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An RXLR effector secreted by *Phytophthora parasitica* is a virulence factor and triggers cell death in various plants

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Running head: A virulent RXLR effector recognized by plants

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SUMMARY

RXLR effectors encoded by *Phytophthora* species play a central role in pathogen-plant interactions. Understanding the biological functions of RXLR effectors are conducive to illuminating pathogenic mechanisms and developing disease-control strategies. However, the virulence function of *Phytophthora parasitica* RXLR effectors is poorly understood. Here, we describe the identification of a *P. parasitica* RXLR effector gene, *PPTG00121* (*PpE4*), which is highly transcribed during the early stages of infection and conserved among various *Phytophthora* species. Live-cell imaging of *P. parasitica* transformants expressing a full-length PpE4 (E4FL)-mCherry protein indicated that PpE4 is secreted and accumulates around haustoria during plant infection. Silencing of *PpE4* in *P. parasitica* resulted in significantly reduced virulence on *Nicotiana benthamiana*. Transient expression of *PpE4* in *N. benthamiana* in turn restored pathogenicity of the *PpE4*-silenced lines. Furthermore, expression of *PpE4* in both *N. benthamiana* and *Arabidopsis thaliana* consistently enhanced plant susceptibility to *P. parasitica*. These results indicate that *PpE4* contributes to pathogen infection. Finally, heterologous expression experiments showed that *PpE4* triggers nonspecific cell death in a variety of plants including tobacco, tomato, potato, and *A. thaliana*. Virus-induced gene silencing assays revealed that *PpE4*-induced cell death is *HSP90*-, *NPK*-, and *SGT1*-dependent, suggesting that PpE4 is recognized by the plant immune system. In conclusion, PpE4 is an important virulence RXLR effector of *P. parasitica* that is conserved across *Phytophthora* species and recognized by a wide range of host plants.

INTRODUCTION

Phytophthora parasitica shares the main features of most *Phytophthora* species; it is a soil-borne pathogen with a wide host range (Meng *et al.*, 2014). It causes tobacco black shank and is listed as one of the Top 10 oomycete pathogens because of its scientific and economic importance (Kamoun *et al.*, 2015). *P. parasitica* serves as a model oomycete pathogen, and its compatible interaction with the model plant *Arabidopsis thaliana* has been established (Attard *et al.*, 2010; Wang *et al.*, 2011b). There have been fewer functional analyses of *P. parasitica* genes (Chang *et al.*, 2015; Evangelisti *et al.*, 2013; Gaulin *et al.*, 2002; Khatib *et al.*, 2004; Meng *et al.*, 2015; Zhang *et al.*, 2012), and these studies are far from being enough to fully understand *P. parasitica* biology, pathogenesis, and plant interaction mechanisms.

During the war between pathogens and hosts, plants evolved two immune systems to defend against invaders: pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) mediated by pattern recognition receptors (PRRs) and effector-triggered immunity (ETI) mediated by resistance (R) proteins that recognize avirulence effectors (AVR) (Dodds & Rathjen, 2010; Jones & Dangl, 2006).

Upon perception of nonself signals (PAMPs or effectors) from pathogens, plant cells activate a complicated signal transduction network. Although the signal transduction pathways implicated in PTI and ETI are different, the downstream cellular events are similar, including a series of cellular responses and also cell death (Dodds & Rathjen, 2010; Pedley & Martin, 2005; Peng *et al.*, 2018).

Although cell death induced by a number of *Phytophthora* RXLR effectors occurs independently of known R proteins, it is likely the result of plant recognition and related to components of the PTI or ETI pathways. Many genes involved in plant immune signaling are required for effector-induced cell death. For example, *MEK2* is required for *Avh238*-triggered cell death (Yang *et al.*, 2017); *MEK2* and *WIPK*

are involved in *Avh241*-induced cell death (Yu *et al.*, 2012); suppressor of G2 allele of *skp1* (*SGT1*) is required for the cell death activity of *PexRD2* (Oh *et al.*, 2009) and *PITG_22798* (Wang *et al.*, 2017a); a specific MAPK cascade is responsible for *Pi_23226*-induced cell death (Lee *et al.*, 2018); and *SGT1*, *HSP90*, *RAR1* and MAPK cascades are required for *PvRXLR16*-induced cell death (Xiang *et al.*, 2017).

During the infection and colonization of plants, pathogens secrete numerous effectors to manipulate plant physiological processes and thereby suppress plant immunity and enhance plant susceptibility.

