INTRODUCTION

In the arms race between plants and microbial plant pathogens, plants develop complex and multilayered immune systems for self-defence: pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs)-triggered immunity (PTI), mediated by pattern recognition receptors (PRRs) and effector-triggered immunity (ETI), mediated by the specific disease resistance (R) proteins that recognize avirulence (AVR) effectors (Chisholm et al., 2006; Jones and Dangl, 2006; Dodds and Rathjen, 2010; Boutrot and Zipfel, 2017; Cesari, 2018; Han, 2018). In plants, perception of PAMPs or effectors activates a complicated signal transduction network, including mitogen-activated protein kinase (MAPK) cascades, and/or chemical...
signalling by plant hormones and transcriptional regulation via transcription factors (Pitzschke et al., 2009; Wang et al., 2019b). The immune activation culminates in a series of physiological changes in the plant, such as reactive oxygen species (ROS) production, cell wall fortification, and the localized rapid cell death known as the hypersensitive response (HR) (Ingle et al., 2006; Franceschetti et al., 2017).

Oomycetes are a group of straminipilous organisms that are phylogenetically distant from true fungi (Göker et al., 2007; Beakes et al., 2012). Notably, phytopathogenic oomycetes are a constant threat to many important crops, rendering enormous crop yield losses globally (Tyler, 2007; Lamour et al., 2012; Fry et al., 2015). The phytopathogenic oomycetes could use the complement effectors, secreted into either apoplastic or cytoplasmic regions, as major virulence factors for successful infection and causing disease symptoms (Tyler et al., 2006; Wang and Wang, 2018a). Among them, the cytoplasmic RXLR effectors that contain an N-terminal signal peptide followed by the conserved Arg-any amino acid-Leu-Arg (RXLR) motif, are a large superfamily of virulence proteins in oomycetes (Rehmany et al., 2005; Whisson et al., 2007). The RXLR motif is located within 30 residues downstream of the secretion signal cleavage site and is frequently followed by a less conserved Asp-Glu-Glu-Arg (dEER) motif (Wawra et al., 2012). It is suggested that the RXLR-dEER motif is involved in translocating effector proteins from haustoria into host cells (Dou et al., 2008; Kale et al., 2010). RXLR effectors have become a focus for studying plant–pathogen interaction in the past decade, with numerous effector genes identified and characterized in Phytophthora and downy mildew species (Tyler et al., 2006; Haas et al., 2009; Baxter et al., 2010; Yin et al., 2017). Many studies have reported that RXLR effectors are involved in the suppression of PTI and/or ETI (Wang et al., 2011; Kong et al., 2017; Fan et al., 2018). Additionally, some of them can trigger immune response–related cell death, for example Phytophthora sojae Avh238 (Yang et al., 2017), Phytophthora capsici Avh1 (Chen et al., 2019), and Plasmopara viticola RXLR16 (Xiang et al., 2017).

After being secreted by oomycete pathogens, the RXLR effectors are transported to a range of subcellular localizations in plant cells, including the nucleus, cytoplasm, or plasma membrane, which often associates with their functions and/or mode of action (McLellan et al., 2013; Xiang et al., 2016). The study of subcellular localization of RXLR effectors from Phytophthora infestans revealed that the nucleocytoplasmic distribution in plant cells is the most common pattern (Wang et al., 2019a). P. sojae Avh238 triggers cell death when it is present in the nucleus (Yang et al., 2017). In addition, plasma membrane localization of P. sojae Avh241 is required for its cell death-inducing activity (Yu et al., 2012).

Peronoschytora litchii is one of the most destructive oomycete pathogens, causing downy blight on litchi fruits as well as tender leaves and panicles rot of litchi plants (Zheng et al., 2019). As a hemibiotrophic pathogen, P. litchii undergoes biotrophic and necrotrophic phases during infection. However, fewer studies have been conducted on the functions of P. litchii genes, hence there is scarcity of information about its pathogenesis and the litchi–P. litchii interaction mechanisms (Jiang et al., 2017, 2018; Kong et al., 2019). The identification and/or investigation of RXLR effectors in P. litchii lags behind that for other Phytophthora and downy mildew species, with only bioinformatics prediction of 245 putative RXLR effector genes (Ye et al., 2016). Therefore, exploring the roles of P. litchii RXLR effectors in host–pathogen interaction could potentially reveal mechanisms underlying oomycete pathogenicity and host resistance, which would be beneficial for developing disease control strategies.

