negative control. **B**, A typical effector candidate (*PBCN_001987*) suppressed BAX-induced cell death. The expression of BAX and *PBCN_001987* was assessed. **C**, Electrolyte leakage from *Nicotiana benthamiana* leaves coexpressing *P. brassicae* secretory proteins genes and BAX. α-HA, HA tag antibody. α-flag, flag tag antibody. * indicates $0.01 < P < 0.05$; and ** indicates $P < 0.01$.

expression. Among these 23 genes, 16 reduced the degree of electrolyte leakage to less than one-half of that found in the negative control. In addition, nine genes reduced the level of electrolyte leakage to less than one-third of that found in the negative control (Fig. 4C).

Twenty-eight secretory proteins suppressed PBCN_002550-induced cell death. PBCN_002550 was the only secretory protein that could induce cell death in both *N. benthamiana* leaves and Chinese cabbage hypocotyls. An additional screen of secretory proteins suppressing PBCN_002550-induced cell death was performed. In contrast to the protocol used for the BAX-induced cell death suppression assay, equal amounts of the genus *Agrobacterium* carrying pGR107-secretory protein-encoding gene constructs and the genus *Agrobacterium* carrying pGR107-PBCN_002550 were coinjected into *N. benthamiana* leaves. Subsequently, the presence of necrosis on *N. benthamiana* leaves was investigated at 7 dpi. Similar to the negative control

(*N. benthamiana* leaves coinjected with the genus *Agrobacterium* carrying pGR107-eGFP and pGR107-PBCN_002550), *N. benthamiana* leaves expressing PBCN_001856, PBCN_008608, and PBCN_009622 showed severe necrosis. Unlike *N. benthamiana* leaves expressing these 3 genes, *N. benthamiana* leaves expressing the other 28 genes exhibited different degrees of necrosis reduction (Fig. 5A). No visible necrosis was observed in *N. benthamiana* leaves expressing PBCN_000619, PBCN_001987, PBCN_4363, PBCN_004708, and PBCN_005126. The typical cell death suppression effect of PBCN_005126 is shown in Figure 5B. The degree of electrolyte leakage from these *N. benthamiana* leaves was also quantified. The necrotic *N. benthamiana* leaves expressing PBCN_001856, PBCN_008608, or PBCN_009622 showed high electrolyte leakage, similar to *N. benthamiana* leaves expressing eGFP, which indicated that these three genes could not suppress electrolyte leakage induced by PBCN_002550. Similar to *N. benthamiana* leaves expressing these three genes, the degree of electrolyte leakage from *N. benthamiana*

Fig. 5. Secretory proteins of *Plasmodiophora brassicae* suppressed PBCN_002550-induced cell death. **A**, Several typical effector candidates suppressed cell death to different degrees. eGFP was used as the negative control. **B**, A typical effector candidate (PBCN_005126) suppressed PBCN_002550-induced cell death. The expression of PBCN_002550 and PBCN_005126 was assessed. **C**, Electrolyte leakage from *Nicotiana benthamiana* leaves coexpressing *P. brassicae* secretory proteins genes and PBCN_002550. α-flag, flag tag antibody. * indicates 0.01 < P < 0.05; and ** indicates P < 0.01.

leaves expressing *PBCN_009214* was not significantly different from the negative control. In contrast to the results obtained for these 4 genes, the electrolyte leakage from *N. benthamiana* leaves expressing the other 27 genes was significantly different ($P < 0.01$) from that of the negative control. Specifically, the electrolyte leakage from *N. benthamiana* leaves expressing 23 genes was reduced to less than one-third of that of the negative control (Fig. 5C).

DISCUSSION

The disease cycle of *P. brassicae* comprises two phases: the primary phase, which occurs in the root hairs, and the secondary phase, which occurs in the root cortex (Kageyama and Asano 2009). According to previous studies, the recognition of *P. brassicae* by the host occurs during the primary phase (McDonald et al. 2014). However, the mechanism through which plants recognize *P. brassicae* and the mechanism through which *P. brassicae* manipulates the plant immune response to achieve successful infection

remain unclear. To reveal these mechanisms, we systematically screened the secretory protein-encoding genes expressed by *P. brassicae* during primary infection and investigated their ability to suppress or trigger plant cell death.