Effectors usually possess dual activities, facilitating infection and triggering plant immunity during plant-microbial interactions (Kamoun, 2006; Kjemtrup *et al.*, 2000; van't Slot & Knogge, 2002). For example, the glycoside hydrolase 12 protein XEG1 is required for *P. sojae* virulence but is also

recognized as a PAMP and triggers cell death and plant immunity (Ma *et al.*, 2015). Necrosis and

ethylene-inducing peptide 1 (Nep1)-like proteins (NLPs), which are conserved virulence factors

widespread in bacterial, oomycete and fungal pathogens, trigger host cell damage-associated plant

immunity and are also recognized as PAMPs (Bohm *et al.*, 2014; Fellbrich *et al.*, 2002; Ottmann *et al.*,

2009; Qutob *et al.*, 2006). Another classic example is the triggering of *R* gene-mediated hypersensitive

response (HR) by AVR effectors that typically exert their virulence function on *R* gene-absent plants

(Kamoun, 2006). In addition to known AVR effectors, a few RXLR effectors, such as *PsAvh241* (Yu *et al.*, 2012), *PsAvh238* (Wang *et al.*, 2011a; Yang *et al.*, 2017) and *PITG_22798* (Wang *et al.*, 2017a),

possess virulence functions even though they induce immune response-related cell death in plants.

RXLR effectors, which exist by the hundreds in each oomycete genome, are among the

best-characterized oomycete effectors (Baxter *et al.*, 2010; Haas *et al.*, 2009; Jiang *et al.*, 2008; Tyler *et al.*,

2006). In recent years, a large number of studies have been carried out to elucidate the biological

functions of RXLR effectors from *Phytophthora infestans*, *P. sojae* and *Hyaloperonospora*

arabidopsis (Anderson *et al.*, 2015; Sharpee & Dean, 2016; Wang *et al.*, 2011a; Whisson *et al.*, 2016; Zheng *et al.*, 2014). However, little is known about RXLR effectors from *P. parasitica*, except PSE1, which has been reported to alter the auxin content and promote infection (Evangelisti *et al.*, 2013). In addition, 172 candidate RXLR effectors have recently been identified in the *P. parasitica* genome, three of which suppress INF1-induced cell death and enhance *P. parasitica* virulence (Dalio *et al.*, 2017). In this study, we investigated the virulence function of the *P. parasitica* RXLR effector gene *PpE4*. We found that *PpE4* is highly expressed during the early stages of infection and is secreted from haustoria. To evaluate the role of *PpE4* in *P. parasitica* pathogenicity, *PpE4*-silenced transformants were created and analyzed. These transformants had reduced ability to infect plants, and transient expression of *PpE4* in *N. benthamiana* restored pathogenicity. To further examine its contribution in promoting pathogen colonization, an inoculation assay was performed after transient or induced *in planta* expression of *PpE4*. Plants expressing *PpE4* were more susceptible to *P. parasitica* infection. *PpE4* also triggered nonspecific cell death in a variety of plants in an *HSP90*-, *NPK*-, and *SGT1*-dependent manner, which suggests that *PpE4* is recognized by the plant immune system. Based on these results we conclude that *PpE4* is a virulence RXLR effector of *P. parasitica* and is recognized by a wide variety of host plants.

RESULTS

***PpE4* encodes a secreted RXLR effector and is highly expressed during the early phase of infection**

Using previous RNA-seq data (Jia *et al.*, 2017), we identified a putative *P. parasitica* RXLR effector gene *PPTG_00121*, named *PpE4*, which was the most highly expressed RXLR effector gene during infection of *Arabidopsis* roots (Fig. S1A, see Supporting Information). Over 70% of the total RXLR

effector transcripts corresponded to *PpE4*, and the FPKM (Fragments Per Kilobase Million) value was over 8000 at 3-6 hours post inoculation (hpi) of *P. parasitica* zoospores (Fig. S1A). To validate the expression profile of *PpE4* during development and plant infection, reverse transcription-quantitative PCR (RT-qPCR) (Bustin *et al.*, 2009) was performed. The expression pattern of *P. parasitica PpE4* during *A. thaliana* root infection initiated with zoospores was consistent with the RNA-seq (Fig. S1B, see Supporting Information). During infection of *Nicotiana benthamiana* leaves, *PpE4* transcripts were rapidly and strongly up-regulated from 3 to 24 hpi, then declined and became barely detectable at 48 hpi, which is similar to that in vegetative hyphae, zoospores, cysts, and germinated cysts before infection (Fig. 1). Biotrophic growth of *P. parasitica* in *N. benthamiana* leaves was dominant before 24 hpi, then the necrotrophic growth with significant cell death became evident (Fig. S2, see Supporting Information). In conclusion, *PpE4* transcripts are strongly induced and predominantly accumulated during the biotrophic phase, at levels hundreds of times higher than that in the mycelium.

To monitor the secretion of *PpE4* during infection, a full-length *PpE4* with its native signal peptide (*E4FL*)-*mCherry* fusion construct was transformed into *P. parasitica* strain 1121, which stably expresses cytoplasmic GFP, via polyethylene glycol (PEG)-CaCl₂-mediated transformation (Bottin *et al.*, 1999). Six transformants showing stable red fluorescence signal and one without that were chosen for RT-qPCR and western blot assays. High levels of *PpE4* transcripts and fusion proteins accumulated in vegetative mycelia of transformants, while no accumulation in E4MC3N4 and strain 1121 (Fig. S3, see Supporting Information). Observation of transformant E4MC4A2 with strong red fluorescence signal showed that the red fluorescence was evenly distributed in mycelia cultured *in vitro* (Fig. 2A), while it was highly enriched in haustoria during infection of *N. benthamiana* leaves at 24 hpi (Fig. 2B). Further detailed observation and the fluorescence intensity analyses of E4MC4A2 (Fig. 2C, D) and

