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The conserved GOLD domain in the *Plasmodiophora brassicae* effector Pb257 is required for triggering cell death and root swelling

Hui Yang^{1,3*†}, Rong Liao^{2†}, Chuang Jin^{1†}, Xiaoya Feng^{1†}, Yang Zhang^{1†}, Xinyue Wang¹, Yuge Zhang¹, Liang Yu¹ and Wenming Wang^{1*}

Abstract

Plasmodiophora brassicae is one of the biggest threats to cruciferous plants and one of the most economically significant diseases worldwide. However, our current understanding of its pathogenic mechanisms remains limited. In this study, we have identified an effector, Pb257, which plays a crucial role in the virulence of *P. brassicae*. The expression pattern analysis revealed continuous induction of Pb257 during both primary and secondary infections. Ectopic expression of Pb257 strongly triggered cell death in *Nicotiana benthamiana* and several other plants, which was dependent on SOBIR1 and the salicylic acid pathway but not BAK1, a central molecular regulator, to mediate classical defense pathways. Overexpression of Pb257 increased susceptibility of *Brassica rapa* to *P. brassicae*. Silencing of the Pb257-encoding gene led to reduce root swelling. Further investigation showed that the conserved GOLD domain within Pb257 was essential for inducing root enlargement, which is similar to the mild symptoms of clubroot, indicating that it is an important effector for promoting root cell enlargement. GOLD domain-containing family proteins were widely present in the kingdoms of protozoa, fungi, and animalia, and Pb257 was clustered into a clade of protozoa, suggesting the encoding gene as a potential marker gene for classification of *P. brassicae*. The GOLD domain-containing proteins are known regulators involved in human cell proliferation and immune system disruption, however, no previous reports have described a pathogen-derived GOLD domain protein with elicitor activity. Our findings demonstrate that Pb257 functions as a crucial effector contributing to the virulence of *P. brassicae* and could be used as a potential molecular target for controlling clubroot disease.

Keywords *Plasmodiophora brassicae*, Effector, Pb257, GOLD domain, HIGS

[†]Hui Yang, Rong Liao, Chuang Jin, Xiaoya Feng and Yang Zhang contributed equally to this work.

*Correspondence:

Hui Yang
yanghui981@126.com
Wenming Wang
j316wenmingwang@sicau.edu.cn

Full list of author information is available at the end of the article



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Background

Plasmodiophora brassicae is a plant pathogenic protozoan, which causes clubroot disease in cruciferous plants (Ludwig-Müller et al. 2015). To date, clubroot disease has a significant impact on *Brassica* crops worldwide. *P. brassicae* is an obligate parasite and need dependent on a plant host to complete its life cycle (Kageyama and Asano 2009). In the soil, resting spores of *P. brassicae* germinate and release primary zoospores to initiate infection of root hairs. Then *P. brassicae* forms zoosporangia within the root hairs and produces secondary zoospores to infect the cortical cells. In the absence of hosts, resting spores can maintain their vitality for many years (Kageyama and Asano 2009).

In susceptible hosts, pathogens can establish a favorable infection by evading or suppressing immune responses (Presti et al. 2015). Similar to other plant pathogens, *P. brassicae* has putative secreted proteins and shares the feature of effectors. The secretory activity of some putative effectors in *P. brassicae* has been experimentally confirmed (Schwelm et al. 2015; Rolfe et al. 2016; Chen et al. 2019; Pérez-López et al. 2020). Pathogens secrete effectors as a strategy to disrupt host defense. Plants possess an innate immune system that recognizes diverse pathogens, triggering effector-triggered immunity (ETI) or pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) to resist pathogen infection (Jones and Dangl 2006; Chisholm et al. 2006). The interaction between hosts and their corresponding pathogens drives coevolutionary processes.

Some pathogen effectors can suppress host defense, while a few effectors initiate host defense (He et al. 2020). For example, among the 33 *P. brassicae* effectors identified, only PBCN_002550 and PBCN_005499 trigger H₂O₂ accumulation in *Nicotiana benthamiana* (Chen et al. 2019). The effect of effectors on plant defense is commonly assessed through transient expression in *N. benthamiana*. For instance, transient expression of *Phytophthora sojae* effector Avh241 or *P. infestans* effector PexRD2 leads to cell death in *N. benthamiana* (Oh et al. 2009; Yu et al. 2012). AvrE1 from *Pseudomonas syringae* pv. *tomato* triggers cell death in tobacco (Badel et al. 2006). DspA/E from *Erwinia amylovora* elicits cell death in *N. benthamiana* (Oh et al. 2007). These effectors influence plant defense and contribute to own virulence.

Up to now, *P. brassicae* has been studied for over 140 years, but the key roles of effectors in *P. brassicae* infection and symptom development remain unclear. Their precise function has not been well defined. Only a few effectors in *P. brassicae* have been reported. Benzoic acid/salicylic acid methyltransferase (PbBSMT) was the first effector to be identified, which can catalyze the conversion of salicylic acid (SA) to methyl salicylate (Me-SA)

to alter the host susceptibility (Bulman et al. 2019). The effector PBZF1 interacts with SnRK1.1 and enhances host susceptibility to *P. brassicae* (Chen et al. 2021). *P. brassicae* effector, SSPbP53, directly interacts with cruciferous papain-like cysteine proteases (PLCPs) to suppress plant defense (Pérez-López et al. 2021). However, the functions of most candidate effectors are unknown.

To date, effectors of various domains have been identified, such as RXLR motifs, chitin-binding domains, and protease/protease inhibitors, etc. In this study, we have identified a novel effector Pb257 from *P. brassicae*, which is a small secreted protein (227 aa) with a conserved GOLD (Golgi dynamics) domain. The GOLD domain-containing protein families are highly conserved across plants, animals, and microorganisms (Pastor-Cantizano et al. 2018). Studies in human medicine have demonstrated that this protein family is involved in the regulation of cell proliferation, extension, differentiation, migration, and diffusion processes. Aberrant expression has been related to cancer development and disruptions in the immune system (Aber et al. 2019). Loss of function of GOLD domain proteins affects cell fate and often results in the abnormalities of morphogenesis. Therefore, we were interested in whether Pb257 interferes with the growth of host roots or defense responses. Identifying and understanding the functions of secreted proteins is an important procedure toward identification of the corresponding resistance proteins in host.

Results

Pb257 trigger cell death and induce immune responses in plant

In previous study, we predicted the potential secreted proteins with an N-terminal signal peptide. Here, several genes were selected and cloned into the plant expression vector based on their putative effector characteristics. The ability of these putative effectors to induce cell death in *N. benthamiana* leaves was analyzed. One of putative effectors, Pb257, strongly triggered cell death at three days post-infiltration (Fig. 1a), otherwise, increased reactive oxygen species were accompanied in the infiltrated leaves (Fig. 1b). Western blot analysis revealed expression of Pb257 in *N. benthamiana* (Fig. 1c), where Pb257-coding gene and *GFP* gene were fused together in the vector, with *GFP* gene having its own start codon. Western blotting detected both the fusion protein of Pb257 and GFP, as well as the individual GFP protein, resulting in two distinct bands.

