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Plasmopara viticola Effector PvRXLR10 Targets a Host Phospholipase VvipPLA-IIδ2 to Suppress Plant Immunity in Grapevine

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ABSTRACT

Plasmopara viticola that causes grapevine downy mildew disease in viticulture regions is among the 10 most relevant pathogens worldwide. It secretes a large arsenal of effectors to facilitate colonisation by perturbing host immunity. However, the underlying mechanisms by which *P. viticola* effectors disturb grapevine defence are still largely unknown. In this study, we report that PvRXLR10, an RXLR effector with a WY domain, promotes *P. viticola* infection in grapevine and *Phytophthora parasitica* colonisation in *Nicotiana benthamiana*. PvRXLR10 interacts with a host patatin-like protein VvipPLA-IIδ2 with phospholipase A2 activity. The WY domain of PvRXLR10 is not responsible for cell death suppression in *N. benthamiana* but is necessary for PvRXLR10 interaction with VvipPLA-IIδ2. Overexpression and RNAi-mediated suppression of *VvipPLA-IIδ2* expression in *Vitis vinifera* consistently showed that this protein positively regulates plant immunity in response to *P. viticola* infection. Interestingly, we found that VvipPLA-IIδ2 partially associates with PvRXLR10 at the endoplasmic reticulum (ER). Reverse transcription-quantitative PCR (RT-qPCR) analysis showed that the expression of *VvipPLA-IIδ2* was suppressed by PvRXLR10 during *P. viticola* infection. The overexpression of *VvipPLA-IIδ2* in *V. vinifera* induced higher expression of genes related to jasmonic acid (JA) biosynthesis, signalling pathways and defence response. The evidence indicates the important roles of VvipPLA-IIδ2 in grapevine immunity and *P. viticola* effector PvRXLR10 targets this protein to promote its infection.

1 | Introduction

Plants are frequently attacked by pathogens in nature, and in order to survive they have evolved a sophisticated immune system. This two-layered innate immune system, comprising pattern-triggered immunity (PTI) and effector-triggered immunity (ETI), is thought to operate by a 'zig-zag' model (Jones and Dangl 2006). In this model, pathogens can secret effectors into host cells to interfere with the first layer of immune defence PTI mediated by plant pattern-recognition receptors (PRRs). To combat pathogen virulence, certain plant species may activate a second tier immune mechanism known as ETI. It was initially thought that PTI and ETI were independent immune systems. However, more recent research has

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revealed that PTI is indispensable to the ETI and PTI-induced defence responses are enhanced by ETI (Ngou et al. 2021). Furthermore, nucleotide-binding leucine-rich repeat receptors (NLRs) and PRRs function synergistically to provide robust immunity (Yuan et al. 2021).

Phytopathogenic oomycetes are responsible for many devastating diseases on a range of important crops (Kamoun et al. 2015). These pathogens achieve their colonisation of plants by disturbing host defences through the action of a series of secreted effector proteins. Based on the target sites, these proteins can be grouped into two classes, apoplastic effectors and cytoplasmic effectors (Rocafort et al. 2020; Fabro 2022). One of the best-studied classes of cytoplasmic effectors in phytopathogenic oomycetes is the RXLR effectors (Tyler et al. 2006; Jiang et al. 2008). The function of the conserved RXLR motif is often associated with secretion and/or translocation of effectors into plant cells (Whisson et al. 2007; Wawra et al. 2017). RXLR effectors use a variety of mechanisms to manipulate plant immunity (Wang et al. 2019) by targeting different host immune proteins. For example, several RXLR effectors of *Phytophthora* have been reported to suppress plant PTI responses by targeting MAPKKKs (Pi22926, PexRD2, Pi17316, PITG20303 and PITG20300 of *P. infestans*) (King et al. 2014; Murphy et al. 2018; Du et al. 2021) or interfere with PTI signalling pathways (Avh331 of *Phytophthora sojae*, SFI3 of *Phytophthora infestans*) (Cheng et al. 2012; He, McLellan, et al. 2019). Two effectors from *P. sojae* (PsAvh163 and PsAvh238) and three effectors from *Hyaloperonospora arabidopsidis* (HaRxL106, HaRxL96 and HaRxL62) have been shown to suppress plant defences by manipulating host phytohormone-mediated signalling (Anderson et al. 2012; Asai et al. 2014; Wirthmueller et al. 2018; Yang et al. 2019). Suppression of programmed cell death (PCD) is another mechanism by which RXLR effectors interfere with plant immunity (Deb et al. 2018; Wang et al. 2011; Xiang et al. 2016). In addition, effectors counter host defence via manipulation of plant transcription (McLellan et al. 2013; Huang et al. 2017; Kong et al. 2017), blocking protein secretion (Bozkurt et al. 2011; Du et al. 2015; Tomczynska et al. 2018; Guo et al. 2019), production of antimicrobial small RNAs and interfering with host RNA silencing (Qiao et al. 2013; Hou et al. 2019) and host autophagy (Dagdas et al. 2016).

Plasmopora viticola is a major grapevine pathogen and is widely spread across all grape-growing regions of the world (Gessler et al. 2011). Because it is a biotrophic pathogen, it cannot be cultured in vitro on artificial media and there is currently no genetic transformation system. As such, research on the functional characterisation of *P. viticola* effectors is lagging behind *Phytophthora* species. However, with the development of bioinformatics in recent years, some progress has been made in the research on the effector proteins of *P. viticola*. Transcriptome and genome analyses have revealed the presence of over 100 RXLR effectors in *P. viticola* (Yin et al. 2015, 2017; Dussert et al. 2016). Three studies preliminarily described the subcellular localisation and potential function of some of these putative RXLR effectors from different *P. viticola* isolates (Xiang et al. 2016; Liu et al. 2018; Chen et al. 2020). Several host targets were also identified to reveal the pathogenic mechanisms (Lan et al. 2019; Liu et al. 2021; Ma et al. 2021). However, the underlying mechanisms of action of most *P. viticola* effectors are still largely unknown.

Lipid-hydrolysing enzymes known as phospholipases are known to play a variety of signalling roles during biotic and abiotic stress responses in plants (Takáč et al. 2019). Based on the hydrolysis of various ester linkages, they can be divided into four major types: phospholipase D (PLD), phospholipase C (PLC), phospholipase A1 (PLA1), and phospholipase A2 (PLA2) (Wang et al. 2012). Patatin-like PLAs (pPLAs) are a subfamily of PLAs with both PLA1 and PLA2 activities (Li and Wang 2014). Recent data have revealed that pPLAs are the most highly represented PLA family in the grapevine genome with 24 members that can be classified into three subgroups (Laureano et al. 2018), following the classification established in well-studied model species such as *Arabidopsis thaliana* (Holk et al. 2002) and *Oryza sativa* (Singh et al. 2012). More importantly, a number of studies have found strong evidence for the involvement of lipid signalling and phospholipases in the immune responses of grapevine to *P. viticola* infection (Guerreiro et al. 2016; Laureano et al. 2018; Cavaco et al. 2021).

Our previous transcriptomic analysis of *P. viticola* revealed that PvRXLR10 is a highly conserved RXLR effector in three different isolates (Yin et al. 2015). Here, we verified that the virulence effector PvRXLR10 promotes *P. parasitica* and *P. viticola* infection in *Nicotiana benthamiana* and grapevine leaves, respectively. Furthermore, we demonstrated that the ER-localised VvipPLA-IIδ2 protein, a patatin-like phospholipase with PLA2 activity, is a host target of this effector. Our analysis also revealed that VvipPLA-IIδ2 positively regulates plant immunity against *P. viticola* infection through the jasmonic acid (JA) signalling pathway. This work adds to the body of knowledge regarding the function of JA in grapevine downy mildew resistance and shows an example of an effector protein interacting with the endoplasmic reticulum (ER)-localised protein to interfere with the host immune response.

2 | Results

2.1 | Characterisation of the Conserved Effector PvRXLR10

The effector PvRxLR10 is a highly conserved RXLR effector in three different *P. viticola* isolates collected from China and Australia (Yin et al. 2015). It is a 136-amino acid protein with a signal peptide (residues 1 to 22), an RXLR motif (residues 42–45), a degenerate dEER motif (residues 51–56) and a putative C-terminal WY domain (Figure 1A). It shares 41% sequence identity (57% similarity) with PHALS_12456 from *Plasmopara halstedii* (Figure S1A). The PvRXLR10 (without signal peptide) construct fused with GFP at the C-terminus or N-terminus was transiently expressed in *N. benthamiana* leaves with an ER marker (Mravec et al. 2009) and a plasma membrane (Kurusu et al. 2012) marker protein to determine the subcellular localisation of PvRXLR10. The stability of each construct was confirmed by immunoblotting (Figure S1B). It showed that the GFP-PvRXLR10 or PvRXLR10-GFP localise to multiple positions of *N. benthamiana* leaf epidermal cells, including plasma membrane, cytoplasmic, nucleus and some association with the ER (Figure 1B and Figure S1C).