In this study, systematic screening identified three P. litchii RXLR effectors, PlAvh23, PlAvh133, and PlAvh142, that are able to induce cell death by transient expression in Nicotiana benthamiana. Further experiments showed that PlAvh142 could induce cell death in different plants, therefore we focused on the investigation of PlAvh142 functions. We found that the internal repeats are indispensable for the cell death–inducing activity. PlAvh142 could localize in both cytoplasm and nucleus in the plant cell, but its cytoplasmic localization was demonstrated to be essential for triggering cell death. Through virus-induced gene silencing (VIGS) assays, we found that cell death triggered by PlAvh142 is dependent on RAR1, SGT1, and HSP90, which suggests that PlAvh142 might be perceived by the innate immune system in plant. Finally, by genetic manipulation we proved that PlAvh142 is important for P. litchii infection to its host plant litchi. The work provides a critical foundation for further dissection of the roles of P. litchii RXLR effectors in interaction with plant immunity.

2 | RESULTS

2.1 | PlAvh142 can induce cell death in plants

To systematically investigate the functions of P. litchii RXLR effectors, 212 out of 245 predicted RXLR effectors (Ye et al., 2016) were successfully cloned and then transiently expressed individually in N. benthamiana to test their cell death–inducing activity. Effector gene cloning and cell-death induction analyses are summarized in Table S1. Three RXLR effectors, PlAvh23, PlAvh133, and PlAvh142, were found to be able to induce cell death at 3–7 days post-agroinfiltration (dpi) (Figure 1a). Among them, PlAvh142 exhibited strong cell death–inducing activity in N. benthamiana, Solanum melongena, and Solanum lycopersicum (Figure 1b). Thus, this effector was selected for further analyses in this study. The cell death activity was further tested by infiltrating Agrobacterium tumefaciens (carrying a green fluorescent protein [GFP]-tagged PlAvh142) with OD600 = 0.001, 0.01, 0.1, and 0.4, respectively, in N. benthamiana leaves (Figure 1b). The results showed that PlAvh142 induced cell death with OD600 = 0.01, 0.1 or 0.4. Western blot assays confirmed the expression of GFP-PlAvh142 in all the agroinfiltrated leaves except for the OD600 of 0.001 (Figure 1c). Sequence analysis indicated that PlAvh142 encodes a protein of 466 amino acids with a signal peptide from 1 to 20 amino acids. Moreover, it harbours the typical N-terminal RXLR-dEER motif (50–71 amino acids) and...
a potential unknown functional C-terminus (Figure S1). Overall, we identified RXLR effectors from *Peronophythora litchii* that could induce plant cell death.

2.2 | Expression of PIAvh142 activates various immune responses in *N. benthamiana*

Cell death triggered by ETI is often considered as a part of the defence response resulting in suppression of disease progress (Balint-Kurti, 2019). In general, some other immune responses may precede this exhibition, including ROS accumulation, callose deposition, and changes in levels of phytohormones (Asai and Yoshioka, 2009; Deb *et al.*, 2018; Bürger and Chory, 2019). Therefore, we assessed whether PIAvh142 could trigger oxidative burst production or callose deposition in *N. benthamiana* leaves via agroinfiltration, with expression of GFP alone as control. There was strong ROS accumulation and massive callose deposition in the PIAvh142-expressing leaves at 36 hr post-agroinfiltration (hpa) (Figure 2a–c). In contrast, the control leaves expressing GFP showed no visible ROS accumulation or callose deposition (Figure 2a–c). To further explore whether PIAvh142 is able to alter hormone signalling pathways in planta, the salicylic acid (SA)-dependent defence pathway marker genes *NbPR1* and *NbPR2*, jasmonic acid (JA)-dependent defence pathways marker
gene NbLOX, and ethylene (ET)-dependent defence pathways marker gene NbERF1 (Dean et al., 2005; Pieterse et al., 2012; Zhang et al., 2017) were chosen for quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis at different time points (0, 12, 24, and 36 hr) after agroinfiltration of N. benthamiana leaves with GFP-PlAvh142 or GFP. We found that NbPR1, NbPR2, NbLOX, and NbERF1...
were significantly up-regulated in the PlAvh142-expressing leaves at 24 and 36 hpa, compared with the GFP-expressing samples (Figure 2d), indicating an induction of phytohormone signalling by PlAvh142 in *N. benthamiana*. Overall, our results suggested that the expression of PlAvh142 can activate various defence responses in planta.