E4MC4A6 (Fig. S4, see Supporting Information) showed that mCherry fluorescence signal accumulated outside the GFP fluorescent haustoria, mainly distributed around the haustorial neck, indicating that E4FL-mCherry accumulates in the extra-haustorial matrix (EHMx) upon secretion from haustoria. By contrast, there was no mCherry fluorescence in strain 1121, and GFP fluorescence distributed evenly in vegetative hyphae as well as in infection hyphae, without specific accumulation at haustoria (Fig. 2). This result is consistent with previous studies of *P. infestans* effectors AVR3a (Whisson *et al.*, 2007), AVR2 (Gilroy *et al.*, 2011), Pi04314 (Wang *et al.*, 2017b), AVR4 and AVRblb1 (van Poppel, 2009), and *P. sojae* effector Avr1b (Liu *et al.*, 2014).

Silencing of *PpE4* attenuates pathogenicity of *P. parasitica*

Inoculation analysis revealed that constitutive expression of *E4FL-mCherry* reduces colonization by *P. parasitica* (Fig. S5, see Supporting Information). To investigate the potential virulence function of *PpE4* in *P. parasitica* pathogenesis, we generated co-silencing transformants as previously described (Meng *et al.*, 2015). A hairpin structure derived from a segment of *GFP* fused with a segment of *PpE4* was constructed and introduced into *P. parasitica* strain 1121 (Fig. S6A, see Supporting Information). Because both *GFP* and *PpE4* were targeted, *PpE4* expression was more likely decreased in the transformants with significantly reduced GFP signal. A total of 173 independent transformants were generated, and 19 with normal colony morphology showed decreased GFP fluorescence, a frequency consistent with previous reports (Meng *et al.*, 2015; Zhang *et al.*, 2012). RT-qPCR experiments revealed that five out of the 19 candidate transformants had obviously reduced *PpE4* expression at 24 hpi compared to strain 1121 (Fig. S6, see Supporting Information). Further pathogenicity analysis showed that three silenced lines (E4S2A6, E4S2B2 and E4S2F5) produced significantly smaller lesions and less hyphae biomass compared with strain 1121, whereas the virulence of the other two lines

(E4S2C4 and E4S2G5) was almost unaffected (Fig. 3A-C). We further confirmed the expression of *PpE4* in these transformants after series of subculturing. The results showed that the expression level of *PpE4* in infected *N. benthamiana* leaves of 15 hpi and 24 hpi remained silenced in transformants E4S2A6, E4S2B2 and E4S2F5, but partially recovered in E4S2C4 and totally recovered in E4S2G5 (Fig. 3D), which were consistent with the results of pathogenicity assay. Therefore, stable silencing of *PpE4* led to attenuated pathogenicity of *P. parasitica*, and restored target gene expression suggested that *PpE4* is important to *P. parasitica*.

Transient expression of *PpE4* in planta restores the pathogenicity of *PpE4*-silenced transformants

To verify that the virulence attenuation of the *PpE4*-silenced lines is due to *PpE4* silencing, three silenced lines were inoculated on mature *PpE4*-expressing (intracellular expression without signal peptide) or *GFP*-expressing leaves, and strain 1121 was inoculated on *GFP*-expressing leaves. The *PpE4*-silenced lines inoculated on *PpE4*-expressing leaves formed significantly larger lesions than those inoculated on *GFP*-expressing leaves, while there was no difference between the size of the lesions on *PpE4*-expressing leaves and those of the control group (1121 inoculated on *GFP*-expressing leaves) (Fig. 4A-C). Western blotting showed that *PpE4* and *GFP* proteins were stably accumulated under low agroinfiltration concentration (OD₆₀₀, 0.01) (Fig. 4D). This indicates that *in planta* expression of *PpE4* is able to restore the virulence of *PpE4*-silenced lines to wild-type levels. In conclusion, *PpE4* positively contributes to pathogenicity of *P. parasitica*.

PpE4* enhances plant susceptibility to *P. parasitica

To further determine whether *PpE4* contributes to *P. parasitica* colonization *in planta*, inoculation was performed on *N. benthamiana* leaves expressing mature *PpE4* on one half and GFP on the other half.

The lesions and *P. parasitica* biomass on the *PpE4*-expressing halves were significantly larger than those on the control (Fig. 5A-C). Stable accumulation of *PpE4* and GFP proteins *in planta* was detected by western blotting (Fig. 5D). These results suggest that transient expression of *PpE4* makes *N. benthamiana* more susceptible to *P. parasitica*.

We also examined the contribution of *PpE4* in the *Arabidopsis*-*P. parasitica* pathosystem.