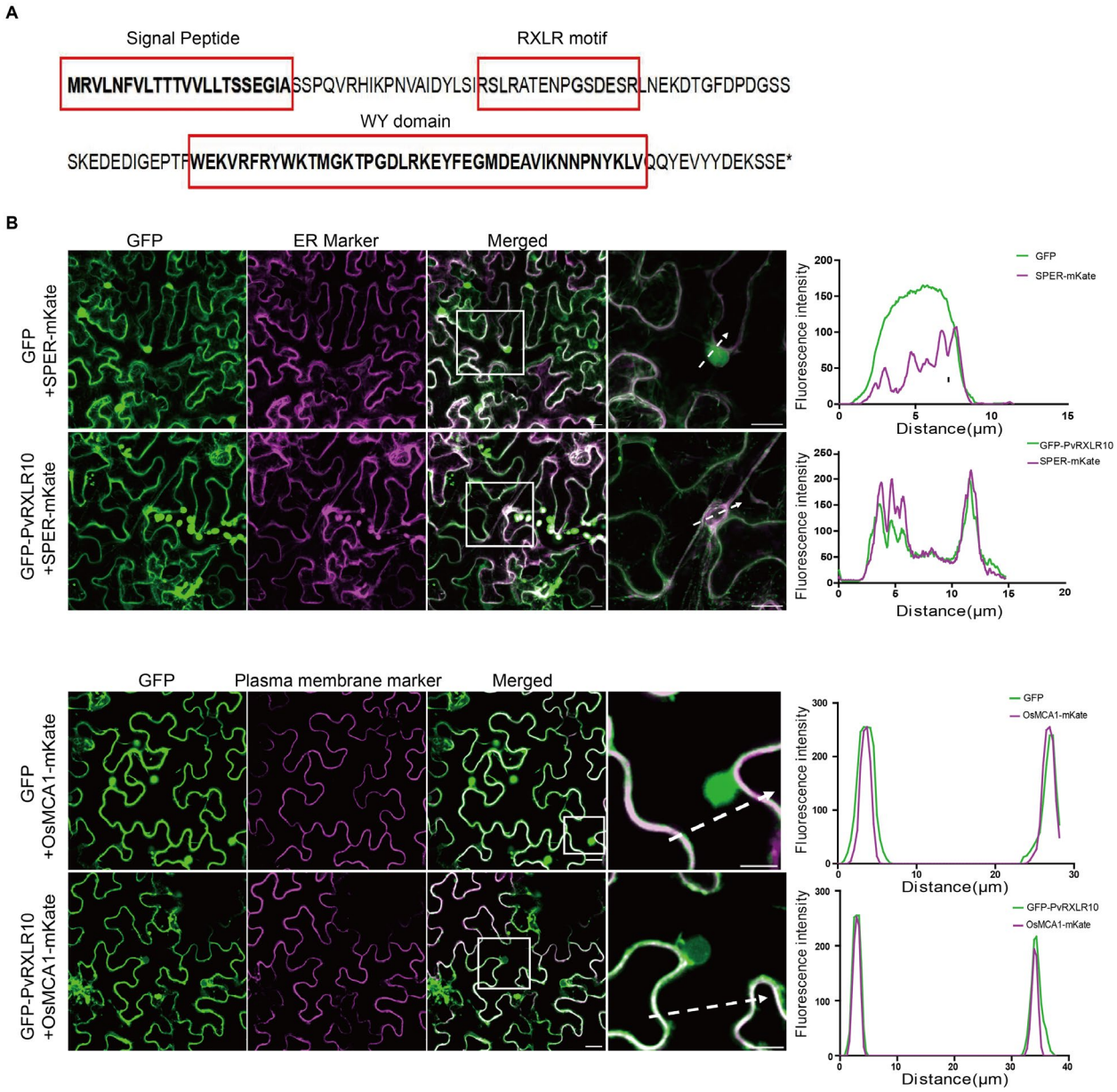

FIGURE 1 | PvRXLR10 is a secreted effector with multiple positions in plant cells. (A) Schematic diagram of the PvRXLR10 protein. The predicted signal peptide, the conserved RXLR-dEER motif and C-terminal WY motif are shown in bold and red box. (B) The confocal projection images of GFP-PvRXLR10 fusion protein and mKate-labelled endoplasmic reticulum (ER) marker protein transiently co-expressed in leaves of *Nicotiana benthamiana* (upper two panels). Single optical section confocal images of GFP-PvRXLR10 fusion protein and mKate-labelled the plasma membrane marker protein OsMCA1 co-expressed in leaves of *N. benthamiana* (lower two panels). Fluorescence was observed by confocal microscopy at 48 h post-infiltration. Fluorescence plots show the relative fluorescence of GFP and mKate along the dotted arrows in the images. Scale bars are 20 μm for the main images and 10 μm for the higher magnification images.

2.2 | PvRXLR10 Suppresses INF1/BAX-Triggered Cell Death and Promotes Pathogen Colonisation

One of the main virulence strategies used by pathogens to facilitate infection is to suppress host immune responses. Many pathogen effectors have been shown to inhibit PCD induced by a range of different cell death-inducing genes in both model plant species and host plants (Wang et al. 2011; Xiang et al. 2016; Liu et al. 2018). To investigate whether PvRXLR10 could suppress cell death like other oomycete effectors, we transiently expressed GFP-PvRXLR10 protein in *N. benthamiana* leaves. As shown in Figure 2A, GFP-PvRxLR10 could completely or partially suppress the cell death elicited by mammalian pro-apoptotic protein BAX or *Phytophthora* infestin 1 (INF1) at 5 days post-infiltration (dpi) in *N. benthamiana*, but it did not suppress cell death induced by *P. viticola* Crinkler protein PvCRN92 (Figure S2). During *P. viticola* infection, the expression of PvRXLR10 significantly up-regulated at zoospores and the early stages (6–48 h post-infection [hpi]) (Figure 2B). These results suggest that PvRXLR10 is associated with *P. viticola* virulence.

To further explore the virulence contribution of PvRXLR10 during *P. viticola* infection, we tested whether it could promote