2.3 The internal repeats are indispensable for PlAvh142-inducing cell death

W, Y or L motifs exist in some RXLR effectors (Win et al., 2012); however, none of them was detected in PlAvh142 (Ye et al., 2016). To further dissect the functions of PlAvh142, the conserved protein domain was analysed and predicted by the web-based program Simple Modular Architecture Research Tool (SMART, http://smart.embl-heidelberg.de/). The prediction results show that in addition to the RXLR region, PlAvh142 comprises two internal repeats (IRs), IR1 (107–234 amino acids) and IR2 (324–457 amino acids), in its C-terminus (Figures 3a and S1). The alignment of IR1 and IR2 showed 25% identity and 52% similarity by BLAST analysis performed on NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). To analyse the role of RXLR and IR motifs in cell death-inducing activity, four truncated PlAvh142 variants were constructed and transiently expressed in *N. benthamiana* (Figure 3a). Our results show that M2 that lacked the RXLR region was still capable of inducing cell death, while either the IR1 (M3) or IR2 (M4) deletion resulted in loss of the ability to induce cell death in *N. benthamiana* (Figure 3a). These experiments suggest that the IRs are indispensable for its cell death induction, but not the RXLR region.

![Figure 3](image-url)  
*Figure 3* Deleting either of the internal repeats in PlAvh142 abolished the ability to trigger cell death. (a) Schematic diagrams of the protein structures of the PlAvh142 deletion mutants are shown in the left. Cell death symptoms in *Nicotiana benthamiana* leaves expressing PlAvh142 deletion mutants are shown in the right. Photographs were taken at 5 days post-agroinfiltration. (b) Western blot confirmation of expression of PlAvh142 mutants using anti-GFP (green fluorescent protein) antibody. The red asterisks indicate protein bands of the correct size. Protein loading was indicated by Ponceau S staining. Similar results were obtained from three independent experiments.
2.4 Cytoplasmic localization is critical for PIAvh142-triggered cell death

To gain insight into the subcellular localization of PIAvh142 in the plant cell, N-terminal GFP-tagged PIAvh142 (without the signal peptide) was transiently expressed in N. benthamiana leaves, and fluorescence was observed by confocal microscopy. The free monomeric red fluorescent protein (RFP) was coexpressed with GFP-PIAvh142 and used as a marker to delineate the nucleus and cytoplasm. GFP-PIAvh142 fusion protein could be detected in both cytoplasm and nucleus of plant cells (Figure 4a). It is documented that the cell death-inducing activity of effectors is often determined by its subcellular location (Du et al., 2015). Hence, to evaluate which subcellular localization of PIAvh142 is essential for the cell death induction, we forced GFP-PIAvh142 to the nucleus or cytoplasm by attaching a nuclear localization signal (NLS) or nuclear export signal (NES), and assessed their cell death-inducing activity, respectively. Constructs with mutated NLS (nls) or NES (nes) fused to GFP-PIAvh142 were included as controls. The green fluorescence signals from NES-fused or NLS-fused GFP-PIAvh142 were detected only in cytoplasm or nucleus, respectively, whereas mutated nls or nes fused GFP-PIAvh142 showed similar fluorescence patterns to GFP-PIAvh142 (Figure 4b). When these PIAvh142 variants were overexpressed in N. benthamiana leaves, GFP-NES-PIAvh142 showed strong cell death as the GFP-PIAvh142 (Figure 4c). In contrast, the cell death was largely attenuated in the leaves expressing GFP-NLS-PIAvh142 (Figure 4c). Moreover, mutated nes and nls did not alter PIAvh142-induced cell death (Figure 4c). Together, these results imply that localization of PIAvh142 in plant cell cytoplasm is critical for its cell death-inducing activity.

2.5 RAR1, SGT1, and HSP90 are required for PIAvh142-induced cell death in N. benthamiana

ETI mediated by intracellular immune receptors usually involves a set of downstream components (Chiang and Coaker, 2015). For example, HSP90, SGT1, RAR1, NDR1, and ED1 are reported to be associated with signal transduction in this process (Shirasu, 2009; Bhattarcharjee et al., 2011; Knapper et al., 2011). In order to determine whether these plant innate immunity components are associated with PIAvh142-induced cell death, virus-induced gene silencing was used to individually knock down these genes in N. benthamiana. Two weeks after inoculation with Agrobacterium carrying the VIGS constructs, PIAvh142 was expressed in these silenced plants and then cell death was scored 5 days later. We observed that silencing of the RAR1, SGT1, or HSP90 significantly compromised the cell death induced by PIAvh142 (Figure 5a,b). However, the cell death proportion in NDR1- or ED1-silenced plants was similar to that of the control plants (Figure 5a,b). The relative expression of these genes was verified by RT-qPCR (Figures 5c and S2c). Immunoblotting assays confirmed the stable expression of GFP-PIAvh142 in the RAR1, SGT1, or HSP90-silenced plants (Figure 5d).