To elucidate whether the defense response triggered by Pb257 is associated with PTI or ETI pathways, we evaluated the expression of defense-related genes *SGT1*, *HSP90*, *Acre31*, *Pti5*, *SIPK*, *CoII*, *EIN2*, and *PDF1.2*. The results were presented in Additional file 1:

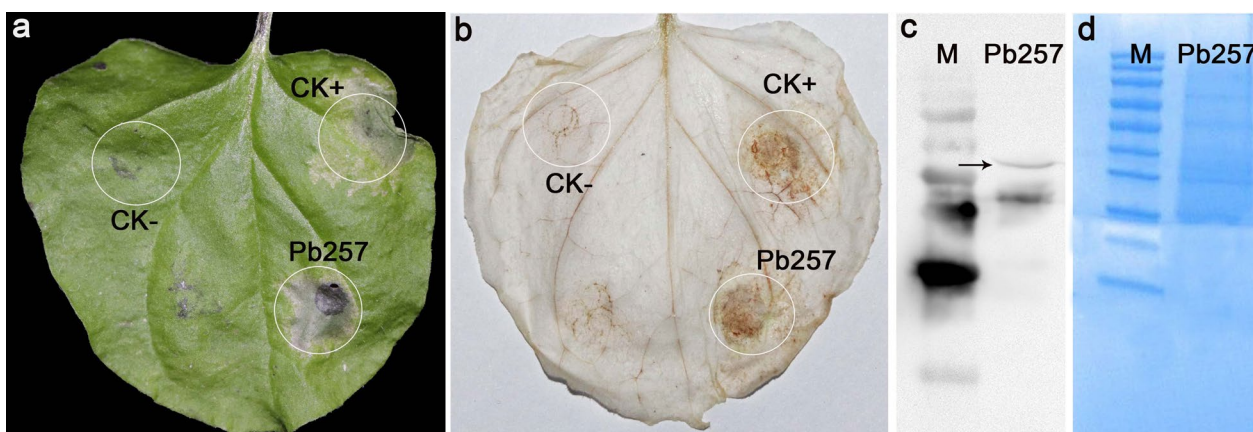


Fig. 1 The putative effector Pb257 in *P. brassicae* induced cell death and ROS burst in *Nicotiana benthamiana* leaves. **a, b** Pb257-infiltrated leaves exhibited cell death at 3 days post-inoculation (dpi), and ROS burst at 1 dpi. GFP was infiltrated as a negative control (CK-). BAX, a mammalian proapoptotic BCL-2 associated X (Bax) protein, was infiltrated as a positive control (CK+), respectively. The experiment was replicated ten times. **c** Expression of Pb257 proteins in infiltrated leaves detected by Western blotting. The anti-GFP antibody was used. The arrow points to the Pb257 protein. **d** The protein electrophoresis gel was stained with Coomassie brilliant blue

Figure S1. All the genes tested were up-regulated after transient expression of Pb257. Taken together, these findings indicate that Pb257 can induce plant immune responses.

Functional validation of the predicted signal peptide of Pb257

Pb257 encodes a protein of 227 amino acids (aa). The first 23 aa residues are a predicted signal peptide (SP). To validate its secretion function, we employed a signal sequence trap system in the yeast. The constructs expressing SUC2 fused with the signal peptide of Pb257 was capable of growing on CMD-W and YPRAA media, indicating successful secretion of invertase (Fig. 2). Additionally, the change from colorless 2,3,5-triphenyltetrazolium chloride (TTC) to insoluble red 1,3,5-triphenylformazan (TPF) further confirmed the secretion of invertase (Fig. 2). These results indicate that Pb257 is a secreted protein.

Signaling peptides of Pb257 are necessary to induce cell death

Signaling peptides are responsible for directing proteins into the different membrane structures. To investigate the role of Pb257 signaling peptide in cell death induction, a Pb257 vector without the signal peptide (Pb257^{24–227} or Pb257-Nsp) was constructed and transiently expressed in *N. benthamiana* leaves. However, Pb257-Nsp failed to induce cell death (Fig. 3a). These results suggested that Pb257-induced cell death was dependent on signaling peptides in *N. benthamiana*.

Pb257 triggers cell death in multiple plant species

To investigate the specificity of Pb257-induced plant defense, we infiltrated Pb257 into the leaves of various plants. Cell death was induced by Pb257 in both nonhost *N. tabacum* and *Solanum lycopersicum*, as well as host *Arabidopsis thaliana*, *Brassica rapa*, and *B. napus* leaves when compared to the control treatments (Fig. 4). The strong induction of cell death suggests that Pb257 can be recognized by multiple different plant species.

Pb257-triggered cell death is dependent on SOBIR1 but not on BAK1

The receptor-like kinases BAK1 and SOBIR1 play crucial roles in pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI). Given that the induction of cell death by Pb257 was dependent on its signaling peptide, we aimed to investigate whether SOBIR1 and BAK1 was involved in Pb257-mediated cell death. The virus-induced gene silencing (VIGS) system was employed to silence *NbBAK1* and *NbSOBIR1* in *N. benthamiana*, respectively. The results showed that Pb257-triggered cell death was abolished in *N. benthamiana* when *NbSOBIR1* was silenced. However, *BAK1*-silenced leaves exhibited cell death in response to transient expression of Pb257 (Fig. 5a). The silencing levels of *NbBAK1* and *NbSOBIR1* were confirmed by RT-qPCR analysis (Fig. 5b).

To further assess the role, we analyzed Pb257-triggered cell death in *A. thaliana* mutants *eds5*, *npr1*, *bak1*, and *NahG*. The Agrobacterium-mediated transient expression of Pb257 failed to induce cell death in *eds5*, *npr1*, and *NahG* mutants (Fig. 5c). The defense signaling is conducted through the salicylic acid pathway. As expected,