Secretory proteins are effector candidates that are critical for pathogen infection. Therefore, the accurate identification of secretory proteins is important for studying their functions. A common identification method involves the combination of predictions using bioinformatics software with yeast signal sequence trap assays (Gu et al. 2011; Krijger et al. 2008; Yeom et al. 2011). This strategy was adopted in this study, and a sensitive D-cutoff value (0.34) was selected for SP sequence prediction using SignalP v4.1.

Surprisingly, we found that the secretory protein *PBCN_002550* can induce cell death in Chinese cabbage hypocotyls. Because *P. brassicae* is an intracellular biotrophic parasite, it needs to absorb nutrients from living hosts, and cell death seems to be detrimental to its propagation. However, cell death-inducing genes are widely spread in plant pathogen species. *Avh241* from *Phytophthora sojae*

TABLE 1. Details of putative secretory proteins

Proteins	e3 homolog	Functional domains	Identity (%)	Functional SignalP in yeast	Induce cell death			Suppress cell death			
					In <i>Nicotiana benthamiana</i>		In Chinese cabbage: necrosis	BAX induced		PBCN_002550 induced	
					Necrosis	Leakage (%)		Necrosis	Leakage (%)	Necrosis	Leakage (%)
PBCN_000548	CEO97203.1	No	100	No	— ^a	—	—	—	—	—	—
PBCN_000619	CEO97274.1	No	98.62	Yes	No	—	—	Yes	13.6 ± 0.03	Yes	16.1 ± 1.6
PBCN_000733	CEO97388.1	recA	99.34	Yes	No	—	—	Yes	38.9 ± 10.7	Yes	18.4 ± 3.3
PBCN_001191	CEO99285.1	No	100	Yes	No	—	—	Yes	46.7 ± 2.0	Yes	17.3 ± 1.4
PBCN_001204	CEO99298.1	ANK	91.45	Yes	No	—	—	Yes	13.3 ± 1.0	Yes	13.7 ± 0.5
PBCN_001442	CEP00388.1	HflC	100	No	—	—	—	—	—	—	—
PBCN_001454	CEP00400.1	No	99.66	No	—	—	—	—	—	—	—
PBCN_001856	CEP01250.1	No	100	Yes	No	—	—	No	78.8 ± 7.2	No	72.8 ± 12.5
PBCN_001987	CEP01381.1	zf-MYND	100	Yes	No	—	—	Yes	23.6 ± 2.1	Yes	14.2 ± 2.1
PBCN_002100	CEP01496.1	SMC_prok_A	100	Yes	No	—	—	Yes	37.4 ± 12.8	Yes	15.1 ± 0.8
PBCN_002462	CEP02197.1	No	98.91	Yes	No	—	—	Yes	34.6 ± 5.7	Yes	18.6 ± 0.5
PBCN_002550	CEP02583.1	No	100	Yes	Yes	69.8 ± 7.4	Yes	—	—	—	—
PBCN_002958	CEP03198.1	ChtBD1	99.42	Yes	No	—	—	Yes	19.6 ± 1.6	Yes	14.6 ± 2.0
PBCN_003236	CEP03476.1	PRE1	100	No	—	—	—	—	—	—	—