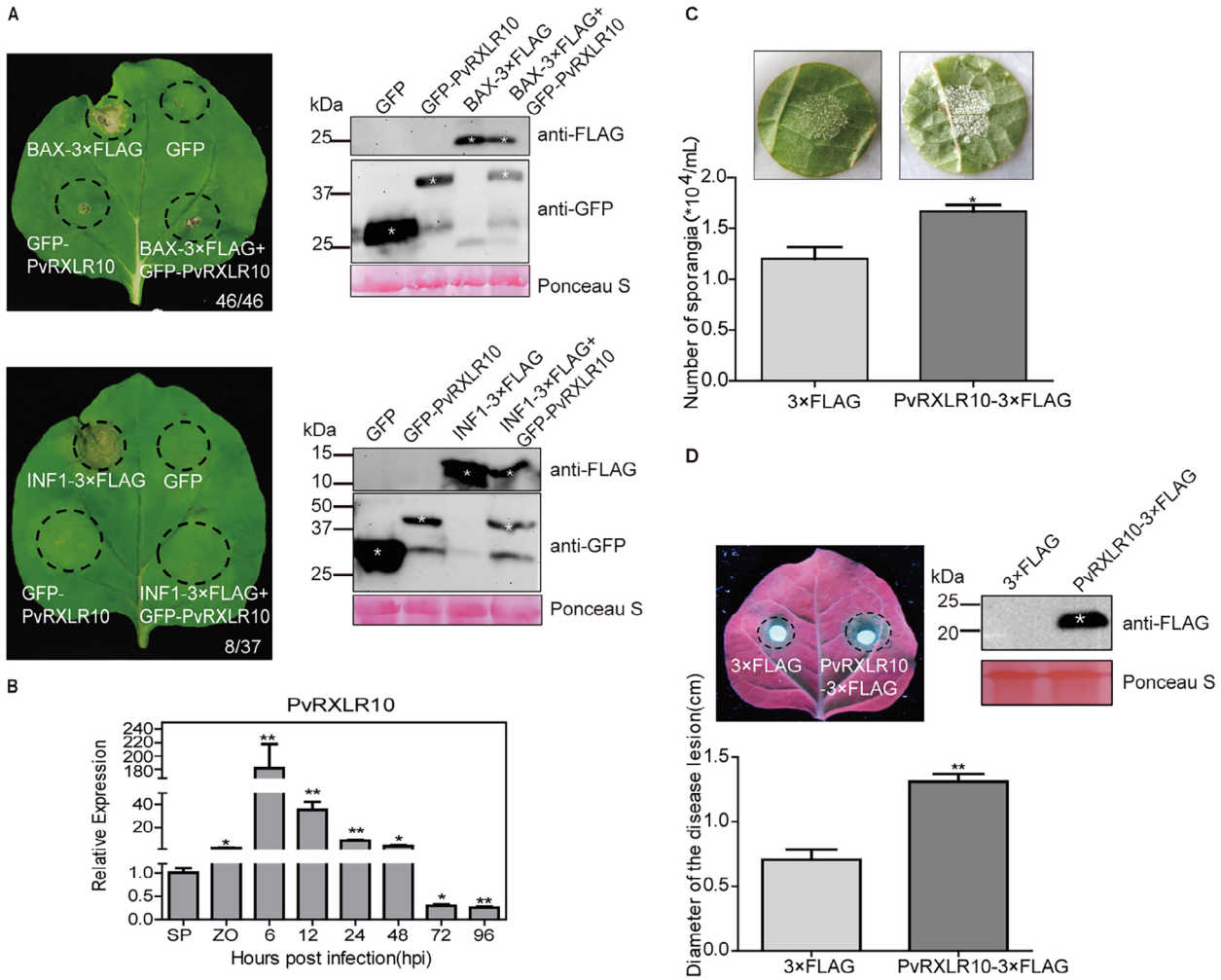

FIGURE 2 | PvRXLR10 promotes pathogen colonisation. (A) PvRXLR10 suppressed cell death induced by INF1 and BAX in *Nicotiana benthamiana*. The ratios at the bottom right corner of the image indicate the number of infiltration sites with no cell death versus the total number of infiltration sites when GFP-PvRXLR10 was co-infiltrated with BAX-3×FLAG or INF1-3×FLAG. Photographs were taken at 5 days post-infiltration (dpi). Protein stability of GFP-PvRXLR10, co-expressed with BAX-3×FLAG or INF1-3×FLAG, was analysed by immunoblotting. Intact fusion proteins are indicated by white asterisks. The size marker is indicated in kDa, and protein loading is indicated by Ponceau S staining. Black dotted circles indicate areas of infiltration. (B) The expression level of *PvRXLR10* was analysed by reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from sporangia (SP), zoospores (ZO) and *Plasmopara viticola*-infected grapevine leaves at 6, 12, 24, 48, 72, 96 h post-inoculation (hpi). The expression levels were normalised with *PvActin* and *PvTubulin*. Error bars indicate SE from three biological replicates. $^{*}p < 0.05$, $^{**}p < 0.01$, significant difference determined by Student's *t* test. (C) PvRXLR10 promoted *P. viticola* infection in *Vitis vinifera* leaves. The number of sporangia on the leaf discs was calculated using thrombocytometry under microscope at 5 dpi. The experiment was repeated three times using 30 leaf discs from three independent biological replicates. Data are the mean ± SE of three independent biological replicates. $^{*}p < 0.05$, significant difference determined by Student's *t* test. (D) Transient expression of PvRXLR10 in *N. benthamiana* leaves followed by *Phytophthora parasitica* infection. The infiltrated regions, expressing PvRXLR10–3×FLAG or 3×FLAG, were inoculated with mycelia plugs of *P. parasitica* at 48 hpi. Lesions were photographed under ultraviolet (UV) light and lesion diameters were measured at 48 hpi, with data (mean ± SE, $n = 30$) analysed from three independent experiments. $^{**}p < 0.01$, Student's *t* test.

P. viticola colonisation in *Vitis vinifera* 'Thompson Seedless' leaves. The young leaves of Thompson Seedless were vacuum-infiltrated with *Agrobacterium tumefaciens* cells carrying PvRXLR10-3×FLAG or 3×FLAG (the control). The infiltrated grapevine leaves were inoculated with *P. viticola* at 5 dpi. Sporulation on leaf discs was quantified at 5 dpi. Compared with FLAG-expressing leaves, the number of sporangia was much higher on leaves expressing PvRXLR10-3×FLAG (Figure 2C), suggesting that PvRXLR10 promotes *P. viticola* infection in grapevine by suppressing basal immunity. In addition, the transient expression of PvRXLR10-3×FLAG in *N. benthamiana* cells significantly enhanced *P. parasitica* colonisation, as shown by larger leaf lesions (Figure 2D).

2.3 | PvRXLR10 Interacts With Host Patatin-Like Protein VvipPLA-IIδ2, a Phospholipase With cPLA2 Activity

To investigate how PvRXLR10 increases *V. vinifera* susceptibility to *P. viticola*, a yeast two-hybrid (Y2H) screen was performed using a cDNA library created from *P. viticola*-infected