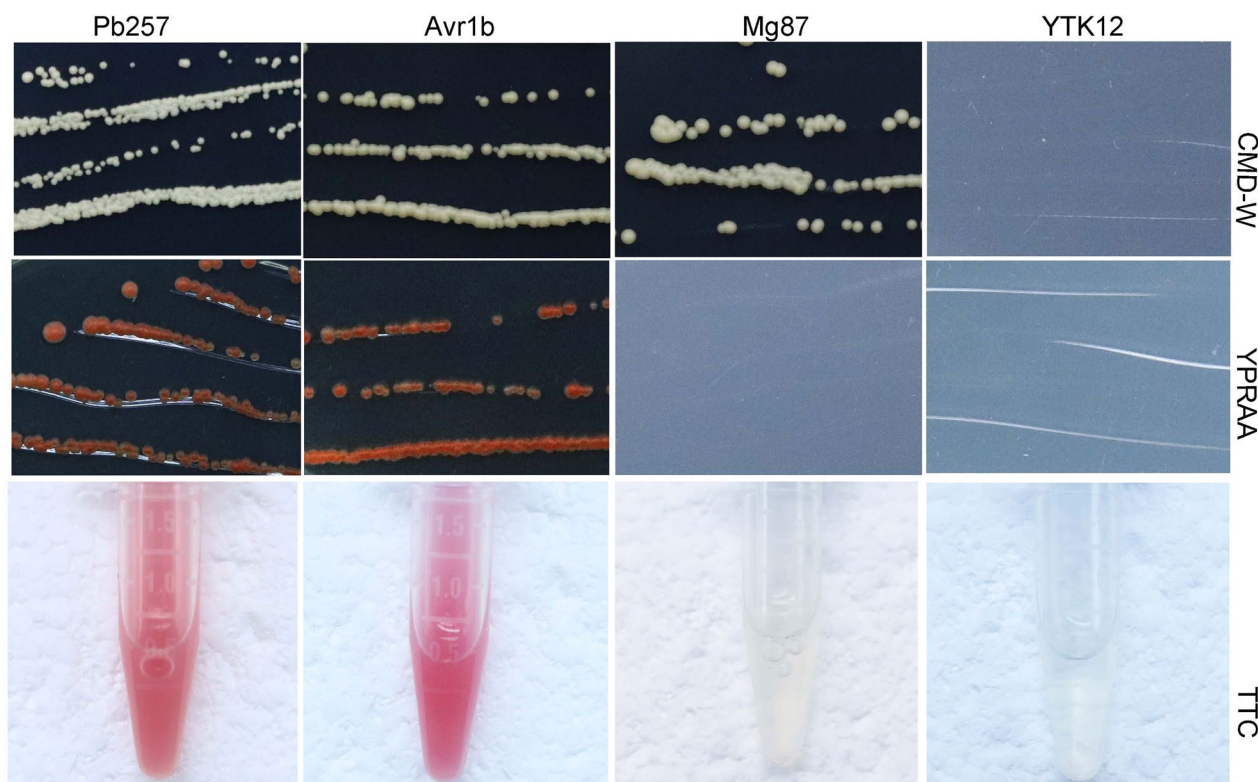


Fig. 2 Validation of the Pb257 signal peptide by a yeast invertase secretion assay. The predicted signal peptide sequence of Pb257 was fused in-frame to the yeast mature invertase sequence in the pSUC2 vector and expressed in YTK12. The functional signal peptide enabled yeast growth on YPRAA medium with raffinose as the sole carbon source (1% yeast extract, 2% peptone, 2% raffinose, and 2 μ g antimycin A per liter) and CMD-W medium (0.67% yeast N base without amino acids, 0.075% tryptophan dropout supplement, 2% sucrose, 0.1% glucose, and 2% agar). N-terminal sequences of *P. sojae* Avr1b and *M. oryzae* Mg87 were used as positive and negative controls, respectively. The untransformed YTK12 did not grow on either CMD-W or YPRAA media. Yeast growth on CMD-W medium was equally viable among the transformed strains

Pb257 still triggered cell death in *bak1* mutants (Fig. 5c). The results demonstrate that the central immune regulator BAK1 is dispensable for Pb257-triggered immune signaling pathway.

Pb257 is a GOLD domain-containing protein that is widely present in various organisms

Since Pb257 contains a conserved GOLD domain, we performed BLAST analyses and identified the significant matches (>30% similarity) in diverse organisms. Subsequently, we conducted phylogenetic tree analysis to study the distribution pattern of GOLD domain-containing proteins among various organisms. The phylogenetic analysis revealed that these proteins were mainly clustered into three clades: protozoa, fungi, and animalia (Additional file 1: Figure S2). The two clusters within protozoa and animalia shared little relatedness, but the clusters within protozoa and fungi shared high relatedness.

Pb257 was clustered into the clade for protozoa, such as, *Naegleria gruberi*, *Polysphondylium violaceum*, *Amphimedon queenslandica*, *Salpingoeca rosetta*,

Cavenderia fasciculata, etc., indicating a common ancestor shared by *P. brassicae* GOLD domain-containing proteins. However, Pb257 of *P. brassicae* (CEO94472) and KMS65477 of *Beta vulgaris* subsp. *vulgaris* were clustered together (Fig. 6). They had the closest genetic relationship with KAA0147869, KAA0151199, and KAA0155311 in *Cafeteria roenbergensis*. *C. roenbergensis* is one of the largest and most complex marine bacterivorous protists, suggesting the potential evolutionary dependence between *P. brassicae* and *Cafeteria*.

The GOLD domain of Pb257 is required for inducing cell death

The conserved GOLD domain may play critical roles in pathogen virulence. Amino acids 44–135 were predicted to constitute the GOLD domain. To explore whether the GOLD domain structure affected the ability of Pb257 to induce cell death, we constructed the deletion mutants Pb257 Δ 44–135 aa (25dG), Pb257 Δ 44–65 aa (25m1), and Pb257 Δ 66–78 aa (25m2). They were individually expressed in *N. benthamiana*. The mutants 25m2