Chemical-inducible transgenic *Arabidopsis* lines in which expression of mature *PpE4* is strictly regulated by estradiol were constructed (Zuo *et al.*, 2000). Wild-type Col-0 and empty vector pER8 transgenic plants were used as controls. The rosette leaves from three homozygous transgenic lines expressing *PpE4* and control plants were infiltrated with 10 μ M 17- β -estradiol to induce *PpE4* expression 12 h before inoculation of *P. parasitica* zoospores. The disease index statistic indicated that the transgenic plants expressing *PpE4* were more susceptible to *P. parasitica* infection than the controls, and the pathogen biomass in these plants was significantly higher than that in the control plants (Fig. 5E-G). Semi-quantitative PCR showed that *PpE4* was expressed upon injection of 17- β -estradiol, while there was no *PpE4* expression in control plants receiving the same treatment (Fig. 5H). These results demonstrate that *PpE4* facilitates *P. parasitica* infection.

***PpE4* triggers cell death in various plants**

When *PpE4* was intracellularly expressed in *N. benthamiana* leaves by agroinfiltration, it triggered cell death at 3 days post infiltration (dpi) (Fig. 6A). To investigate whether this cell death is species-specific, *PpE4* was transiently expressed in several Solanaceae plants, including three tobacco species, tomato and potato. We found that *PpE4* triggered cell death in all tested Solanaceae plants (Fig. 6A). No *Arabidopsis* transgenic plants were recovered when the 35S promoter was used to drive *PpE4* expression, implying that *PpE4* is lethal to *Arabidopsis* cells. Using estradiol-inducible transgenic plants, we found that *PpE4* triggered cell death on *Arabidopsis* leaves 4 days after induction by 17- β -estradiol, while no cell death occurred in the control plants (empty vector pER8 transgenic plants and Col-0) (Fig. 6B). These results indicate that *PpE4* triggers nonspecific cell death in a variety of plants.

According to the protein secondary structure predicted by Phyre2 (Kelley *et al.*, 2015), *PpE4* contains four α -helices downstream of the signal peptide (Fig. S7, see Supporting Information). To identify which domains are crucial for cell death-inducing activity, we successively deleted the α -helices to construct a series of deletion mutants and transiently expressed them in *N. benthamiana* (Fig. 6C-E). E4FL with its native signal peptide could also induce cell death. However, the cell death occurred more slowly and weakly than that of mature protein. As shown in Figure 6, E4M1, E4M4, E4M7, and E4M8 still maintain cell death-inducing activity, which indicates that the first α -helix, the RXLR motif and the last 13 amino acids were not required for cell death induction. Deletion of the RXLR-DEER domain as well as the second α -helix abolished cell death-inducing ability. Since DEER motif is in α -helix 2, its deletion may destroy the structure of α -helix 2. Moreover, E4M5 and E4M6 were unable to induce cell death which indicated that α -helix 3 and part of α -helix 4 are necessary to cell death induction (Fig. 6C,

D). In short, E4M8, with residues 56-121, is sufficient to maintain the integrity of the protein tertiary structure and trigger cell death.

PpE4*-induced cell death requires *HSP90*, *NPK* and *SGT1

Cell death induced by a number of pathogen effectors is considered to be the outcome of recognition by the plant immune system, either PTI or ETI, and this process is complex, involving a variety of receptors and signal transduction pathways (Lee *et al.*, 2018; Wang *et al.*, 2011a; Wang *et al.*, 2017a; Xiang *et al.*, 2017; Yu *et al.*, 2012; Yang *et al.*, 2017). To determine which signaling pathway is involved in *PpE4*-induced cell death, VIGS was used to silence a series of genes in *N. benthamiana*, including genes responsible for R protein function such as *HSP90*, *SGT1*, and *RAR1* (Kanzaki *et al.*, 2003; Takahashi *et al.*, 2003; Zhang *et al.*, 2004); genes associated with the activation of the TIR-NB-LRR and CC-NB-LRR R proteins, *EDS1* and *NDR1* (Knepper *et al.*, 2011; Oh *et al.*, 2014), respectively; the receptor-like kinases *BAK1* and *SOBIR1* (Chaparro-Garcia *et al.*, 2011; Liebrand *et al.*, 2013; Liebrand *et al.*, 2014); the transcription factors *MYB1* and *WRKY3*; and the MAPK cascade genes *NPK*, *MEK1*, *MEK2* and *SIPK* (Jin *et al.*, 2002; Liu *et al.*, 2004b). Cell death was scored after transient expression of *PpE4* in these silenced plants. *PpE4*-induced cell death was almost abolished in *HSP90*- and *NPK*-silenced plants and significantly attenuated in *SGT1*-silenced plants compared with *GFP*-silenced plants (Fig. 7A, B). Western blot assay showed that *PpE4* protein was stably accumulated in the silenced plants (Fig. 7D). In addition, cell death was slightly, but significantly, compromised in *BAK1*-silenced plants, whereas cell death was not affected in plants with silenced expression of other genes (Fig. S8A, B, see Supporting Information). RT-qPCR assays confirmed that there was a

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significant reduction in the transcript levels of the targeted genes in silenced plants compared with the levels in *GFP*-silenced plants (Figs 7C and S8C, see Supporting Information). In summary, *HSP90*, *NPK* and *SGT1* are required for *PpE4*-induced cell death.