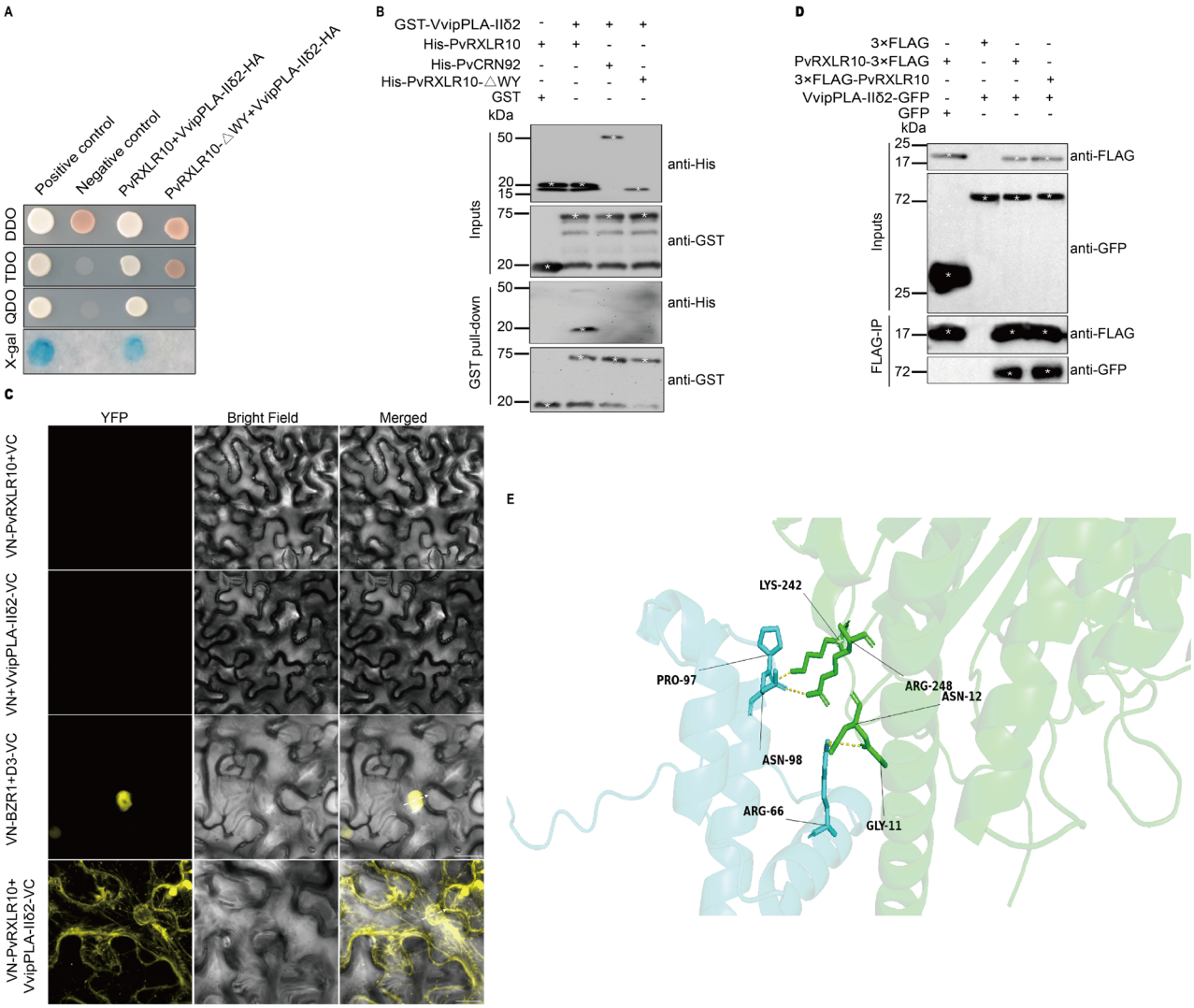

FIGURE 3 | PvRXLR10 interacts with VvipPLA-IIδ2 in vitro and in vivo. (A) PvPXLR10 interacts with VvipPLA-IIδ2 in yeast two-hybrid assay and the WY domain of PvPXLR10 is necessary for the interaction. DDO: SD/−Trp/−Leu, TDO: SD/−Trp/−Leu/−His, QDO: SD/−Trp/−Leu/−His/−Ade. The p53 (bait, a human tumour antigen) and LargeT (prey, the SV40 large T antigen) co-transformants were used as a positive control. Blank vectors pGAD-HA and pLexA-p53 co-transformants were used as negative controls. The plates were photographed at 3 days post-incubation. (B) Glutathione S-transferase (GST) pull-down assay shows interaction between PvPXLR10 and VvipPLA-IIδ2. GST-VvipPLA-IIδ2, His-PvRXLR10, His-PvCRN92, His-PvRXLR10-ΔWY and GST proteins were expressed in *Escherichia coli* Rosetta (DE3). The pull-down inputs and co-precipitation samples were detected by western blot. White asterisks indicate protein bands of the correct size. The experiment was performed two times with similar results. (C) Bimolecular fluorescence complementation (BiFC) assay confirming that PvRXLR10 interacts with VvipPLA-IIδ2 at endoplasmic reticulum (ER)-like structure. We fused the N-terminus of Venus (VN) to the N-terminus of PvRXLR10, and the C-terminus of Venus (VC) to the C terminus of VvipPLA-IIδ2. The combinations of VN-PvRXLR10/VvipPLA-IIδ2-VC, VN-PvRXLR10/pGreenII-62-SK-VC, pGreenII-62-SK-VN/VvipPLA-IIδ2-VC and VN-BZR1/D3-VC were co-agroinfiltrated into *Nicotiana benthamiana* leaves and pictures were taken at 48 h post-infiltration (hpi). The interaction between BZR1 and D3, two proteins from rice (Fang et al. 2020), acted as a positive control. The dotted arrow indicates the path used to generate the fluorescence intensity profile shown in Figure S3A,B. Scale bar represents 20 μm (the upper two panels) and 10 μm (the lower two panels). Three biological replicates, each with two leaves for observation. (D) PvRXLR10 interacted with VvipPLA-IIδ2 in planta. PvRXLR10-3×FLAG or 3×FLAG-PvRXLR10 was co-expressed with VvipPLA-IIδ2-GFP or GFP in *N. benthamiana* leaves, and total protein was extracted at 48 hpi. Immunoprecipitations were performed using FLAG-trap beads. Tagged proteins were detected by western blot. The expected proteins are indicated by white asterisks. Similar results were obtained in three independent experiments. (E) Structural model of the PvRXLR10-VvipPLA-IIδ2 complex generated with AlphaFold3. PvRXLR10 and VvipPLA-IIδ2 are shown in cyan and light green, respectively. The predicted key contact amino acids on PvRXLR10 and VvipPLA-IIδ2 are labelled in the picture. Hydrogen bonds are indicated by dotted lines.

V. vinifera 'Cabernet Sauvignon' leaves to identify the putative host targets of PvRXLR10. Thirteen yeast colonies were recovered from selection plates, two of which were aligned to patatin-like protein 1 encoded by the *VvipPLA-IIδ2* gene (XM_002277282) (Laureano et al. 2018) and three of which were aligned to NAC domain-containing protein 91 (XM_010664637) (Table S1). An additional pairwise Y2H assay was performed using mature PvRXLR10 protein as the bait and VvipPLA-IIδ2 and VvNAC91 as the prey, respectively. The results proved that PvRXLR10 interacts with VvipPLA-IIδ2

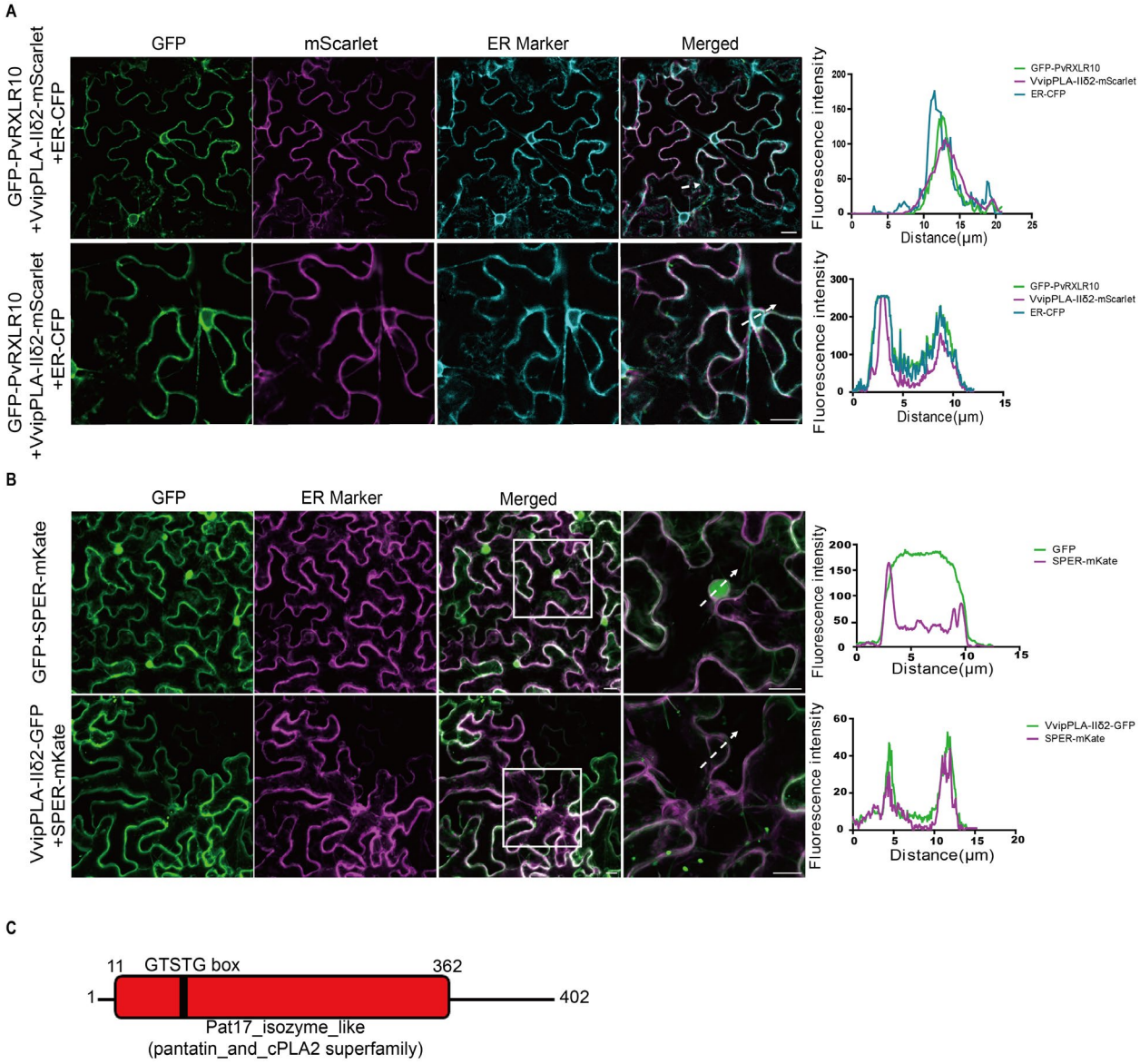

FIGURE 4 | VvipPLA-IIδ2 is a predicted phospholipase that partially associates with PvRXLR10 at the endoplasmic reticulum (ER). Co-localization of the GFP-PvRXLR10, VvipPLA-IIδ2-mScarlet and a CFP-labelled ER marker (A) and co-localization of VvipPLA-IIδ2-GFP and the mKate-labelled ER marker (B) in *Nicotiana benthamiana* leaves. The confocal images were taken at 48 h post-infiltration. Three biological replicates, each with two leaves for observation. Fluorescence plots show the relative fluorescence of GFP, mScarlet, CFP and mKate along the dotted arrows in the images. Scale bars are 20 μm for the main images and 10 μm for the higher magnification images. (C) Schematic diagram of the VvipPLA-IIδ2 protein. The predicted domain of patatin_and_cPLA2 subfamily with a 'GTSTG' esterase box is shown in red.