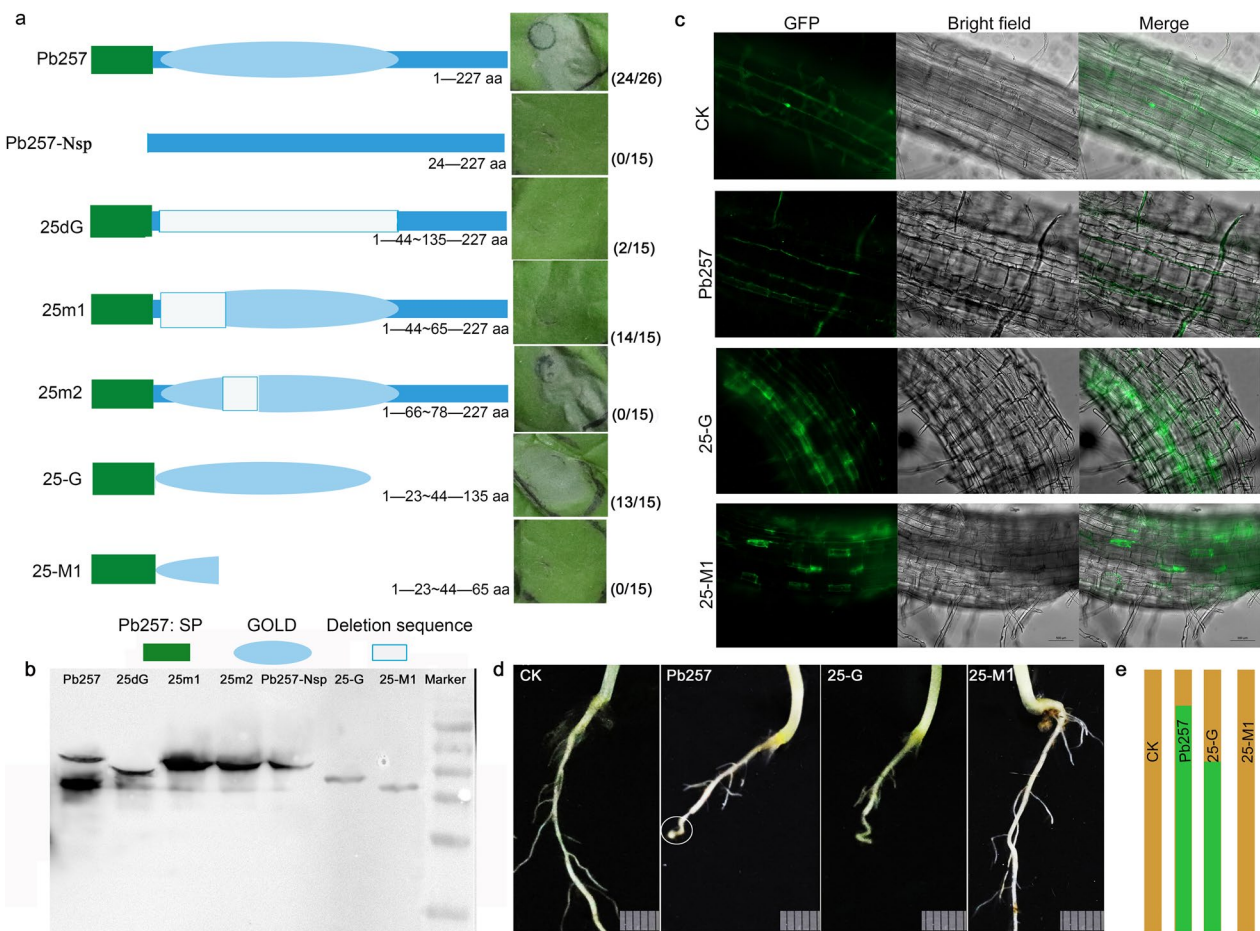


Fig. 3 The GOLD domain of Pb257 is required for induced cell death and root swelling. **a** Various truncation mutants were constructed and transiently expressed through agroinfiltration in *N. benthamiana* leaves. Photographs were taken at 4 days after agroinfiltration. Numbers e.g., 24/26, indicate that 24 of 26 infiltrated leaves exhibiting cell-death. **b** Expression of Pb257 and the proteins of various truncation mutants in infiltrated leaves were detected by Western blotting. The anti-GFP antibody was used. **c** The root expressing of various truncation mutants were checked under a confocal laser scanning microscope. Green fluorescent protein (GFP) was used as a control (CK), the fusion proteins of GFP with Pb257, the GOLD domain of Pb257 (25-G), and 44–65 aa of the GOLD domain (25-M1), respectively, were expressed in the roots of *B. rapa*. Green fluorescence appeared in root hair and root epidermis. **d** The growth of roots after control treatment (CK) or transient over-expression of Pb257, 25-G, and 25-M1 in *B. rapa*. Photographs were taken for 17-day seedlings. Small gall appearing at the root tip is marked by the circle. The experiment was replicated three times. **e** Statistics on plants with swollen or stubby roots. Panels on right show the proportion of roots where small galls or stubby were present (green) and absent (orange). 50–60 seedlings in each treatment group were used for analysis. Significant difference was determined by Student's *t* test. ****P* < 0.001

were still capable of triggering cell death. In contrast, the mutants 25dG and 25m1 failed to induce cell death (Fig. 3a). The results indicates that the GOLD domain and the 44–65 aa segment of the GOLD domain play a key role. Therefore, individual fusions of the two segments with the signal peptide of Pb257 were constructed and then transiently expressed in *N. benthamiana*. Only the fusion with the complete GOLD domain (25-G) could induce cell death, while the fusion with the partial sequence of the GOLD domain (25-M1) failed (Fig. 3a). The results show that the GOLD domain is crucial for the full function of Pb257.

The GOLD domain of Pb257 is required for root swelling

Since *P. brassicae* can cause root swelling, we speculate that GOLD domain-containing proteins may play roles in promoting plant growth. Therefore, Pb257, 25-G, and 25-M1 were transiently expressed in *B. rapa* via *A. tumefaciens*-mediated infiltration. Fluorescence of the GFP fusion proteins was detected at 72 hpi (Fig. 3c), which showed that they could be expressed in the roots. In the cabbages overexpressing Pb257 and 25-G, the main roots became shorter, thicker, and slightly swollen, with reduced lateral roots in 17-day seedlings (Fig. 3d), similar to mild symptoms of clubroot. The cabbages

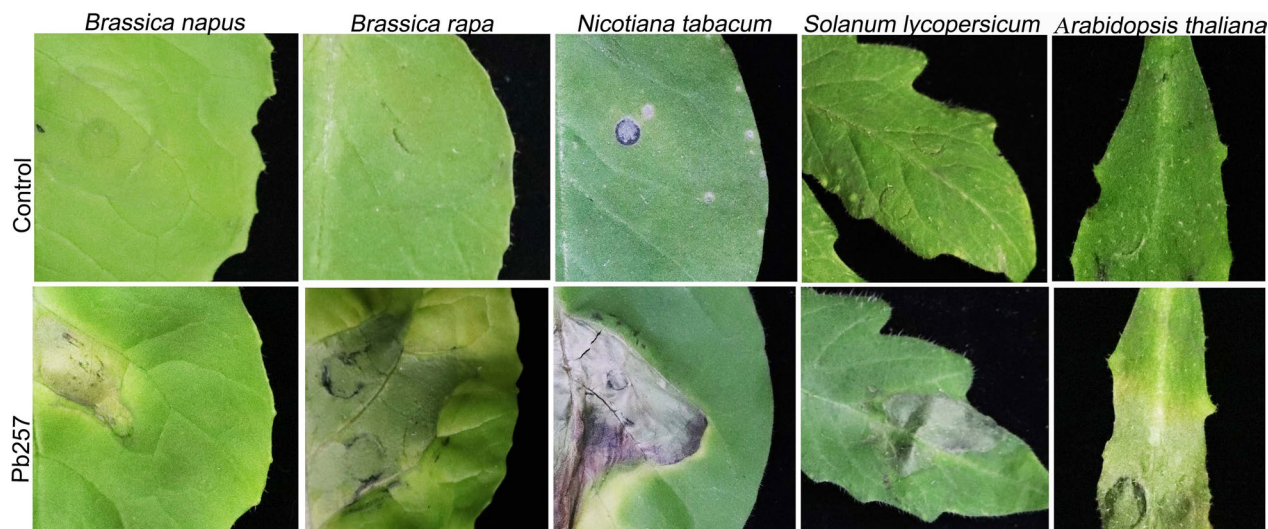


Fig. 4 The putative effector Pb257 induces cell death in multiple plants. Pb257-infiltrated leaves exhibited cell death at 5 days post infiltration (dpi) in *B. napus*, *B. rapa*, *N. tabacum*, *S. lycopersicum*, and *A. thaliana*. Green fluorescent protein (GFP) was used as a negative control. Infiltration of a concentration ($OD_{600}=0.5$) of *Agrobacterium* containing Pb257 expression construct into various plant leaves. Pb257 can induce varying degrees of cell death. The experiment was replicated three times

overexpressing 25-M1 showed no difference from those with the control treatment. The finding suggests that the GOLD domain protein exerts a pivotal influence on the root growth of the host.

Pb257 is required for virulence of *P. brassicae*

To determine the involvement of Pb257 in *P. brassicae* development or infection, we quantified the expression level of Pb257 using reverse transcription-quantitative PCR. Notably, Pb257 exhibited significant up-regulation at 4 and 10 dpi and was still up-regulated at 16 dpi and 30 dpi (Fig. 7c). The expression pattern strongly suggests a crucial role during the primary and secondary infection stages.