DISCUSSION

PpE4 is highly induced during infection and secreted from haustoria

As previously reported, functionally important RXLR effectors are usually induced *in planta*, whereas only a few RXLR effectors, which contribute the vast majority of RXLR effector transcripts, are considered to be crucial for pathogen pathogenicity (Wang *et al.*, 2011a). Here, we found that the effector gene *PpE4* is highly up-regulated, accounting for more than 70% of total RXLR effector transcripts during the early stages of infection (Figs 1 and S1). The relative expression of *PpE4* in the transformants was hundreds of times higher than that in strain 1121 in vegetative mycelia. But only three transformants exhibited a bit higher expression level than strain 1121 at 36 hpi (Fig. S3B and C), indicating that *PpE4* is extraordinarily highly induced during infection and it's not easy to get it over-expressed during infection. This implies that *PpE4* has a critical role during *P. parasitica* infection. RXLR effectors usually accumulate in the EHMx and are especially concentrated at the haustorial neck upon secretion (Gilroy *et al.*, 2011; Liu *et al.*, 2014; van Poppel, 2009; Wang *et al.*, 2017b; Whisson *et al.*, 2007). In this study, we demonstrated that E4FL-mCherry fusion proteins accumulate substantially in the EHMx after secretion from haustoria during infection, even though expression of these proteins was driven by constitutive promoters (Figs 2, S3 and S4).

***PpE4* contributes to infection even though it triggers cell death in plants**

In virulence assays of *PpE4*-silenced transformants, three stable silenced lines showed attenuated pathogenicity that could be restored by transient expression of *PpE4* in plant. However, the virulence of the other two unstable transformants, E4S2C4 and E4S2G5, was not attenuated, in which *PpE4* expression was initially silenced but restored after series of subculturing (Figs 3 and 4). These results indicated that stable silencing of *PpE4* did affect pathogenicity of *P. parasitica*. The restored expression of *PpE4* in the silencing transformants and its low-frequency of co-silencing with GFP suggested its importance and tightly regulated expression in *P. parasitica*, similar to a case of previous report (Meng *et al.*, 2015). We also found that *E4FL-mCherry*-expressing transformants displayed decreased virulence (Fig. S5). It is possible that the virulence reduction was attributed to *PpE4*-recognition by plant immune system, as the overexpression of *PpE4* likely led to partially relieve of its suppression by other effectors. Considering that *PpE4* is lowly transcribed in mycelia and zoospores, it is more likely that the constitutive overexpression of *PpE4* during the pre-infection stage disrupted its original expression pattern and affected its pathogenicity. Similarly, premature expression of *Avh238* also affected the ability of *P. sojae* to infect plants; thus, the timing of effector expression is crucial for pathogenicity (Wang *et al.*, 2011a).

In addition to its contribution to infection, we also found that *PpE4* triggers nonspecific cell death in two major eudicots (Fig. 6). Cell death plays a vital but ambiguous role in plant-pathogen interactions, especially those involving biotrophic and hemibiotrophic pathogens. Hemibiotrophs require living cells to establish colonization, and cell death would not be preferred at early stages of infection. During rapid expansion in plants the invaders induce host cell death to facilitate the transition from biotrophy to necrotrophy (Qutob *et al.*, 2002). However, cell death induced by recognition of PAMPs or AVRs

emerging at the very beginning of infection usually abolishes pathogen invasion. Therefore, the timing and intensity of cell death is under sophisticated regulation during plant-pathogen interactions; actually, the one who controls cell death wins (Coll *et al.*, 2011; Kabbage *et al.*, 2013).

Many RXLR effectors have been demonstrated to promote pathogen colonization when transiently expressed in plants, such as AVR1 (Du *et al.*, 2015a; Du *et al.*, 2015b), PexRD2 (King *et al.*, 2014), PITG_22798 (Wang *et al.*, 2017a), Avh241 (Yu *et al.*, 2012) and Avh238 (Yang *et al.*, 2017). However, these effectors could also induce HR when detected by corresponding R proteins or recognized by unknown mechanisms in plants. To eliminate the influence of cell death during the inoculation process, we reduced the concentration of the *Agrobacterium tumefaciens* suspension to OD₆₀₀ value of 0.01 to delay and weaken cell death (Wang *et al.*, 2017a). As a result, *PpE4* enhanced infection when transiently or stably expressed in plants, in spite of its cell death-inducing activity (Figs 5 and 6). Three hypotheses may explain this result. First, although *PpE4* is highly transcribed at early stages of infection, the accumulation of PpE4 protein in plant cells via translocation from the pathogen may be insufficient to induce cell death under natural conditions. Second, considering the cases of Avh241 and Avh238, where cell death could be suppressed by other immediate-early expressed effectors (Wang *et al.*, 2011a), we suspect that *PpE4*-induced cell death may be suppressed by other cooperative effectors. Thus, *PpE4* possibly manifest its virulence function and enhance colonization when its cell death activity is blocked. Finally, it is possible that the cell death occurs just in time to promote the transition into necrotrophy, enabling earlier occurrence of necrotrophy phase. In this situation, cell death triggered by its intracellular expression is beneficial to pathogen infection, making it a virulence factor. In any case, *PpE4* exhibits dual functions; it contributes to *P. parasitica* virulence while triggering recognition-related cell death in the host plant.