PBCN_003620	CEO94807.1	No	99.14	Yes	No	—	—	No	74.9 ± 0.3	Yes	44.7 ± 16.8
PBCN_004344	CEO95618.1	No	91.4	Yes	No	—	—	Yes	22.1 ± 2.1	Yes	14.7 ± 3.2
PBCN_004363	CEO95637.1	No	100	Yes	No	—	—	Yes	20.6 ± 1.2	Yes	11.1 ± 1.0
PBCN_004660	CEO95970.1	No	100	No	—	—	—	—	—	—	—
PBCN_004708	CEO96018.1	Ribosomal_P2	100	No	—	—	—	—	—	—	—
PBCN_004763	CEO96073.1	PRK14510	100	Yes	No	—	—	Yes	15.7 ± 1.9	Yes	10.9 ± 0.7
PBCN_005031	CEO96360.1	No	100	Yes	No	—	—	Yes	27.9 ± 3.7	Yes	11.3 ± 1.2
PBCN_005126	CEO96517.1	vWFA	100	Yes	No	—	—	Yes	23.1 ± 2.6	Yes	9.6 ± 1.4
PBCN_005440	CEO96836.1	No	98.87	Yes	No	—	—	No	72.9 ± 7.2	Yes	37.9 ± 13.6
PBCN_005456	CEO96852.1	Ank_2, WD40	99.61	Yes	No	—	—	Yes	14.3 ± 1.0	Yes	21.4 ± 4.7
PBCN_005499	CEO96895.1	No	100	Yes	Yes	82.6 ± 2.9	No	—	—	—	—
PBCN_005691	CEO97087.1	No	97.5	No	—	—	—	—	—	—	—
PBCN_006649	CEO98535.1	Ank_4	90.71	Yes	No	—	—	Yes	17.1 ± 1.2	Yes	19.6 ± 4.5
PBCN_006655	CEO98541.1	ANK	99.68	No	—	—	—	—	—	—	—
PBCN_006697	CEO98583.1	ANK	98.43	Yes	No	—	—	Yes	35.7 ± 12.7	Yes	48.2 ± 12.1
PBCN_006785	CEO98671.1	eIF3_subunit	99.22	Yes	No	—	—	Yes	37.4 ± 10.4	Yes	16.4 ± 3.4
PBCN_007081	CEO98967.1	Tyrosinase	100	Yes	No	—	—	No	68.3 ± 11.6	Yes	26.6 ± 6.4
PBCN_007090	CEO98976.1	CDA1	100	Yes	No	—	—	Yes	33.2 ± 6.7	Yes	13.8 ± 1.8
PBCN_007206	CEO99092.1	pntA	99.46	Yes	No	—	—	Yes	30.1 ± 1.5	Yes	15.6 ± 3.7
PBCN_007663	CEO99929.1	alkPPc	100	No	—	—	—	—	—	—	—
PBCN_007856	CEP00122.1	zf-RING_2	100	No	—	—	—	—	—	—	—
PBCN_007868	CEP00134.1	Lactamase_B	98.02	Yes	No	—	—	Yes	13.2 ± 2.2	Yes	14.9 ± 1.7
PBCN_008279	CEP00967.1	No	99.13	Yes	No	—	—	Yes	55.6 ± 4.8	Yes	20.4 ± 0.8
PBCN_008608	CEP01666.1	No	100	Yes	No	—	—	No	76.6 ± 3.5	No	69.6 ± 11.0
PBCN_009214	CEP02996.1	RING	100	Yes	No	—	—	Yes	24.4 ± 3.9	Yes	57.2 ± 27
PBCN_009249	CEP03031.1	ANK	82.59	Yes	No	—	—	Yes	22.9 ± 0.6	Yes	12.3 ± 1.3
PBCN_009251	CEP03033.1	ANK	87.17	Yes	No	—	—	No	83.9 ± 5.5	Yes	23.2 ± 5.1
PBCN_009331	CEP03113.1	CLECT	100	Yes	No	—	—	Yes	46.2 ± 17.0	Yes	14.3 ± 0.8
PBCN_009387	CEP03502.1	Ribosomal_L22	100	No	—	—	—	—	—	—	—
PBCN_009622	CEO95090.1	No	100	Yes	No	—	—	No	73.6 ± 4.5	No	67.0 ± 7.6
PBCN_009670	CEO95403.1	C2H2 Zn finger	100	No	—	—	—	—	—	—	—

^a Dashes indicate unknown.