To investigate the role of Pb257 in *P. brassicae* virulence, we utilized agrobacterium-mediated transient over-expression and host-induced gene silencing (HIGS) techniques in *B. rapa* to alter the transcript level of Pb257. Because the signal peptide was required for Pb257-induced cell death, Pb257 fused with its signal peptide Pb257-O was transiently expressed in *B. rapa*. Seedlings exhibiting different expression levels of Pb257 could be infected by *P. brassicae*. At 7 dpi, the number of empty zoosporangia in the Pb257-O seedlings was greater than that in the other seedlings (Fig. 7a), indicating the maturation and release of secondary zoospores. At 14 dpi, little or no zoosporangia could be observed in root hair and epidermal cells, resting spores were observed in the root cortex of the seedlings with transient over-expression of Pb257. The numbers of zoosporangia in the seedlings of Pb257-HIGS had no obvious

difference when compared with those in the control-treated seedlings, and most of them were zoosporangia. The results indicated that Pb257 could promote *P. brassicae* infection and development. At 30 dpi, the roots of cabbages with silenced *Pb257* showed reduced virulence when compared with those of the Pb257-O and control-treated cabbages (Fig. 7a). We also recorded the cabbage disease index (DSI). The DSI values of seedlings in the control, Pb257-O, and Pb257-HIGS groups were 51, 62, and 39, respectively. Consistent results were obtained in *B. napus* (data not shown).

Subsequently, we then assessed the impact of *Pb257* mRNA level on *P. brassicae* infection. The analysis revealed an up-regulation of Pb257 expression in *B. rapa* roots transiently expressing Pb257-O, while a reduction was observed in *B. rapa* roots transiently expressing Pb257-HIGS compared with the roots of control *B. rapa* (Fig. 7d). These findings uncover the involvement of Pb257 in promoting *P. brassicae* virulence.

Discussion

Pathogen effectors play an important role in the successful infection of the host, and serve as important mediators interfering with the defense response of the respective hosts (He et al. 2020). Transcriptome sequencing data have revealed that certain candidate effectors of *P. brassicae* are induced during primary infection or secondary infection, suggesting their potential significance (Chen et al. 2019; Pérez-López et al. 2020). The identification and characterization of virulence effectors are

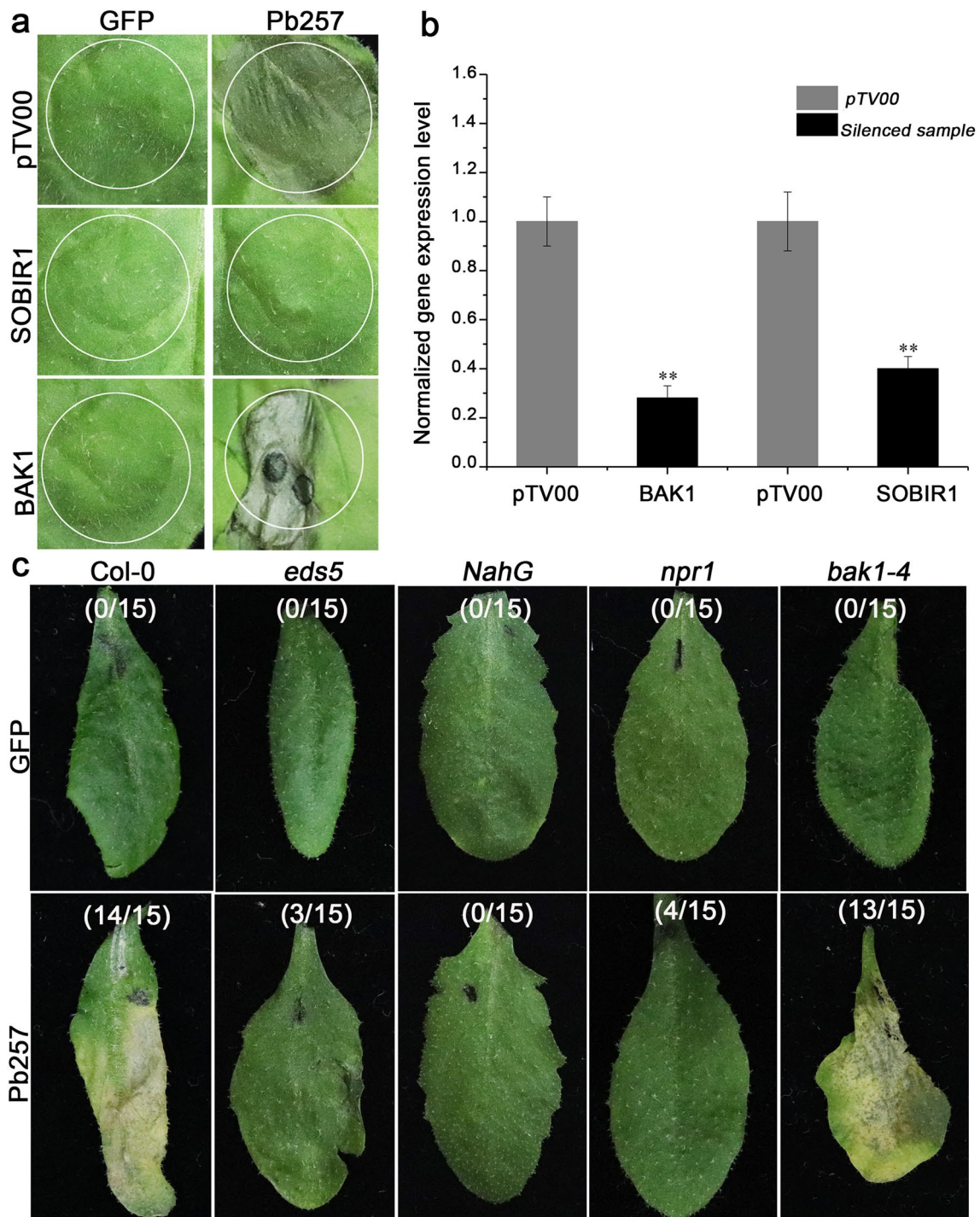


Fig. 5 Analysis of dependent genes and pathway of Pb257 induced cell death. **a** Representative photographs of Pb257-induced cell death in *A. thaliana eds5, npr1, bak1-4, NahG* mutant leaves, and wild-type leaves at 4 days post-infiltration. Numbers e.g., 14/15, indicate that 14 of 15 infiltrated leaves exhibiting cell-death. **b** Representative photographs of Pb257-induced cell death in silenced *N. benthamiana* leaves at 4 days post-infiltration. Pb257 was transiently expressed in *N. benthamiana* leaves silenced for pTV00 (control), *BAK1*, or *SOBIR1*. GFP was control proteins. The experiment was replicated ten times. **c** The transcript expression levels of *SOBIR1* and *BAK1* in the corresponding infiltration area of leaves were taken to analyze by qRT-PCR. Statistical analysis was performed by one-way ANOVA, followed by Tukey's multiple comparison test. Error bars are the standard deviation (SD) of three independent replicates (** $p < 0.01$)