***PpE4*-triggered cell death may be related to plant recognition**

In this study, we applied VIGS technology to demonstrate that *PpE4*-induced cell death requires *HSP90*, *NPK*, and *SGT1* (Fig. 7). As previously reported, HSP90 often forms a complex with co-chaperones RAR1 and SGT1 to maintain the function of NB-LRR proteins (Kadota *et al.*, 2010; Shirasu, 2009). In addition to being involved in R3a-AVR3a-mediated HR and INF1-triggered cell death (Bos *et al.*, 2006; Chapman *et al.*, 2014; Kanzaki *et al.*, 2003), HSP90 and SGT1 are required in both *N*- and *Rx*-mediated defense responses against viruses (Boter *et al.*, 2007; Liu *et al.*, 2004a; Lu *et al.*, 2003). However, only SGT1 is required for PITG_22798 and Rpiblb2-AVRblb2-triggered HR (Oh *et al.*, 2014; Wang *et al.*, 2017a). *NPK1* is the *Nicotiana* homolog of human *MEKK1* and encodes a MAP kinase kinase kinase that is involved in responses mediated by the resistance genes *N*, *Bs2*, and *Rx* (Jin *et al.*, 2002; Liu *et al.*, 2004b; Soyano *et al.*, 2003). The fact that *HSP90*, *NPK*, and *SGT1* are involved in either *R* gene- or *PRR*-mediated immune signaling, suggests that *PpE4*-triggered cell death is possibly the consequence of plant recognition. But this recognition is not mediated by either BAK1- or SOBIR1-associated cell surface receptors, or by EDS1- or NDR1-associated R proteins, because cell death was only slightly affected in *BAK1*-silenced plants and not significantly affected in *SOBIR1*-, *EDS1*-, or *NDR1*-silenced plants (Fig. S8). According to previous study, *PpE4* shows moderate sequence similarity to *P. sojae* effector Avh238 (Yang *et al.*, 2017). Although they are significantly divergent in the C-terminal region, both *PpE4* and Avh238 trigger nonspecific cell death in various plants, whereas the cell death mechanism may be distinct since different genes are responsible for mediating cell death induced by each effector (Yang *et al.*, 2017). Different components of the PTI or ETI pathways have been reported to be specifically involved in cell death induced by different RXLR effectors, which indicates that there are distinct recognition mechanisms and complicated signaling

pathways responsible for each effector (Bos *et al.*, 2006; Lee *et al.*, 2018; Oh *et al.*, 2009; Wang *et al.*, 2017a; Xiang *et al.*, 2017; Yang *et al.*, 2017; Yu *et al.*, 2012). However, it is still unclear how these effectors are recognized in plants.

It is worthwhile to elucidate the cell death induction mechanism of the early-induced RXLR effectors.

Studies of the biological functions and host targets of RXLR effectors are conducive to illuminating pathogenic mechanisms and developing disease-control strategies against pathogens such as *P. parasitica* that have a broad host range.

EXPERIMENTAL PROCEDURES

Plant and *Phytophthora* cultivation

Arabidopsis thaliana seeds were sterilized and sown on 0.8% agar plates containing half-strength Murashige and Skoog nutrient solution, followed by a one-week incubation in a growth chamber as described previously (Wang *et al.*, 2011b). Then the seedlings were transferred into a matrix containing soil and vermiculite and grown in a 22-25°C climate chamber with a photoperiod of 14 h light, 10 h dark and 70% relative humidity for 4 weeks. *Nicotiana benthamiana*, tobacco, and tomato seeds and potato tubers were routinely cultured in the matrix in a climate chamber for about 5 to 6 weeks under the same conditions used for growing *Arabidopsis*. The *Phytophthora parasitica* strain and transformants were cultured on 5% (v/v) carrot juice agar (CA) medium with 0.01% (w/v) CaCO₃ and 0.002% (w/v) β-sitosterol for 4 days at 23°C. Then 5% CA plugs with fresh mycelia were cultured in carrot broth for 4 days. To produce sporangia, carrot broth was replaced with Petri solution [Ca(NO₃)₂, 0.4 g/L;

KH₂PO₄, 0.15 g/L; Mg(NO₃)₂, 0.15 g/L; CaCl₂, 0.06 g/L], and the culture was cultivated for another 5 days. Zoospores were released by chilling and recovering as described previously (Wang *et al.*, 2011b).

Total RNA extraction and RT-qPCR analyses

Total RNA of different samples was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed using the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China) according to the product manuals. RT-qPCR was performed using 5 µl of a 1:10 dilution of first-strand cDNA and SYBR Green mix (CWBio, Beijing, China) on a QuantStudio™ 3 Real-Time PCR System (Thermo Scientific, USA). Gene-specific primers were designed online

(<http://sg.idtdna.com/PrimerQuest/Home/Index>), and the specificity was examined by performing dissociation curve assays. The previously described internal controls were chosen as follows:

ubiquitin-conjugating enzyme (*PpUBC*) and 40S ribosomal protein S3A (*PpWS21*) genes for *P.*

parasitica (Yan & Liou, 2006); the *AtUBC9* gene for *A. thaliana*; and the β-actin gene for *N.*

benthamiana. For the biomass assay, primers specific to *PpUBC*, *AtUBC9* and *NbF-box* were used for quantitative PCR.