(Figure 3A) but does not interact with VvNAC91 (Figure S3) in yeast cells. Further pull-down assays were performed to confirm the physical interaction between PvRXLR10 and VvipPLA-IIδ2. Glutathione S-transferase (GST) tag or GST-VvipPLA-IIδ2 protein, purified from *Escherichia coli* Rosetta (DE3), was immobilised on glutathione agarose beads and then incubated with His-PvRxLR10 and His-PvCRN92 (as a negative control), respectively. His-tagged PvRxLR10 proteins were specifically detected in GST-VvipPLA-IIδ2 pull-down products (Figure 3B), indicating a physical interaction between PvRXLR10 and VvipPLA-IIδ2 in vitro. To further confirm whether this interaction also occurs in planta, we performed a bimolecular fluorescence complementation (BiFC) assay and co-immunoprecipitation (Co-IP) assays in *N. benthamiana*. In the BiFC assay, PvRXLR10 interacted with VvipPLA-IIδ2 based on the yellow fluorescent signals detected by confocal laser microscopy (Figure 3C). Co-IP assays were carried out using 3×FLAG-PvRXLR10, PvRXLR10-3×FLAG and VvipPLA-IIδ2-GFP constructs, with 3×FLAG tag or GFP tag used as negative controls. As shown in Figure 3D, VvipPLA-IIδ2-GFP was immunoprecipitated in the presence of 3×FLAG-PvRXLR10 and PvRXLR10-3×FLAG, but not with the 3×FLAG and GFP tag controls, whereas all proteins were detectable in the relevant input fractions. The co-localisation of GFP-PvRXLR10 and VvipPLA-IIδ2-mScarlet showed partially overlapped with the CFP-labelled ER marker in the peri-nuclear ER and the ER network (Figure 4A). For further confirmation, VvipPLA-IIδ2-GFP was co-expressed with an mKate-labelled ER marker protein in *N. benthamiana* leaves. Two proteins partially overlapped at the perinuclear

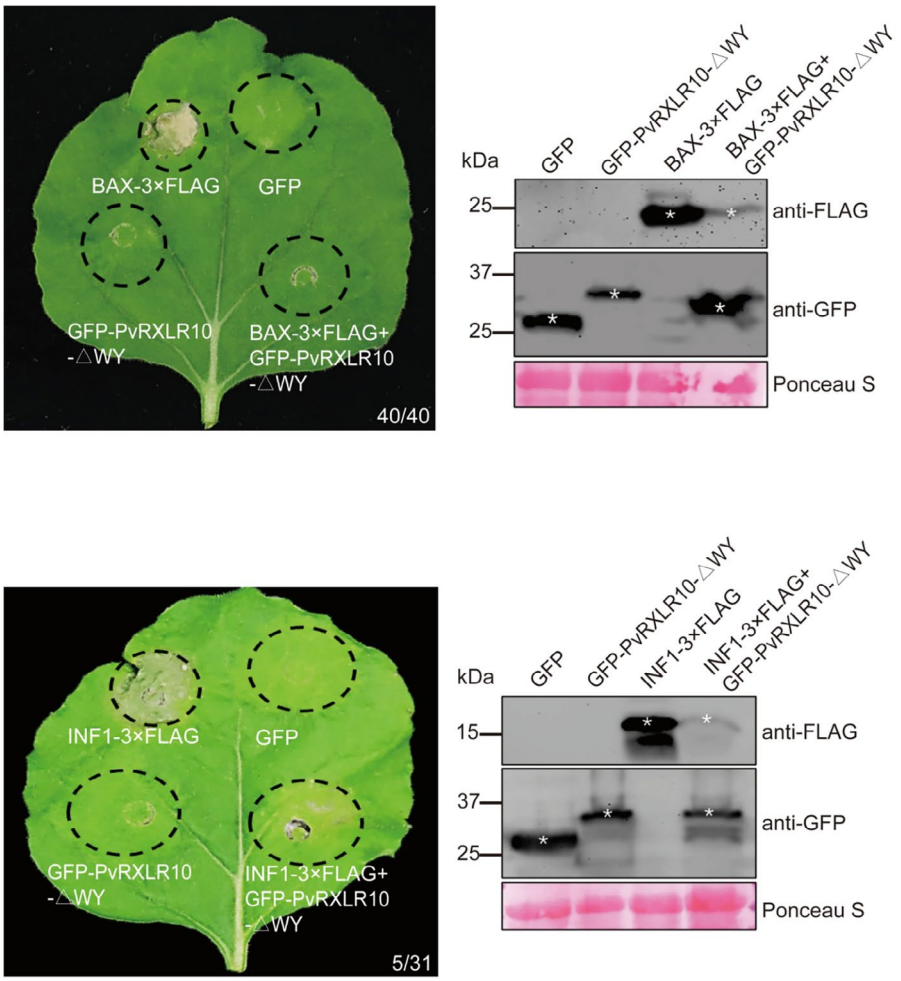

FIGURE 5 | The WY domain of PvRXLR10 is not necessary for cell death suppression. PvRXLR10-ΔWY (ΔWY, the deletion of WY domain) suppressed cell death induced by INF1 and BAX in *Nicotiana benthamiana*. The ratios at the bottom right corner of the image indicate the number of infiltration sites with no cell death versus the total number of infiltration sites when GFP-PvRXLR10-ΔWY was co-infiltrated with BAX-3×FLAG or INF1-3×FLAG. Photographs were taken at 5 days post-infiltration. Protein stability of GFP-PvRXLR10-ΔWY, co-expressed with BAX-3×FLAG or INF1-3×FLAG, was analysed by immunoblotting. The size marker is indicated in kDa, and protein loading is indicated by Ponceau S staining. Black dotted circles indicate areas of infiltration.

ER and several bright GFP spots were observed in cytoplasm (Figure 4B). The stability of each construct and proteins co-expressed was confirmed by immunoblotting (Figure S4).

The full-length *VvipPLA-IIδ2* cDNA consists of a 1209 bp coding region encoding a 402 amino acid protein. The predicted molecular mass and pI of VvipPLA-IIδ2 protein are 44.20 kDa and 5.48, respectively. A search for conserved domains identified a Pat17_isozyme-like domain (EC 3.1.1.4) at residues 11–362, which belongs to Patatin_and_cPLA2 subfamily with a 'GTSTG' esterase box (Figure 4C). To further investigate whether VvipPLA-IIδ2 has phospholipase A2 activity, the full-length VvipPLA-IIδ2 protein was fused to GST and expressed in *Escherichia coli* BL21. SDS-PAGE and immunoblotting results showed that VvipPLA-IIδ2-GST was detected at a molecular mass of around 48 kDa and purified protein without GST-tag was obtained (Figure S5). The phospholipase A2 activity assay revealed that the VvipPLA-IIδ2 protein exhibited a PLA2 activity of 14.9 nmol/min/mL, which was calculated following the formula provided by the cPLA2 assay kit. It indicated that VvipPLA-IIδ2 has phospholipase A2 activity to catalyse the hydrolysis of fatty acids at the *sn-2* position of glycerophospholipids.

2.4 | The WY Domain of PvRXLR10 Is Not Necessary for Cell Death Suppression but Is Required for Interaction With VvipPLA-IIδ2

Sequence analysis of the predicted PvRXLR10 protein identified that the C-terminal of this protein contains a single copy of the WY motif (83–123 amino acids) (Figure 1A). To examine whether the WY motif is involved in cell death suppression, a PvRXLR10-ΔWY (ΔWY, the deletion of the WY domain) mutant construct was transiently expressed in *N. benthamiana* leaves. The leaves infiltrated with PvRXLR10-ΔWY showed similar BAX or INF1-induced cell death suppression ability as the leaves infected with PvRXLR10 (Figure 5), indicating that the WY domain of PvRXLR10 is not required for cell death suppression. To further determine whether the WY domain is indispensable for the interaction between PvRXLR10 and VvipPLA-IIδ2, we conducted a Y2H assay and a GST pull-down assay. The results showed that colonies of yeast