Other signalling pathway components of plant innate immunity, including BAK1, SOBR1, MEK1, MEK2, MAP3Kα, WIPK, SIPK, WRKY2, and MYB1, were also individually knocked down in N. benthamiana. Nevertheless, there was no obvious difference in cell death proportion in these silenced plants compared with the control (Figure S2). Taken together, these results show that RAR1, SGT1, and HSP90 are required for the cell death induced by PIAvh142 in N. benthamiana.

2.6 PIAvh142 is up-regulated in P. litchii zoospores and during the early phase of infection

In order to investigate the biological function of PIAvh142 in P. litchii development and pathogenicity, we assessed the expression profile of PIAvh142 during different stages including mycelial growth, zoospore development, and infection of litchi leaf. The results showed that PIAvh142 was highly up-regulated in zoospores and infection stage (at 1.5, 3, 6, 12 or 24 h post-inoculation [hpi]) in comparison to mycelia. The highest expression peak appeared at 3 hpi, and then rapidly declined (Figure 6). However, the relative expression level at 24 hpi was still several fold higher than that of the mycelia. The accumulation of PIAvh142 transcript in zoospores and infection phase suggests that it might play a role in P. litchii infection.

2.7 PIAvh142 contributes to P. litchii virulence

To further explore the possible role played by PIAvh142 during P. litchii infection to its native host litchi, we generated the PIAvh142 knockout mutants using the CRISPR/Cas9 gene editing system. The single guide (sg) RNA targeted PIAvh142 were designed using the web tool EuPaGDT (http://grna.ctegd.uga.edu/) and gene replacement strategy schematically displayed in Figure 7a. Finally, we successfully generated three mutants, T14, T22, and T46, in which PIAvh142 was replaced by the NPTII gene as verified by PCR amplification (Figure 7b) and confirmed by Sanger sequencing. At the same time, two PIAvh142-overexpression mutants (OE7 and OE10) were also obtained and verified by RT-qPCR (Figure S3). The mycelial growth of all mutants mentioned above was identical to that of wild-type strain SHS3 (Figure S4).

Next, we inoculated the tender leaves of litchi plants with 100 zoospores from the wild-type strain, knockout mutants, or overexpressed mutants. An unsuccessful knockout transformant T37 was included, serving as negative control. We observed that the PIAvh142 knockout mutants caused fewer disease symptoms in litchi leaves at 48 hpi compared to that of the wild-type strain (Figure 7c,d). In contrast, the overexpressed mutants caused more severe disease symptoms (Figure 7c,d). These results indicate that PIAvh142 contribute to the P. litchii virulence during infection in its native host litchi.
FIGURE 4 Cytoplasmic localization is critical for PIAvh142-induced cell death. (a) Confocal microscopy imaging shows that green fluorescent protein (GFP)-tagged PIAvh142 localizes to both the nucleus and the cytoplasm. GFP-tagged PIAvh142 was transiently coexpressed with red fluorescent protein (RFP) via agroinfiltration in Nicotiana benthamiana with an OD$_{600}$ of 0.1. Images are from GFP channel (left panel), RFP channel (middle panel), and the overlay (right panel) in N. benthamiana leaf cells. Scale bar, 20 μm. Photographs were taken at 36 hr post-agroinfiltration (hpa). (b) Confocal microscopy images showing the subcellular localization of PIAvh142 attached with the nuclear localization signal (NLS) and nuclear export signal (NES), and the mutant forms nes and nls. The fusion constructs were agroinfiltrated at a final OD$_{600}$ of 0.1. Photographs were taken at 36 hpa. Scale bars, 20 μm. (c) PIAvh142 could still induce cell death when localized to the cytoplasm. Strains with NLS-, NES-, nls- or nes-tagged PIAvh142 were agroinfiltrated at a final OD$_{600}$ of 0.1. Cell death triggered by NLS-targeted PIAvh142 is delayed and weak. Photographs were taken at 5 days post-agroinfiltration. Representative images for each construct were selected from three biological repeats, each of which contained at least five leaves for agroinfiltration.
Like other biotrophic and hemibiotrophic oomycete or fungal pathogens, *P. litchii* must overcome plant defences to establish host colonization. Pathogen effectors play important roles in subverting plant immunity. In oomycetes, much attention has been focused on identification and functional analysis of RXLR effectors over the past decade (Wang et al., 2011, 2019; Xiong et al., 2014; Huang et al., 2019).