Vector construction

All the primers and vectors used in this study are listed in Table S1 (see Supporting Information). The gene fragments were amplified using PrimeStar polymerase (TaKaRa) and digested using appropriate restriction endonucleases (Promega, Madison, WI, USA) followed by ligation into vectors using T4 DNA ligase (Promega). The *PpE4* and *GFP* co-silencing hairpin vector pTH210::E4S was constructed

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in reference to a previous study (Meng *et al.*, 2015). First, the *SpeI*- and *ClaI*-digested *GFP* fragment and *ClaI*-digested kanamycin resistance gene linker were ligated into *SpeI*-linearized pBluescript II KS to generate the GFP-linker-GFP hairpin structure. Then the *BamHI*- and *SpeI*-digested *PpE4* fragment and the *SpeI*-released GFP-linker-GFP fragment were ligated into *BamHI*-linearized pBluescript II KS to generate the PpE4-GFP-linker-GFP-PpE4 co-silencing hairpin structure. Finally, the co-silencing hairpin structure, which was blunt-ended by *Pfu* DNA Polymerase (Promega) after being digested by *BamHI*, was inserted into *SmaI*-linearized plasmid pTH210 (Judelson *et al.*, 1991). To construct the overexpression vector, *E4FL* was inserted into pMCherryH (*Ham34* promoter) after being digested with *AgeI* and *NheI*, or fused with *mCherry* by overlapping PCR and then inserted into pTH210 (*Hsp70* promoter) after being digested with *ApaI* and *KpnI*. The signal peptide of PpE4 was predicted using the SignalP 4.1 online server (<http://www.cbs.dtu.dk/services/SignalP/>) (Nielsen, 2017). *E4FL*, mature *PpE4* without a signal peptide, and its deletion mutants were ligated into pCAMBIA1307-3×Flag and pER8 vector. For the VIGS assay, primers were designed in reference to previous studies: *EDS1* and *RAR1* (Liu *et al.*, 2002); *SGT1*, *HSP90*, and *NPK* (Jin *et al.*, 2002); *NDR1*, *MEK2*, *SIPK*, *MEK1*, *MYB1*, and *WRKY3* (Liu *et al.*, 2004b); *BAK1* (Yang *et al.*, 2017); and *SOBIR1* (Liebrand *et al.*, 2013). Fragments amplified from *N. benthamiana* cDNA were cloned into the binary vector pTRV2. All constructs were sequenced by Genscript China.

***Agrobacterium tumefaciens*-mediated transient expression**

A. *tumefaciens* GV3101 strains carrying the respective constructs were cultured in Luria-Bertani medium supplemented with the appropriate antibiotics at 28°C for one day, then harvested and suspended in infiltration buffer [10 mM 2-(N-morpholine)-ethane sulfonic acid (MES), 10 mM MgCl₂, pH 5.6, and 200 μM acetosyringone] to an appropriate concentration. For inoculation of *P. parasitica* after transient expression, infiltrations were performed at a final optical density (OD₆₀₀) of 0.01, otherwise an OD₆₀₀ value of 0.4 was used. After incubation for 1 hour at 28°C, the *A. tumefaciens* suspensions were infiltrated into plant leaves using needleless syringes (Meng *et al.*, 2015). Cell death was observed at 3-5 dpi in *N. benthamiana* and tobacco species and 5-8 dpi in *S. lycopersicum* and *S. tuberosum*. For western blot analysis, proteins were extracted at 2 dpi. All experiments were repeated at least three times.

B.

Virus-induced gene silencing assay in *N. benthamiana*

A. *tumefaciens* GV3101 strains carrying different pTRV2 constructs were mixed with pTRV1 in equal ratios to a final OD₆₀₀ of 0.25. pTRV2::*GFP* was used as a control, and pTRV2::*PDS* was used to visualize the silencing process. The lower leaves of 4-leaf stage *N. benthamiana* plants were infiltrated as previously described (Liu *et al.*, 2002; Ratcliff *et al.*, 2001), then the degree of cell death and gene silencing efficiency were analyzed in the upper leaves at 16-20 dpi.

Transformation of *Arabidopsis thaliana*

- A. *tumefaciens* carrying the empty vector pER8 or pER8::3×*Flag-PpE4* were cultured and suspended in a solution of 5% sucrose and 0.02% Silwet L-77 (GE Healthcare, Sweden). *Arabidopsis* ecotype Col-0 was transformed by dipping in the suspension as previously described (Clough & Bent, 1998). The kanamycin-resistant seedlings were screened on selective medium and planted in soil. Then the expression level of *PpE4* in transgenic plants after induction by 17-β-estradiol was determined by semi-quantitative PCR (Zuo *et al.*, 2000).