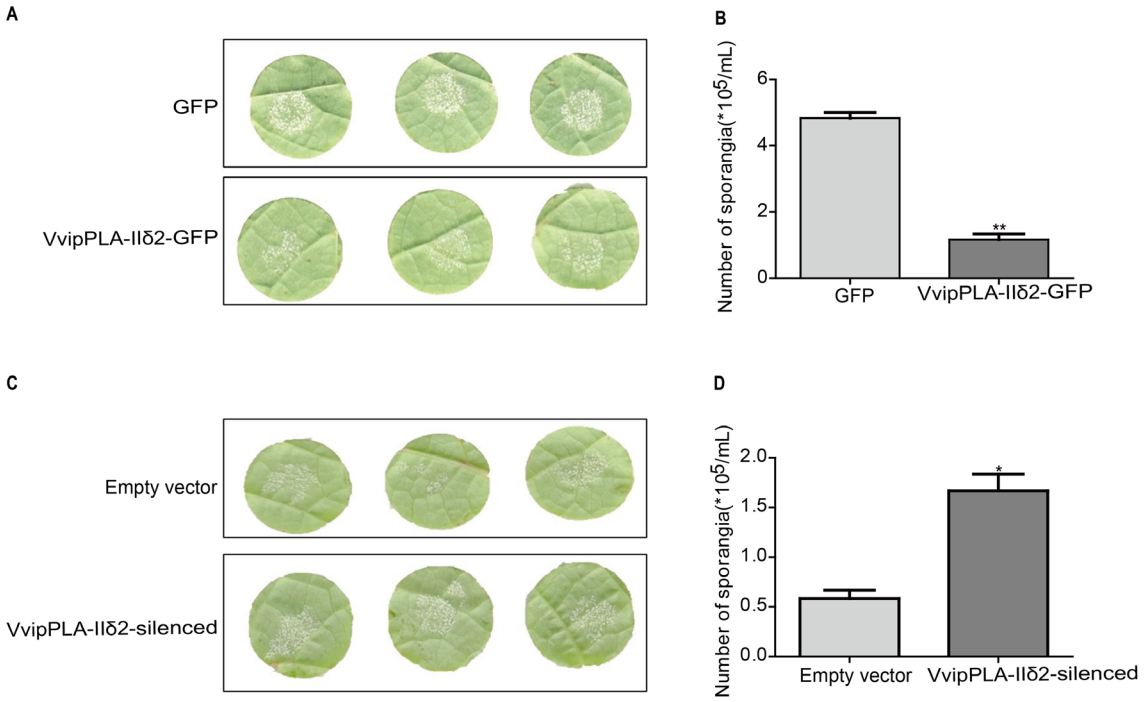

FIGURE 6 | VvipPLA-IIδ2 contributes to plant resistance against *Plasmopara viticola*. (A) Grapevine leaves overexpressing *VvipPLA-IIδ2-GFP* showed enhanced resistance to *P. viticola* infection. Images were taken at 6 days post-inoculation (dpi). (B) The number of *P. viticola* sporangia on the infected leaf surface of *GFP*- and *VvipPLA-IIδ2-GFP* overexpressing leaves was measured at 6 dpi. Data presented in the graph are the mean ± SE ($n = 33$) of three biological replicates (**$p < 0.01$, Student's *t* test). The experiment was repeated four times. (C) The *VvipPLA-IIδ2*-silenced grapevine leaves showed enhanced susceptibility to *P. viticola* infection. Images were taken at 5 dpi. (D) The number of *P. viticola* sporangia on the infected leaf discs of empty vector and *VvipPLA-IIδ2*-silenced leaves was measured at 5 dpi. Error bars are SE ($n = 16$), and the graph represented the combined data from three biological replicates (*$p < 0.05$, Student's *t* test). The experiment was repeated twice.

strain NMY51 expressing both pLexA-PvRXLR10-ΔWY and pGAD-VvipPLA-IIδ2-HA were unable to grow on the QDO/X/A plates (Figure 3A) and no His-tagged PvRXLR10-ΔWY protein was detected in the GST-VvipPLA-IIδ2 pull-down product (Figure 3B), indicating that the WY domain of PvRXLR10 is necessary for the interaction between PvRXLR10 and VvipPLA-IIδ2. The structural model of the PvRXLR10-VvipPLA-IIδ2 complex generated using AhlphaFold3 (Abramson et al. 2024) also showed that the WY domain is a key region for their interaction, with 66R, 98N and 97P within the WY domain being the predicted contact amino acids (hydrogen bonds) on PvRXLR10 (Figure 3E).

2.5 | VvipPLA-IIδ2 Contributes to Plant Resistance Against *P. viticola*

To test whether VvipPLA-IIδ2 contributes to resistance to *P. viticola*, the plasmid of pBWA(V)HS-VvipPLA-IIδ2-GFP or empty vector pBWA(V)HS-GFP was transiently transformed into leaves of three independent grapevine plants of cultivar Thompson Seedless by *Agrobacterium*-mediated vacuum infiltration. The infiltrated leaves were further inoculated with *P. viticola* suspension. Compared with GFP control leaves, the leaves overexpressing *VvipPLA-IIδ2* showed enhanced resistance with a significantly smaller number of sporangia (**$p < 0.01$, Figure 6A,B). To further confirm the function of VvipPLA-IIδ2, transient silencing of *VvipPLA-IIδ2* by agroinfiltration with a construct for RNA interference (RNAi) was used to reduce the *VvipPLA-IIδ2* expression levels in Thompson Seedless leaves. RT-qPCR assay of *VvipPLA-IIδ2*-silenced leaves of three independent plants revealed that the expression level of the *VvipPLA-IIδ2* gene was significantly reduced compared to the RNAi-empty vector control (*$p < 0.05$; Figure S6). After inoculation with *P. viticola*, the number of sporangia produced on the leaf discs of *VvipPLA-IIδ2*-silenced plants was significantly increased compared to the control leaves containing empty vector (*$p < 0.05$, Figure 6C,D). These data suggest that the overexpression of *VvipPLA-IIδ2* enhanced the resistance of grapevine to *P. viticola*.

2.6 | Analysis of JA Marker Genes Expression in *VvipPLA-IIδ2*-Overexpressed Plants

Patatin-like PLAs are involved in the release of linolenic acid from the membrane lipids (Li et al. 2011). 12-oxo-phytodienoic acid (12-OPDA), as the precursor of JA, is synthesised from α-linolenic acid (18:3). Given that VvipPLA-IIδ2 is a phospholipase with cPLA2 activity, we hypothesised that it would affect JA biosynthesis and signalling pathways. To further investigate this hypothesis, we conducted an RT-qPCR expression analysis of JA biosynthesis and signalling associated genes in the leaves of plants overexpressing *VvipPLA-IIδ2*. Compared with GFP control plants, the six genes *AOS*, *AOC*, *AOC1*, *AOC2*, *OPR3* and *JAR1*, which are involved in jasmonic acid synthesis, increased their expression significantly in the *VvipPLA-IIδ2*-overexpressing grapevine leaves (Figure 7A). Moreover, the transcript levels of one JA signalling pathway

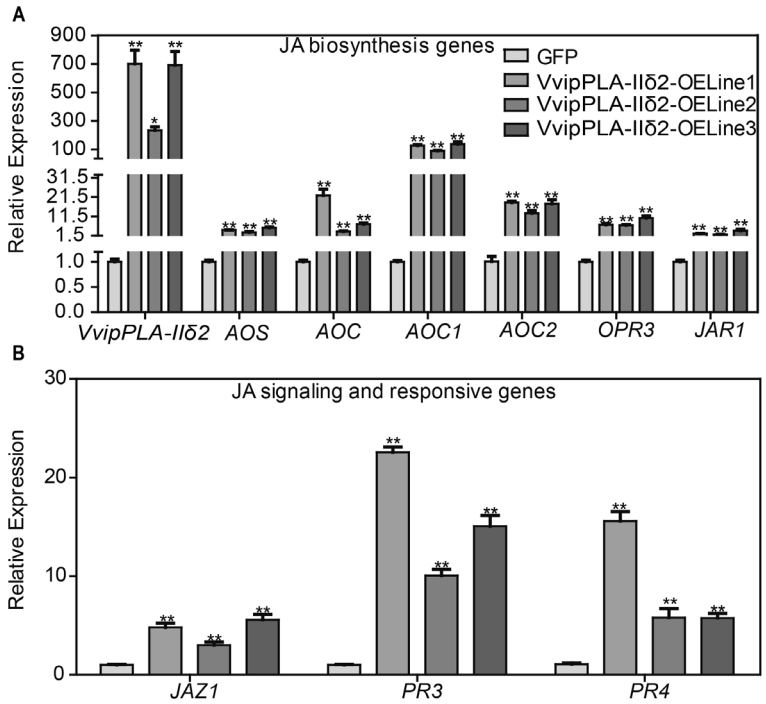

FIGURE 7 | *VvipPLA-IIδ2* overexpression alters the expression of jasmonic acid (JA) pathway-related genes. The relative level of expression of six genes *AOS, AOC, AOC1, AOC2, OPR3* and *JAR1*, which are associated with the JA biosynthesis (A) and the relative expression of JA signalling pathway gene *JAZ1* and two JA-responsive genes *PR3* and *PR4* (B) in *GFP* control plants and *VvipPLA-IIδ2*-transiently overexpressing grapevine plants at 5 days post-infiltration. *VvEF1α, VvGAPDH* and *VvCYP* were used as the standard internal genes. Data represent the mean ± SE of three independent biological repeats (*$p < 0.05$, **$p < 0.01$, Student's *t* test).

gene, *JAZ1*, and two JA-responsive genes, *PR3* and *PR4*, also showed an up-regulation (Figure 7B). To exclude the possibility that the response from JA-associated genes is a result of ER stress, the expression of two ER stress response genes, *CRT1* and *BIP3*, was measured. The RT-qPCR results revealed that the expression of *CRT1* and *BIP3* was not significantly different from the control in two out of three different biological replicates (Figure S7).

3 | Discussion

The oomycete *P. viticola* is an obligate biotrophic pathogen that causes widespread damage to the grape industry (Gessler et al. 2011). In our previous studies, a large number of effectors were identified and characterised in *P. viticola* based on transcriptomic and genomic data (Xiang et al. 2016; Yin et al. 2015, 2017; Liu et al. 2018). PvRXLR10 is a highly conserved RXLR effector across multiple *P. viticola* isolates with a single WY domain in the C-terminus. In this study, we demonstrated that PvRXLR10 localises mainly to the ER. As previously demonstrated for other effectors (Wang et al. 2011; Yin et al. 2015; Liu et al. 2018), PvRXLR10 was able to suppress PCD induced by BAX and INF1 in *N. benthamiana*. We also found that PvRXLR10 promoted *P. viticola* infection in grapevine and enhanced *P. parasitica* colonisation in *N. benthamiana*. VvipPLA-IIδ2 was identified and proved to be a host target of this effector.