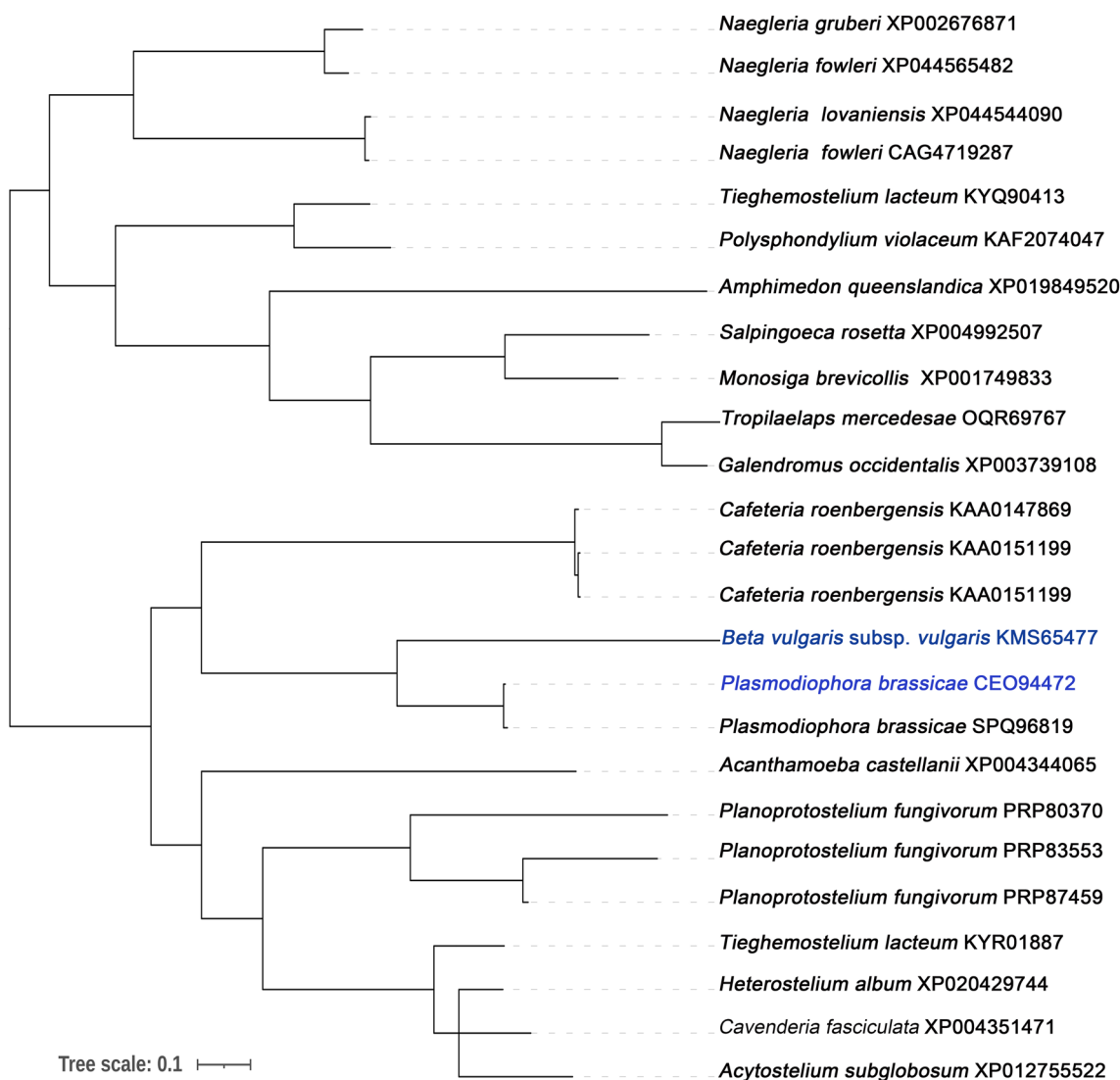


Fig. 6 ML tree of GOLD domain-containing protein families

pivotal for elucidating the pathogenic mechanisms of *P. brassicae*.

Agroinfiltration assays were conducted in both hosts and nonhosts, including *N. benthamiana*, *S. lycopersicum*, *N. tabacum*, *A. thaliana*, *B. napus*, and *B. rapa* to evaluate the effect of Pb257 on cell death induction. The effector-induced cell death has been reported in various pathogens, such as *P. sojae*, *P. syringae*, and *Magnaporthe oryzae*. Avh241 triggers cell death in host soybean and non-host tobacco, while Avh238 induces cell death in host soybean as well as several nonhost plants e.g. tomato, tobacco, potato, and eggplant (Badel et al. 2006; Ham et al. 2008; Yang et al. 2017). The possible explanations could be that the effector-triggered cell death in different plant species might be an effect on its virulence

targets or a conserved host target. The effectors can be recognized by plant PRRs. For example, *P. sojae* effector PsXEG1 was a PAMP (Ma et al. 2015), which could be recognized by a receptor-like protein in *N. benthamiana* (Wang et al. 2018). Pb257 may be detected by a cell surface pattern receptor, because NbSOBIR1 was required for Pb257-mediated cell death. Pb257 failed to induce cell death in *A. thaliana eds5*, *npr1*, and NahG mutants, suggesting that the classical salicylic acid (SA) pathway plays a key role in the defense response induced by Pb257. Plant resistance to biotrophic pathogens is believed to be mediated by SA signaling pathway (Javed et al. 2023).

Some effectors, such as AEP and XEG1 in *P. sojae* triggered cell death in *N. benthamiana* depending on BAK1 (Ma et al. 2015; Xu et al. 2021). BAK1 acts as a