Although the genome sequence of *P. litchii* has been published with 245 RXLR predicted (Ye et al., 2016), the function of these effectors during *P. litchii* infection remains unknown. In this study, we used the *N. benthamiana* model system for systematic screening of *P. litchii* RXLR effectors with plant cell death-inducing activity. Here we reported that three effectors identified were able to trigger cell death in *N. benthamiana*, among which PlAvh142 could trigger plant cell death in a broad spectrum of plants and therefore it was chosen for further investigation. Unfortunately, we failed to test whether PlAvh142 could trigger cell death in its host plant, as we were not successful in a particle bombardment assay on litchi leaves despite repeated attempts.

Bioinformatic analysis showed that about 15% of the 245 predicted *P. litchii* RXLR effectors harbour IRs (Table S1), and PlAvh142 contains two IRs in its C-terminal region. IRs exist widely in both eukaryotes and
prokaryotes, and are considered to be involved in protein–protein interaction (Andrade et al., 2001; Pawson and Nash, 2003; Björklund et al., 2006). A recent report showed that P. sojae RXLR effector PsAvh23 contains two IRs and at least one IR that is required for its interaction with host target protein (Kong et al., 2017). In this study we proved that both IRs in PlAvh142 were required for its cell death-inducing activity. To our knowledge, this is the first report on the requirement of IRs for RXLR inducing plant cell death. However, it remains unclear whether these two IRs are required for interaction (if any) between PlAvh142 and its target protein(s) in the plant cells.

RXLR effectors could localize in different compartments of the host cells, in correspondence to their various molecular/cellular functions during the host–pathogen interaction (Liu et al., 2018; Wang et al., 2019a). Thus, perception of effector proteins by the cognate receptor(s) is frequently associated with their localized position, for example activation of R1-mediated HR and resistance required localization of the R1/AVR1 pair in the nucleus, although both AVR1 and its cognate R protein R1 could be observed in cytoplasm and nucleus (Du et al., 2015). In this study we found that localization of PlAvh142 in the cytoplasm of the plant cell was sufficient and essential for inducing cell death, which is different from many other identified cell death-inducing RXLR effectors shown to be localized to the plasma membrane or nucleus in plant cells (Yu et al., 2012; Asai et al., 2018; Yin et al., 2019). A weak cell death was observed in plant cells expressing the nuclear localized PlAvh142 variant, which may be due to a small amount of protein residue in the cytoplasm. We infer that the cell death triggered by PlAvh142 effector may depend on its recognition in the cytoplasm of the plant cell.

A conserved chaperone complex consisting of HSP90, SGT1, and RAR1 is known to stabilize and sustain NLR-mediated ETI responses (Azevedo et al., 2002; Shirasu, 2009), and is required for plant cell death triggered by PvAvh74 (Yin et al., 2019). SGT1 and HSP90, rather than RAR1, are required for PpE4-triggered cell death (Huang et al., 2019). In the cases of P. infestans AVR-blb2 and PITG_22798, cell death-inducing activity is dependent on SGT1 (Oha et al., 2014; Wang et al., 2017). Besides, SGT1 is also involved in PTI and plant defence against viruses (Huitema et al., 2005; Boter et al., 2007). In the present study, silencing of RAR1, SGT1, and HSP90 in N. benthamiana resulted in abolishing PlAvh142-inducing cell death, suggesting that PlAvh142-triggered cell death is possibly the consequence of plant perception and mediated by the HSR (HSP90, SGT1, and RAR1) complex. However, we silenced the other two well-known components, EDS1 and NDR1, of NLR signal transduction in N. benthamiana, and found no change in plant cell death induced by PlAvh142. Likewise, neither did we find any of the tested MAP kinases or transcription factors involved in the PlAvh142-induced cell death. These results corroborate the fact that plants have multiple pathways for mediating cell death in response to different effectors. Combining the results of VIGS assays and PlAvh142-induced cell death in various plants, we infer that a conserved recognition mechanism or function may
underlie this effector. We speculate that either PlAvh142 targets a conserved and critical plant protein guarded by NLR genes or direct NLR recognition is conserved in various plants. Despite this, we cannot rule out the possibility that cell death triggered by PlAvh142 is mediated by an unknown mechanism.