The WY domain was originally identified in RXLR effectors of *Phytophthora* species via bioinformatic analysis (Jiang et al. 2008). They were later found to be prevalent not only in *Phytophthora* species but also other oomycete pathogens such as downy mildews (Boutemy et al. 2011; Derevnina et al. 2015; Ye et al. 2016; Klein et al. 2020; Purayannur et al. 2020). Furthermore, WY domain-containing effectors that lacked a canonical RXLR motif were also present in the genome or secretome of *Peronospora tabacina* (Derevnina et al. 2015), *P. halstedii* (Sharma et al. 2015; Pecrix et al. 2019), *P. viticola* (Combier et al. 2019), and *Bremia lactucae* (Wood et al. 2020). Conservation of this domain in oomycetes implies that it is critical for effector function. The WY domain of *P. sojae* Avr1b, PSR1 and PSR2 is required for their avirulence function in *P. sojae* infection (Dou et al. 2008; Zhang et al. 2019; He, Ye, et al. 2019). Furthermore, sequence mutations of *P. infestans* effectors Avr3a, PexRD2 and *P. sojae* effector PSR2 revealed that the WY domains of these effectors mediate the interaction with their host target proteins the E3 ligase CMPG1 (Bos et al. 2010), MAPKKK (King et al. 2014) and DRB4 (He, Ye, et al. 2019), respectively. Consistent with this observation, our experiments revealed that the WY domain of PvRXLR10 is also required for interaction with VvipPLA-IIδ2. It may mean that the WY domain is directly involved in physical contact with VvipPLA-IIδ2 or affects the correct conformation of PvRXLR10 to bind to VvipPLA-IIδ2 more efficiently. This binding may affect the normal function of VvipPLA-IIδ2 by altering its spatial conformation or its interaction with other proteins. However, contrary to the findings with Avr1b, deletion of the WY domain of PvRXLR10 had no effect on its ability to suppress PCD in *N. benthamiana*. We speculate that PvRXLR10 may mediate interaction(s) with additional host target(s) to form a larger protein complex to suppress cell

death. Alternatively, other domains or motifs of PvRXLR10 are associated with PCD inhibition. In addition, the WY domain has been shown to be required for RNA silencing suppression activity of PSR1 (Zhang et al. 2019) and PSR2 (He, Ye, et al. 2019). Altogether, these findings suggest the WY domain plays diverse roles in effector–host interaction.

The ER is an organelle with important functions involved in calcium homeostasis (Kim et al. 2008) and protein folding (Braakman and Hebert 2013) in eukaryotic cells. Increasing evidence suggests that the ER also contributes to plant immunity in different ways. For example, the biosynthesis and proper folding of plant pattern recognition receptors (PRRs) rely on N-glycosylation and the ER quality-control (ERQC) system (Saijo et al. 2009; Li et al. 2009). The ER membrane-localised IRE1 protein plays an important role in the secretion of PR proteins upon salicylic acid (SA) treatment (Moreno et al. 2012).

Under adverse environmental conditions, like biotic stress, host plant ER stress responses are induced (Howell 2013). Several effectors from plant pathogens have been shown to target host ER proteins as part of their infection strategy (Jing and Wang 2020). PsAvh262 was firstly reported to suppress ER stress-mediated cell death by targeting BiP protein to promote *P. sojae* infection (Jing et al. 2016). It was recently shown that type III effector HopD1 of *Pseudomonas syringae* and RXLR effector Pi03192 of *P. infestans* can manipulate plant immunity by targeting NAC transcription factors localised to ER (McLellan et al. 2013; Block et al. 2014). In this study, we also identified NAC transcription factors as potential target proteins for PVRXLR10 through library screening. Although pairwise Y2H experiments have confirmed that there is no interaction between them in yeast, it does not rule out an interaction in plants. We will be investigating separately in the future. Recent work has also demonstrated that an RXLR effector PcAvr3a12 from *Phytophthora capsici* suppresses ER-resident protein AtFKBP15-2-mediated plant immunity by inhibiting its peptidyl-prolyl cis-trans isomerase activity (Fan et al. 2018). In the current study, the ER is not only the localisation site of VvipPLA-IIδ2 but also one of the target sites of PvRXLR10. These findings deepen our understanding of the ER as a key battleground in pathogen infection.

Plant phospholipase As (PLAs) can be divided into three groups, PLA1, PLA2, and patatin-like PLA (pPLA), which catalyse the hydrolysis of acyl groups from the *sn-1* and/or *sn-2* position (Chen et al. 2013). Here, we showed that VvipPLA-IIδ2 is a patatin-like phospholipase A with cPLA2 activity. A total of 41 PLAs were previously identified and characterised in the *V. vinifera* genome, of which 24 belong to the pPLA family. The *VvipPLA-IIδ2* gene was reported to increase its expression 12 hpi after pathogen challenge. This suggests that VvipPLA-IIδ2 plays an important defensive role in the early stages of downy mildew infection. It is consistent with our findings that VvipPLA-IIδ2 enhanced grapevine resistance to *P. viticola*.

Increasing evidence suggests that pPLA proteins (PLPs) are involved in plant defence responses to multiple pathogens. However, whether the *PLP* genes contribute positively or negatively to plant resistance depends on the different lifestyles of the pathogens. For example, *Nicotiana attenuata* NaPLP and *Arabidopsis* AtPLP2 protein play negative roles in resistance to the necrotrophic fungal pathogens *Alternaria alternata* and *Botrytis cinerea* (La Camera et al. 2005; Cheng et al. 2019). In contrast, AtPLP2 contributes to resistance to cucumber mosaic virus, an obligate biotrophic parasite (La Camera et al. 2005). Similarly, overexpression of the pepper *CaPLP1* gene and the cotton *GhPLP2* gene in *Arabidopsis* conferred enhanced resistance to the biotrophic pathogen *H. arabidopsidis*, the hemibiotrophic pathogen *P. syringae* pv. *tomato* and *Verticillium dahliae* (Kim et al. 2014; Zhu et al. 2021). But *GhPLP2*-silenced cotton plants were more susceptible to *V. dahliae* infection (Zhu et al. 2021). The results of our study also show that overexpression of *VvipPLA-IIδ2* enhanced the resistance of grapevine to the biotrophic downy mildew pathogen *P. viticola*, whereas reduced expression of *VvipPLA-IIδ2* diminished the resistance of grapevine to this pathogen. All these findings indicate that plant patatin-like PLA proteins positively regulate plant defence against pathogens with a biotrophic phase in their lifecycle.

In addition to ER stress-mediated plant immunity, the ER is also an important site for phospholipid synthesis (Lagace and Ridgway 2013). pPLAs are involved in the release of linolenic acid from complex membrane lipids (Aloulou et al. 2018). The octadecane pathway starting from α-linolenic acid (18:3) is one of the JA synthesis pathways. α-linolenic acid is catalysed by a series of enzymes to synthesise 12-oxo-phytodienoic acid (12-OPDA) in the chloroplast, which is the precursor of JA (Ruan et al. 2019). Several studies have provided evidence for a JA-signalling role in grapevine defence against *P. viticola* (Figueiredo et al. 2015; Guerreiro et al. 2016). These findings prompted us to question whether VvipPLA-IIδ2 mediates immunity against *P. viticola* via the JA signalling pathway. Our RT-qPCR results showed that the expression of genes involved in JA biosynthesis (*AOS*, *AOC*, *AOC1*, *AOC2, OPR3* and *JAR1*), signalling (*JAZ1*) and response (*PR3* and *PR4*) was significantly increased in *VvipPLA-IIδ2*-overexpressing lines. These results suggest that in *VvipPLA-IIδ2*-overexpressing plants, the synthesis of JA and JA-Ile was increased, and the JA signalling pathway was activated. Other studies also reported an early up-regulation of JA biosynthesis genes (*LOXO*, *LOX*, *AOS*, *AOC*, and *OPR3*), a later activation of JA signalling and responsive genes (*JAR1*, *COI1*, *JAZ1*, *PR14*) and a higher content of JA in the resistant genotype after *P. viticola* inoculation (Polesani et al. 2010; Marchive et al. 2013; Gauthier et al. 2014; Figueiredo et al. 2015; Li et al. 2015). Therefore, VvipPLA-IIδ2 may be involved in the JA biosynthesis and signalling in grapevine, which contributes to the plant defence against *P. viticola*. Although *PLP* genes play nearly opposite contributions in defence against pathogens with different nutritional modalities, several earlier studies have also demonstrated that PLPs mediate plant immunity by the regulation of fatty acid accumulation and the JA pathway (La Camera et al. 2009; Kim et al. 2014; Zhu et al. 2021).