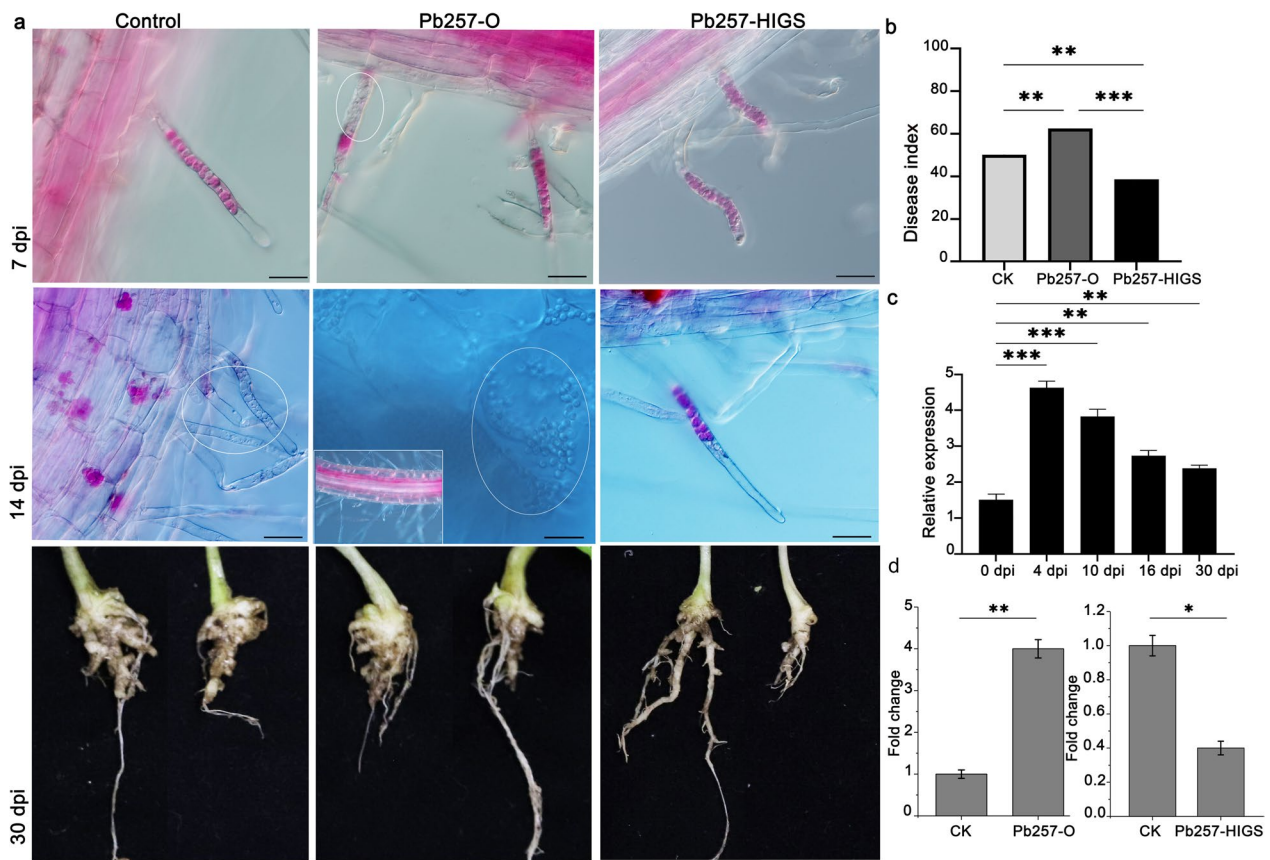


Fig. 7 Pb257 promotes *P. brassicae* infection in *B. rapa*. **a** Images of infected root hairs and epidermal cells at 7 dpi and 14 dpi, respectively, and symptoms at 30 dpi. The transient overexpression of Pb257 fused to the signal peptide (Pb257), host-induced gene silencing (HIGS) of Pb257 (Pb257-HIGS). **b** The disease index of *B. rapa* inoculated with *P. brassicae*. 50–60 seedlings in each treatment group were used for analysis. Significant difference was determined by Student’s *t* test. (** $p < 0.01$; *** $p < 0.001$). **c** qRT-PCR analysis of Pb257 transcript levels at different time points. The constitutively expressed gene *PbActin* was used as an internal reference. The experiment was replicated three times. **d** Detection of Pb257 expression levels by qRT-PCR at 8 days post-inoculation *P. brassicae*. Empty vector treatment was used as a control (CK), Pb257-O means transient overexpression of Pb257, Pb257-HIGS means the host-induced gene silencing (HIGS) of Pb257. Statistical analysis was performed by one-way ANOVA, followed by Tukey’s multiple comparison test. Error bars are the standard deviation (SD) of three independent replicates (* $p < 0.05$; ** $p < 0.01$)

central molecular regulator by forming complexes with numerous PRRs to mediate classical defense pathways. However, Pb257-triggered cell death is independent of BAK1, suggesting that Pb257 is recognized by plants via a distinct pathway. Together, these results indicate that plants employ multiple pathways to regulate the defense response against different effectors. The effectors produced by pathogens can target various host factors to interfere with host defense.

Observation on the infection of *P. brassicae* revealed an increased number of empty zoospangia in the root hairs at 7 dpi and the formation of resting spores in the root cortex at 14 dpi in the seedlings with transient Pb257 over-expression. The results indicated that Pb257 could promote the development of *P. brassicae*. Pb257 expression was significantly up-regulated during the primary infection stage, indicating its potential role in

promoting nutrient absorption from hosts and facilitating the diffusion and movement of *P. brassicae*. As previously reported, *P. sojae* effector XEG1 was significantly up-regulated during the biotrophic stage of infection, and meanwhile induced plant cell death during early infection. The triggered-cell death is a critical creation of nutrition source prior to the development of haustoria, as well as facilitates the breaking of physical barriers which is conducive to invasion of host tissues (Ma et al. 2015). In addition, the other effectors, AEP1 in *P. sojae* and Fg12 in *Fusarium graminearum*, which trigger cell death in *N. benthamiana*, contribute to pathogen virulence (Ma et al. 2015; Xu et al. 2021). These effectors, which induce cell death, likely play additional roles beyond their influence on the plant defense response.

Sequence analysis showed that Pb257 had a conserved GOLD domain (44–135 aa). GOLD domain proteins

including the members of the p24 (TMED/p24/ gp25L/ emp24) family, have been identified across various species. Medical studies in humans have shown that the expression levels of TMED proteins tightly regulate cell survival, migration, and proliferation (Aber et al. 2019). For example, TMED2 is significantly upregulated in tissues of breast cancer, ovarian cancer, and other cancers, and plays an important role in proliferation (Ge et al. 2020). TMED3 promotes proliferation, migration, and invasion of breast cancer cells (Pei et al. 2019). Moreover, TMED family members are known to be key players in innate immune signaling pathways (Connolly et al. 2013). These reports suggest that the GOLD domain-containing protein Pb257 in *P. brassicae* may serve a similar function. Deletion or partial mutation of the GOLD domain resulted in loss of function for triggering cell death and root swelling. A comprehensive analysis of the amino acid sequence was conducted by utilizing Phyre2. Notably, the examined amino acid sequences lacking residues 44–65, resulting in the prediction of a distinct secondary structures compared with the complete Pb257 and sequences lacking residues 46–78. The lacking residues 44–65 is speculated to potentially impact its function by perturbing its secondary structure.

The roots of plants infected by *P. brassicae* exhibit hypertrophy and swelling as a result of the interference caused by *P. brassicae* with the host's growth hormone pathways (Schuller and Ludwig-Müller 2016). Considering that the roots of *P. brassicae* become short and thick after treatment with Pb257, it is speculated that Pb257 may exert a significant influence on the host's growth hormone pathway.

Based on the phylogenetic analysis of the GOLD protein family, Pb257 from *P. brassicae* belongs to protozoa rather than fungi, which is consistent with current classification system. The results indicate that Pb257-encoding gene can be potentially used as a molecular marker for identifying *P. brassicae*.

Conclusions

Overall, Pb257 is an important effector protein that plays a crucial role in the virulence of *P. brassicae*. Due to its unique and conserved nature in *P. brassicae*, it could be a potential molecular target for developing effective strategies to control clubroot disease.

Methods

Preparation and inoculation of resting spores

P. brassicae was isolated from Chengdu in China, which was identified as pathotype 4 based on the differentials of Williams (1996). Resting spores of *P. brassicae* were prepared according to a previous method (Ji et al. 2014).

The resting spores were adjusted to a concentration of 1×10^7 spores/mL in sterile water, and stored at 4°C.

Morphological characterization

The phloxine B powder (S19236, Yuanye Biotech, China) was dissolved in 100 mL sterilized water to prepare 0.5% phloxine dye solution. The solution was stored in a tinfoil-wrapped bottle. The roots infected by *P. brassicae* were stained with the 0.5% phloxine solution for one minute, followed by washing away the background color of the roots with sterile water. The plasmodia, zoospore, and resting spores of *P. brassicae* exhibited a red stain under observation using a Carl Zeiss microscope (GmbH37081, Gottingen, Germany).