Hemibiotrophic pathogens need to keep the host cell alive before establishing their colonization in the biotrophic stage, and later trigger cell death to promote the necrotrophic infection (Qutob et al., 2002). Some apoplastic or cytoplasmic effectors from Phytophthora pathogens display elicitor activity, that is, they could trigger plant immunity responses, and concurrently contribute to the virulence or promote pathogen colonization. Examples include PsXEG1 (Ma et al., 2015, 2017; Wang et al., 2018b), Avh238 (Yang et al., 2017), and PpE4 (Huang et al., 2019). Another similar example was reported in the cell death-inducing RXLR effector, PcAvh1, which is a virulence factor of P. capsici as its deletion mutants displayed reduced pathogenicity in contrast to the more aggressive overexpression mutants (Chen et al., 2019). Such a seemingly contradictory phenomenon was also observed for PlAvh142 in this study, and we raise the following hypotheses in an attempt to explain this. First, the accumulation of PlAvh142 protein during P. litchii infection to the native host plant may be insufficient to trigger cell death and/ or immune responses under natural conditions. Second, cell death triggered by one RXLR effector could be suppressed by the other cooperative effectors during pathogen infection, which has been already reported (Wang et al., 2011). Therefore, PlAvh142 is still able to enhance colonization and execute its virulence function when its elicitor activity is blocked. Alternatively, the cell death induced by PlAvh142 may contribute to the transition from biotrophy to necrotrophy and thus positively regulate P. litchii virulence. Although it is generally accepted that RXLR effectors facilitate pathogen infection mainly by modulating plant immune system, the functional relationship (if any) between PlAvh142’s cell death-inducing activity and contribution to virulence awaits elucidation; alternatively, a mechanism(s) other than cell death induction underlying PlAvh142 virulence function needs to be uncovered.

Overall, we report here, for the first time, that an RXLR effector secreted by P. litchii acts as an elicitor that triggers immune responses in plants. The possible mechanism involved in perception of PlAvh142 will be very useful for exploring the potential resistance genes or materials for the litchi plant, which provides insights into novel disease control strategies. The next step is to identify the potential PlAvh142-interacting protein(s) to reveal the functions of RXLR effector in litchi–P. litchii interaction, for a better understanding of the biological functions of RXLR effectors.

4 | EXPERIMENTAL PROCEDURES

4.1 | Microbial strains, plant material, and culture conditions

P. litchii strain SHS3 was cultured on carrot juice agar (CJA) medium (juice from 200 g carrot topped up to 1 L, 15 g agar/L for solid medium) at 25 °C in the dark. Escherichia coli DH5α was cultured at 37 °C in Luria Bertani (LB) medium and used for cloning and propagation of recombinant plasmids. A. tumefaciens GV3101 used for transient expression was cultured at 28 °C in LB medium using appropriate antibiotics. N. benthamiana was maintained in the greenhouse at 22–25 °C with a photoperiod of 18 hr light/6 hr darkness.

4.2 | Plasmid construction

All the primers used in this study are listed in Table S2. The PCR fragments were amplified by Phanta Max Super-Fidelity DNA Polymerase (Vazyme). For pVX, vectors and gene (without signal peptide) fragments were digested by SmaI and NotI (New England Biolabs) in the appropriate conditions. The digested fragments were linked to the linearized pVX by T4 DNA ligase (Takara). For pBINGFP2 and pTRV2, vectors and gene fragments (without signal peptide for pBINGFP2) were digested by SmaI and EcoRI, respectively (New England Biolabs) in the appropriate conditions. The fragments were linked to the linearized pBINGFP2 by ClonExpress MultiS One Step Cloning Kit (Vazyme). To construct the overexpression vector, the full length of PlAvh142 coding sequence was linked to the linearized pTORmRFP4, which were digested by Clal and BsrWI (New England Biolabs). The vectors pYF2.3G-RibosqRNA and pBluescript II KS used for knock-out of PlAvh142 by CRISPR/Cas9 were generated as described previously (Fang and Tyler, 2016).

4.3 | RNA extraction, cDNA synthesis, and expression analysis of PlAvh142

Mycelia and litchi leaves infected with zoospores suspension of P. litchii were harvested at the indicated time points and RNA was extracted using All-in-One DNA/RNA Mini-preps Kit (Bio Basic) according to the recommended protocol. All cDNAs were synthesized from total RNA by PrimeScript RT Master Mix (Takara). RT-qPCR was performed in 20 μl reactions that included 20 ng cDNA, 0.4 μM gene-specific primer of PlAvh142, 10 μl SYBR Premix Ex Taq II (Takara) and 6.4 μl dH2O. The RT-qPCRs were performed on qTOWER2 Real-Time PCR thermal cyclers (Analytik Jena) under the following conditions: 95 °C for 2 min, 40 cycles at 95 °C for 30 s, and 60 °C for 30 s to calculate cycle threshold values, followed by a dissociation programme of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s to obtain melt curves. The relative expression values were determined using Actin from P. litchii as reference gene and calculated with the formula 2−ΔΔct.