In summary, our study revealed that the interaction between PvRXLR10 and phospholipase VvipPLA-IIδ2 of the host plant grapevine occurs at the ER. VvipPLA-IIδ2 plays an active role in the defence of grapes against downy mildew. We speculate that the effector PvRXLR10 of *P. viticola* may indirectly affect the JA signalling pathway by interacting with VvipPLA-IIδ2 or affecting its phospholipase activity, regulating phospholipid metabolites, and then inhibiting the defence response of plants and promoting

its successful infection. This discovery not only enriches our understanding of pathogen–host interactions, but also reveals the potential mechanism of VvipPLA-IIδ2 in plant immunity, providing a theoretical basis for the development of novel disease resistance strategies. To gain a deeper insight into the specific mechanisms of this interaction, future studies might employ live-cell imaging, biochemical analysis, gene editing technology and other methods to reveal the molecular details of the interaction between PvRXLR10 and VvipPLA-IIδ2 such as exploring how the activity of VvipPLA-IIδ2 is regulated by this effector protein.

4 | Experimental Procedures

4.1 | Plant Material and Pathogen Growth Condition

The potted *N. benthamiana* and grapevine (*V. vinifera* 'Thompson Seedless') plants were grown in a greenhouse at 25°C under 16 h light/8 h dark photoperiod. Five-week-old *Nicotiana* plants were used for infection assays. The tissue culture seedlings of *V. vinifera* 'Thomson Seedless' were cultured in vitro on woody plant medium (Phytotech) with 20 mg/μL IBA. *P. viticola* inoculation was performed as described by Yin et al. (2015). *P. parasitica* isolate was routinely cultured in the dark at 25°C on potato dextrose agar (PDA) plates.

4.2 | Plasmid Construction

The sequence encoded mature PvRXLR10 protein (without the signal peptide) was amplified from the mixed cDNA from *P. viticola*-infected grapevine leaves at different time points. For cell death suppression assays in *N. benthamiana*, GFP, INF1, BAX, PvCRN92, PvRXLR10 and PvRXLR10-ΔWY (deleted the coding sequence of 83–123 amino acids) sequences were inserted into the pBWA(V)HS vector (#REC10-I, Biorun Biosciences Co. Ltd.) driven by the CaMV 35S promoter. For subcellular location and co-localisation assays, the coding sequence of mature PvRXLR10 and VvipPLA-IIδ2 were cloned into the N-terminus or C-terminus of GFP/mScarlet in the vector pBWA(V)HS. The plasmid of ER marker SPER-mKate and ER-CFP transformed into *Agrobacterium tumefaciens* GV3101 was provided by Wuhan BioRun Biosciences Co. Ltd. To generate constructs for BiFC, the coding sequences of mature PvRXLR10 and VvipPLA-IIδ2 were cloned into pGreenII-62-SK-VN/VC vectors. To generate constructs for pull-down assays, the coding sequences of desired genes were fused with either GST or His tag and cloned into the pGEX-6P-1 or pET28a vector. For Co-IP assay, PvRXLR10 fused with 3×FLAG tag and VvipPLA-IIδ2 fused with GFP tag was inserted into the pBWA(V)HS vector. To generate the RNAi construct for knocking down *VvipPLA-IIδ2* in *V. vinifera*, a 200-bp fragment of *VvipPLA-IIδ2* was amplified and inserted into the pBWA(V)HS-RNAi vector to form hairpin RNA constructs. All primers are listed in Table S2.

4.3 | cDNA Library Screen and Yeast Two-Hybrid Assay

Detached leaf discs were inoculated with 35 μL droplets of 3×10^5/mL sporangia suspension. The infected leaf discs were sampled at different time points and snap-frozen in liquid nitrogen and stored at −80°C in preparation for RNA extraction. The cDNA library was constructed by Dualsystems Biotech. The library screen and the yeast two-hybrid assays were performed using the DUAL hybrid Kit (Dualsystems Biotech) according to the protocol provided by the kit instructions. The sequence of *PvRXLR10*, *PvRXLR10*-ΔWY, or *VvipPLA-IIδ2* was inserted into the bait vector pLexA-N to generate a fusion protein as bait. To confirm the interactions between PvRXLR10 and VvipPLA-IIδ2, the *VvipPLA-IIδ2* gene was cloned into the prey vector pGAD-HA to generate a fusion protein as prey. All constructs were transformed into the yeast strain NMY51. The positive transformants were selected on different selective media with or without 3-aminotriazole (3AT) and X-gal when needed.

4.4 | Assays of Leaf Agro-Infiltration and Infection in *N. benthamiana* and *V. vinifera*

Leaf agro-infiltration in *N. benthamiana* was performed as described in our previous study (Yin et al. 2015). For cell death suppression assays, a suspension of *A. tumefaciens* cells ($OD_{600}=0.5$) carrying GFP or GFP-PvRXLR10 was infiltrated into *N. benthamiana* leaves first. The same site was re-infiltrated with *A. tumefaciens* cultures carrying either the BAX-3×FLAG, INF1-3×FLAG or PvCRN92-3×FLAG after 24 h. The development of leaf necrosis symptoms was monitored 4 to 7 days after infiltration and photographed. *P. parasitica* infection assays were performed by mycelia agar disc (5-mm diameter) inoculation of the isolate 2-1 on at least 10 independent *N. benthamiana* detached leaves (4–5 weeks old). The lesion diameter of inoculated leaves was measured, and the infected leaves were photographed under UV light at 36 hpi. These assays were repeated at least three times.

Agro-infiltration of grapevine leaves for fast transient assays of gene expression was performed according to the method previously described by Ben-Amar et al. (2013) with modification. Vacuum agro-infiltration was conducted on non-detached expanded leaves of tissue culture seedlings, which were transplanted into pots for about 3 weeks. For tests of disease resistance to *P. viticola*, leaf discs (15 mm) were inoculated with 30 μL droplets of 3×10^5/mL sporangia. The test was repeated at least three times and three plants were used in each test.

4.5 | RNA Extraction and RT-qPCR

Plasmopora viticola-infected leaf discs were collected, and total RNA was extracted by using Spectrum plant total RNA extraction kit (Sigma-Aldrich) according to the manufacturer's instructions. The mixed cDNA was synthesised with the PrimeScript IV 1st strand cDNA Synthesis Mix (TaKaRa). Real-time PCR was performed by using a LightCycler 480 SYBR Green I Master (Roche) on a LightCycler 480 System II (Roche) following the manufacturer's instructions. Three genes of grapevine (*VvEF1α*, *VvGAPDH* and *VvCYP*) and *PvActin* and *PvTubulin* genes of *P. viticola* were used as internal control for normalisation. Three technical repeats and three biological replicates of each reaction were performed. The relative gene expression was

quantified using $2^{-\Delta\Delta Ct}$ method. The primers used for qPCR are listed in Table S1.

4.6 | Confocal Microscopy

Agrobacterium tumefaciens containing indicated vectors was infiltrated or co-infiltrated into leaves of 4-week-old *N. benthamiana* plants. Fluorescent signals were observed and imaged at 2 dpi using a Nikon C2-ER or Olympus FV3000 confocal laser scanning microscope (488 nm excitation/500–540 nm emission for GFP, 561 nm excitation/570–620 nm emission for mKate and mScarlet, 488 nm excitation/508–548 nm emission for YFP, mScarlet, 445 nm excitation/459–496 nm emission for CFP).

4.7 | Pull-Down and co-IP

Escherichia coli Rosetta (DE3) was used to express His-PvRXLR10, His-PvCRN92, His-PvRXLR10-ΔWY, GST and GST-VvipPLA-IIδ2 proteins. The soluble GST-fusion proteins were incubated with 50 μL glutathione agarose beads (Solarbio) at 4°C for 2 h. The beads were washed three times and incubated with His-tagged proteins at 4°C overnight. The beads were washed three times, and the presence of His-tagged protein was detected by western blotting using anti-His antibody.

Plasmid of empty vector 3×FLAG, PvRXLR10-3×FLAG and 3×FLAG-PvRXLR10 constructs were expressed together with VvipPLA-IIδ2-GFP in *N. benthamiana* leaves by agro-infiltration. Leaf samples were harvested 48 hpi and ground to fine powder in liquid nitrogen. Proteins were extracted using RIPA lysis buffer (50 mM Tris–HCl, pH 8, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 1% SDS) with 1 mM dithiothreitol (DTT), protease inhibitor cocktail, and 1 mM phenylmethyl sulphonyl fluoride. 3×FLAG-tagged PvRXLR10 was immunoprecipitated using anti-FLAG M2 Magnetic Beads. The resulting samples were separated by SDS-PAGE and detected by western blotting. Immunoprecipitated FLAG fusions and co-immunoprecipitated GFP fusions were detected using appropriate antisera (Abcam).

4.8 | Phospholipase A2 Assay

The full-length cDNA sequence of VvipPLA-IIδ2 was cloned into the GST fusion protein expression vector pGEX-6p-1. The GST-fusion protein was expressed in *E. coli* BL21 and purified following the manufacturer's instructions. Phospholipase A2 activity was measured using the cPLA2 assay kit (Cayman) following the manufacturer's instructions.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All relevant data can be found within this manuscript and the Figures S1–S7 and Tables S1 and S2.

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Supporting Information

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