Assessment of secretory activities directed by signal peptides from putative secretory proteins in yeast

The secretory function of the signal peptide was confirmed by using a yeast signal sequence trap assay (Jacobs et al. 1997). The signal peptide sequence of Pb257 was amplified using a high-fidelity DNA polymerase (TransGen Biotech, China). The primer sequence is listed in Additional file 2: Table S1. Subsequently, the purified PCR products were inserted into the linearized Psc2 plasmid. These recombinant vectors were then transformed into *Saccharomyces cerevisiae* yeast strain YTK12, which was cultured on the CMD-W medium (0.075% tryptophan dropout supplement, 2% sucrose, 0.67% yeast N base without amino acids, 0.1% glucose, and 2% agar) as well as YPRAA medium (1% yeast extract, 2% peptone, 2% raffinose, and 2 µg/mL antimycin A). If the strains can grow on both CMD-W and YPEAA media, it indicates that the signaling peptide may possess secretory function. Otherwise, the 2,3,5-triphenyltetrazolium chloride (TTC) assay was performed to further verify the secretory activity of signal peptide.

Virus-induced gene silencing (VIGS) assay in *N. benthamiana*

The cDNA sequences of *NbBAK1* and *NbSOBIR1* were retrieved from the NCBI website, and approximately 300-bp fragments were selected for amplification, the primer sequences are provided in Additional file 2: Table S1. Subsequently, PCR products were digested with *EcoRI* and *BamHI* restriction enzymes and then cloned into the pTRV2 vector using the ClonExpress II One Step Cloning Kit (Vazyme, Nanjing, China). The recombinant vector was transformed into *A. tumefaciens* GV3101. All recombinant vectors were validated through sequencing by Sangon Biotech (Shanghai, China).

The *A. tumefaciens* GV3101 strains containing pTRV1 or pTRV2 vector were cultured at 28°C for 36 h. Then the bacterial suspension was centrifuged and

the precipitation was suspended with MMA solution (10 mM MES, 10 mM MgCl₂, and 150 mM acetosyringone), and adjusted to a concentration of OD600 about 0.5. Then the bacterial solution of pTRV1 was mixed with pTRV2 in equal proportions. *N. benthamiana* leaves of the four-leaf stage were infiltrated. At 6 and 21 days post-infiltration, RT-qPCR was used to detect the expression levels of the *NbBAK1* and *NbSOBIR1* genes to determine their silencing efficiency. The experiment was repeated five times.

Plasmid construction of Pb257 and deletion mutagenesis of Pb257

RNA extraction and cDNA synthesis was performed as reported previously (Jin et al. 2021). The full-length sequence of Pb257 was amplified using a high-fidelity enzyme (TransGen Biotech, Beijing, China). The primer design of Pb257 was based on *P. brassicae* PBRA_000257 (Accession: CDSF01000 001.1). The PCR product was cloned into the pBIB-BASTA-35S-GWR-green fluorescent protein (*GFP*) vector, which was used to generate mutant nucleotide sequences of Pb257. Primer sequences are listed in Additional file 2: Table S1.

Gene function verification by host-induced gene silencing (HIGS) and transient over-expression

A 312-bp forward fragment of Pb257 was inserted into the *XhoI/KpnI* site of the pKANNIBAL vector, and then the reverse fragment was inserted into the *HindIII/XbaI* site of the same vector. The sequences upstream of the forward fragments and downstream of the reverse fragments contained a *BsrGI* restriction enzyme site. The fragment in the pKANNIBAL vector released by *BsrGI* digestion was inserted into the *BsrGI/BsrGI* site of the pBIB-BASTA-35S-GWR-GFP vector. The recombinant vector was transformed into *A. tumefaciens* strain GV3101. The bacterial suspension containing Pb257 sequence was centrifuged and then resuspended in MMA solution (10 mM MgCl₂, 10 mM MES, and 150 mM Acetosyringone, PH 5.7). The bacterial solution was adjusted to an OD600 concentration of 0.8. The seeds were mixed with this solution and gently shaken for 10–20 h (Yue et al. 2021; Zhang et al. 2023). The transient expression of Pb257, 25-G, and 25-M1 in *B. rapa* was achieved through *A. tumefaciens*-mediated infiltration using the same method.

The treated seeds were planted in sterilized soil and grown at 25°C under a 16 h light/8 h dark cycle. 500 µL of the above solution was dripped into the soil around the roots of 10-days-old seedlings. Root samples were pulled out at 20 days after treatment to determine the silencing efficiency. Seven-day-old seedlings were inoculated with *P. brassicae*. 400 µL of suspension of *P. brassicae*

was injected into the soil around the roots of each seedling. Disease index was determined at 25–30 days post-inoculation. Clubroot severity (disease severity index, DSI) was assessed based on a standard 0–4 scale (Siemens et al. 2002). At least 50 plants were assessed in each experiment.

Oxygen burst detection

Reactive oxygen species in *N. benthamiana* leaves were detected using 3, 3'-diaminobenzidine stain (Sangon Biotech, China) according to a previously described method (Hans et al. 1997).

The phylogenetic analysis

We constructed phylogenetic trees by Bayesian inference (BI), included in MrBayes 3.2.5, and maximum likelihood (ML), included in the software MEGA7 (Kumar et al. 2016; Ronquist et al. 2012). The robustness of the topology of the ML trees was assessed using 1000 bootstrap replicates. BI was run for 5 million generations of Markov chains sampled every 100 generations to establish the convergence of all parameters. The detailed legend of EMP24_GP25L proteins family were listed in Additional file 2: Table S2.

Quantitative reverse transcription-polymerase chain reaction

RNA extraction was performed as above mentioned (Jin et al. 2021). RNA was extracted from seedling roots mixed with resting spores used for inoculation to assess the transcript levels of Pb257 at 0 dpi. Additionally, RNA extraction was performed from the seedling roots at 4, 10, 16, and 30 days post-inoculation with *P. brassicae* to assess the transcript levels of Pb257 in host plants following infection. cDNA was synthesized according to the previously described method (Jin et al. 2021). The values for the relative expression levels were determined by using an internal reference gene *actin* in *B. napus*. Fluorescent quantitative PCR was performed using a Bio-Rad Real-Time PCR System (Bio-Rad, USA). We used the 2^{-ΔΔCt} algorithm to calculate the relative expression levels of genes (Livak and Schmittgen 2001). The primer sequences are listed in Additional file 2: Table S1. Three biological replicates were used. Statistical analysis was performed by one-way ANOVA, followed by Tukey's multiple comparison test.

Abbreviations

GOLD	Golgi dynamics
HIGS	Host-induced gene silencing
GFP	Green fluorescent protein
ETI	Effector-triggered immunity
PTI	Pathogen-associated molecular pattern (PAMP)-triggered immunity

RT-qPCR Quantitative reverse transcription-polymerase chain reaction

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s42483-024-00236-x>.

Additional file 1: Figure S1. Expression profiling of the indicated defense-related genes in Pb257-expressing *N. benthamiana* leaves. **Figure S2.** ML tree of GOLD domain-containing protein families.

Additional file 2: Table S1. The primers sequence. **Table S2.** The detailed legend of EMP24_GP25L proteins family.

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Author contributions

HY and WW designed the project. HY, RL, CJ, XF, YZ, XW, and YuZ performed the experiments. LY prepared the seedlings. HY wrote the manuscript. All authors read and approved the manuscript.

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Availability of data and materials

All data generated during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹State Key Laboratory of Crop Gene Exploration and Utilization in Southwest China, Sichuan Agricultural University, Chengdu 611130, China. ²The Agricultural Comprehensive Service Center, Agriculture and Rural Bureau of Xindu District, Chengdu 610500, China. ³College of Agronomy, Sichuan Agricultural University, Chengdu 611130, China.

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