(Yu et al. 2012), PvRxLR16 from *Plasmopara viticola* (Xiang et al. 2016), and Las5315mp from '*Candidatus Liberibacter asiaticus*' (Pitino et al. 2016) reportedly induce cell death in *N. benthamiana* and other plants. These results suggest that these genes might have other functions. Moreover, because the plant materials used in these studies were susceptible cultivars, these gene products might be recognized not by resistant genes but by some intracellular nucleotide-binding domains or NB-LRR immune receptors (Pitino et al. 2016).

In addition to PBCN_002550, the secretory protein PBCN_005499 induced cell death in *N. benthamiana* but not in Chinese cabbage hypocotyls. Chinese cabbage is the host of *P. brassicae*, whereas *N. benthamiana* is a nonhost. The different responses induced by PBCN_005449 reflected the different interactions of *P. brassicae* with host and nonhost plants.

Although *P. brassicae* has secretory proteins that can induce cell death in the host, cell death might not occur. Of the 31 tested secretory proteins, 28 suppressed PBCN_002550-induced cell death, and 24 suppressed BAX-induced cell death. This finding indicated that *P. brassicae* encodes a large number of proteins that can suppress plant immunity. This phenomenon has also been found in other biotrophic pathogens. Of the 83 tested secretory proteins from *P. viticola*, 62 suppressed elicitor-induced cell death (Liu et al. 2018). Seventy-eight of the 95 tested secretory proteins from *Heterodera avenae* suppressed BAX-induced cell death (Chen et al. 2018). For these biotrophic parasites, the possession of a large number of proteins that can suppress cell death might benefit propagation and colonization.

Among the 28 proteins that suppressed PBCN_002550-induced cell death (PBCN_003620, PBCN_005440, PBCN_008279, and PBCN_009251 could not suppress BAX-induced cell death), 18 proteins contained functional domains (Table 1). Some of these domains have been reported to be involved in pathogen infection, plant disease, and tolerance to biotic and abiotic stresses. PBCN_007081 contains a tyrosinase domain that can catalyze tyrosine to DOPA (Langfelder et al. 2003). L-DOPA reportedly reduces reaction oxygen species in soybean roots (Soares et al. 2011). An MYND zinc finger domain is found in PBCN_001987, and an RING-H2 zinc finger is found in PBCN_009214. Some proteins containing the RING-H2 zinc finger reportedly have functions in tolerance to abiotic stresses (Ko et al. 2006; Sahin-Cevik and Moore 2006; Zeng et al. 2014). PBCN_009251, PBCN_001204, PBCN_005456, PBCN_006649, PBCN_006697, and PBCN_009249 contain ankyrin repeats, which mediate protein-protein interactions in widely diverse families of proteins (Sedgwick and Smerdon 1999). Some ankyrin repeat-containing proteins from plants are involved in disease resistance, antioxidation metabolism, reactive oxygen production, and biotic and abiotic stresses (Böttner et al. 2009; Li et al. 2013; Sakamoto et al. 2008; Yan et al. 2002). In addition to the ankyrin repeat domain, a WD40 domain is found in PBCN_005456. Some proteins containing a WD40 domain are reportedly associated with plant tolerance to abiotic stresses and control the early stages of infection by *Rhizobia* in legumes (Kiss et al. 2009; Kong et al. 2015).

In conclusion, the functions of *P. brassicae* secretory proteins expressed during the primary infection stage in the induction and suppression of plant immunity were investigated. The results showed that only two secretory proteins induced plant immunity and that most of the secretory proteins suppressed plant immunity. These findings expanded our understanding of the functions of *P. brassicae* secretory proteins in interactions between *P. brassicae* and plants. Whether the effects of these secretory proteins in plant cell death are beneficial for *P. brassicae* infection and the mechanism through which these secretory proteins manipulate plant immunity remain unclear and are worth additional investigation/research. Future studies will focus on the targets of these secretory proteins and the functions and molecular mechanisms of these secretory proteins in *P. brassicae* infection.

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