4.4 | Agroinfection assay in N. benthamiana

The PlAvh142 gene was amplified from P. litchii cDNA and then cloned into the PVX vector pGR107 and pBINGFP2, respectively. The recombinant plasmid was introduced into A. tumefaciens microorganisms, which will be cultured on the solid medium (juice from 200 g carrot topped up to 1 L, 15 g agar/L for solid medium) at 22–25 °C with a photoperiod of 18 hr light/6 hr darkness.

4.4 | Agroinfection assay in N. benthamiana

The PlAvh142 gene was amplified from P. litchii cDNA and then cloned into the PVX vector pGR107 and pBINGFP2, respectively. The recombinant plasmid was introduced into A. tumefaciens
GV3101 by heat shock. For cell death induction experiments, *A. tumefaciens* carrying the respective recombinant plasmids was cultured in LB medium at 28 °C with shaking at 200 rpm for 48 hr. The cultures were harvested and washed three times with 10 mM MgCl₂, then resuspended in 10 mM MgCl₂ to achieve final concentrations before agroinfiltration in *N. benthamiana* leaves. Symptoms were visually monitored and photographs were taken after 3–8 days. The experiments were repeated at least three times.

### 4.5 Callose and ROS staining

To observe callose deposition and ROS accumulation in planta, the whole leaves of *N. benthamiana* were harvested at 36 hpa. For callose deposition assay, leaves were stained with 0.01% aniline blue in 150 mM K₂HPO₄ buffer 1–2 hr after destaining in 96% ethanol (Sohn et al., 2007) and subsequently imaged by Olympus BX53 microscopy system. For ROS accumulation assays, leaves were visualized using dianiminobenzidine-HCl solution (1 mg/ml, pH 3.8) in darkness for 8–12 hr and subsequently destained with 96% ethanol (Zhang et al., 2017). The quantification of callose deposition and ROS accumulation was calculated using ImageJ software. At least three leaves were tested in each independent experiment. The experiments were repeated at least three times.

### 4.6 Virus-induced gene silencing assays in *N. benthamiana*

The cultured *A. tumefaciens* strains carrying pTRV2::BAK1, pTRV2::SOBIR1, pTRV2::MEK1, pTRV2::MEK2, pTRV2::MAP3Kα, pTRV2::SIPK, pTRV2::WIPK, pTRV2::WRKY2, pTRV2::RAR1, pTRV2::HSP90, pTRV2::SGT1, pTRV2::NDR1, pTRV2::EDS1, pTRV2::MYB, pTRV2::GUS, and pTRV1 were resuspended in the agroinfiltration buffer described above. Each pTRV2 and pTRV1 was mixed in equal ratios with a final OD₆00 of 0.2 for each. pTRV2::GUS was used as a control. The three primary leaves of 4-leaf-stage *N. benthamiana* plants were infiltrated by the mixtures. The gene silencing efficiency was analysed by RT-qPCR and PI-Avh142 was agroinfiltrated at 16–20 days after infiltration with *A. tumefaciens* carrying the VIGS constructs. The degree of cell death was monitored at 5 days after PI-Avh142 was agroinfiltrated. The experiments were repeated at least three times.

### 4.7 Protein extraction and western blot analysis

The leaves of *N. benthamiana* that were infiltrated with *A. tumefaciens* were ground into powder in liquid nitrogen and vigorously mixed with 0.5 ml of precooled radioimmunoprecipitation assay buffer (RIPA buffer) (250 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate [SDS] (Thermo Fisher Scientific)). After 5 min of incubation on ice, the samples were centrifuged at 14,000 × g for 15 min to obtain the supernatant. After adding loading buffer and boiling for 5 min, total proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels. The quantification of callose deposition and ROS accumulation was calculated using ImageJ software. At least three leaves were tested in each independent experiment. The experiments were repeated at least three times.

### 4.8 Confocal microscopy

For fluorescence observations, patches of *N. benthamiana* leaves were cut after 2 dpa and used for confocal imaging on a Nikon A1 laser scanning microscope with a 40× objective lens. RFP or GFP fluorescence was observed at an excitation wavelength of 561 or 488 nm, respectively.

### 4.9 Transformation of *P. litchii*

To generate knockout and overexpressing transformants, *P. litchii* protoplasts were transformed using the polyethylene glycol (PEG)–CaCl₂-mediated method as described previously (Fang and Tyler, 2016; Jiang et al., 2017). For knockout experiment, plasmids pYF2-PsNSL3–hSpCas9, pYF2.3G–RibosgRNA::PlAvh142, and pBluescriptII KS::PlAvh142 were cotransformed into protoplasts of strain SHS3. For the overexpression experiment, plasmid pTOmRef4::PlAvh142 was transformed into protoplasts of strain SHS3. The transformed protoplasts were regenerated overnight, and the recovered mycelia were selected on CJA medium with 30 µg/ml G418. After 2–3 days, the primary transformants were transferred to new selective medium, named sequentially and maintained for subsequent analyses.

### 4.10 Pathogenicity assays

For pathogenicity assays, zoospores were inoculated on the tender leaves of litchi (Guiwei), which were collected from the litchi orchard in South China Agricultural University, Guangzhou, Guangdong province. One hundred zoospores of each strain were inoculated on the center of the tender leaf, and kept at 80% humidity in 12 hr light/12 hr darkness at 25 °C. Each strain was tested on no fewer than 10 leaves. The symptoms were observed and the lesion diameter was measured at 48 hpi. The experiments were repeated at least three times.
ACKNOWLEDGMENTS
We thank Prof. Brett Tyler (Oregon State University, Corvallis, OR, USA) for kindly providing the CRISPR/Cas9 vectors and Prof. Wang Yuanchao (Nanjing Agricultural University) for providing vectors used in transient expression in N. benthamiana. We are grateful for the critical reading our manuscript by Prof. Shaobin Zhong (North Dakota State University) and Dr. Stephen Dela Ahator (South China Agricultural University). This research was supported by grants to G.K. from the National Natural Science Foundation of China (31701771), Z.J. from the earmarked fund for the China Agriculture Research System (CARS-32-07), and G.K. from the Natural Science Foundation of Guangdong Province, China (2017A030310310).

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID
Zide Jiang https://orcid.org/0000-0002-1274-4627

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SUPPORTING INFORMATION

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

FIGURE S1 Protein sequences of PIAvh142. Different regions are marked with bold lines.

FIGURE S2 Analysis of the components involved in PIAvh142-induced cell death. Nicotiana benthamiana leaves were agroinfiltrated with pTRV2 constructs targeting BAK1, SOBIR1, EDS1, NDR1, MEK1, MEK2, MAP3Kα, SIPK, WIPK, WRKY2, and MYB; pTRV2::GFP was used as a control. (a) Representative images of PIAvh142-induced cell death in silenced N. benthamiana leaves at 5 days post-agroinfiltration (dpa). Agrobacterium tumefaciens carrying PIAvh142 was infiltrated into the upper leaves of silenced plants at 16–20 dpa of TRV constructs. (B) Quantification of cell death in N. benthamiana leaves scored at 5 dpa. The degree of cell death was divided into three levels: no cell death, weak cell death, and strong cell death. Asterisks indicate significant differences from green fluorescence protein (GFP)-silenced plants (Wilcoxon rank-sum test: ***, p < .001). (C) The transcript abundance of the genes in corresponding silenced plants was detected by RT-qPCR. The constitutive expression gene NbEF1α was used as internal reference. Error bars represent the SD of three biological replicates. Similar results were obtained from three independent experiments.

FIGURE S3 The relative expression level of PIAvh142-overexpressing mutants. RT-qPCR was used to determine the overexpressing level of the mutants. The relative expression level was calibrated to the levels for the wild type that set as 1. The constitutive expression gene PIActin was used as internal reference. Error bars represent the SD of three biological replicates.

FIGURE S4 The phenotype of PIAvh142 mutants were identical to wild type. (a) Colony morphology of PIAvh142 mutants grown on carrot juice agar medium after 5 days. (b) Growth rates of PIAvh142 mutants. Letters represent significant differences (p < .05; Duncan’s multiple range test). Similar results were obtained from three independent experiments.

TABLE S1 Screening of cell death-inducing RXLR effectors in Peronosphythora litchii

TABLE S2 Primers used in this study

How to cite this article: Situ J, Jiang L, Fan X, et al. An RXLR effector PIAvh142 from Peronosphythora litchii triggers plant cell death and contributes to virulence. Molecular Plant Pathology. 2020;21:415–428. https://doi.org/10.1111/mpp.12905