P. parasitica transformation

To generate silencing and overexpressing transformants, *P. parasitica* protoplasts were transformed using the PEG-CaCl₂-mediated method as described previously (Bottin *et al.*, 1999; Meng *et al.*, 2015). The silencing and overexpression plasmids (pTH210::E4S, pMCherryH::E4FL and pTH210::E4FL-mCherry) were linearized by *Bam*HI and separately co-transformed with linearized pTH209 into protoplasts of strain 1121, which stably expresses hyphal cytoplasmic GFP. The transformed protoplasts were regenerated overnight, then the recovered mycelia were selected on 5% CA medium with 4 μg/mL geneticin and 100 μg/mL hygromycin. After 3-7 days, the primary transformants were transferred to new selective medium in 6-well plates and named sequentially and maintained for subsequent analyses.

Fluorescence microscopy

To identify *PpE4* and *GFP* co-silencing transformants, transformants with attenuated GFP signal were identified using an Olympus BX-51TRF fluorescence microscope (Olympus, Tokyo, Japan) with the GFP filter (BP450-480). For *E4FL-mCherry*-expressing lines, the putative transformants were observed under the mCherry filter (BP520-550). Images of vegetative mycelia and infection hyphae in *N. benthamiana* leaves were captured on an Olympus IX83-FV1200 confocal microscope with 488 nm excitation and a 500-530 nm emission spectrum for GFP. For mCherry, the emission spectrum was acquired between 595-625 nm under 559 nm excitation to eliminate potential autofluorescence from *P. parasitica* hyphae and cell damage. The detached *N. benthamiana* leaves inoculated with the transformants and the control strain were incubated at 23°C for 12-48 h to allow penetration and formation of intercellular hyphae with haustoria. The control strain and transformants were observed under the same conditions.

Inoculation of *P. parasitica*

N. benthamiana leaves were detached 24 h after agroinfiltration and kept in a plastic tray covered with moist filter paper. The petioles were wrapped with wet cotton, then the leaves were inoculated with 1000 zoospores of *P. parasitica* strain 1121 and incubated in a growth chamber at 23°C. For pathogenicity assays of *P. parasitica* transformants, fresh mycelia of transformants and the control strain grown on 5% CA agar plugs were inoculated on each side of detached *N. benthamiana* leaves. More than 15 leaves were used in each assay. At 36-48 hpi the hyphae expansion was marked under fluorescence microscope to measure lesion diameter. Total DNA was extracted from identical areas on

each side of the leaf, and the biomass was calculated by the DNA ratio of *P. parasitica* in infected tissues using quantitative PCR (Meng *et al.*, 2015). The rosette leaves of wild-type *Arabidopsis* Col-0 and T3 homozygous pER8 and pER8::3×*Flag-PpE4* transgenic plants were injected with 10 μM 17-β-estradiol (Zuo *et al.*, 2000). After 12 h, the treated leaves were detached and placed in a plastic tray with wet cotton covering the petioles. Then 2000 zoospores were dropped on the abaxial surface of each leaf. About 25 leaves from more than 15 plants of each line were analyzed for each assay. Disease severity index (DSI) was recorded at 48 hpi, with grade 1 being no visible symptom and few hyphae colonized on the leaf surface, grade 2 being developed restricted water-soaked lesions with diameter less than 2 mm, grade 3 being developed water-soaked lesions smaller than the inoculation sites with abundant hyphae colonized, and grade 4 being developed large lesions with massive hyphae spreading beyond the inoculation sites. The expansion of *P. parasitica* hyphae was visualized by trypan blue staining and *P. parasitica* biomass was determined in equal amounts of inoculated leaves by quantitative PCR.

Western blot analysis

Mycelia or plant leaves were ground into powder in liquid nitrogen and vigorously mixed with a double volume of precooled RIPA lysis buffer [50 mM Tris (pH7.4), 150 mM NaCl, 1% TritonX-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM EDTA, 10 mM DTT, 1% (w/v) protease inhibitor cocktail (Sigma, St. Louis, MO, USA)]. After 20 minutes of incubation on ice, the sample was centrifuged at 20,000 g for 15 min to obtain the supernatant. After adding loading buffer and boiling for 5 min, total proteins were separated by SDS-PAGE. Then the

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proteins were transferred to PVDF membranes (Roche) followed by blocking in 10% skim milk (BD, Sparks, MD, USA) dissolved in Tris buffered saline (TBS; pH 7.2). Mouse anti-Flag monoclonal antibody (Abbkine, Redlands, CA, USA) and mouse anti-mCherry monoclonal antibody (Abbkine) were used at a 1: 2,000 dilutions to detect the corresponding fusion proteins. The membranes were washed and incubated with a goat anti-mouse antibody (Abbkine). Protein bands were visualized by chemiluminescence using the eECL Western blot kit (CWBio), and photos were taken under the ChemiDOC™ XRS+ imaging system (Bio Rad, USA).

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SUPPORTING INFORMATION

Table S1 Primers and vectors used in this study.

Fig. S1 Expression pattern of *PpE4* during *P. parasitica* infection of *Arabidopsis*. (A) FPKM (Fragments Per Kilobase Million) value of *PpE4* and other RXLR effector genes from RNA-seq data. The sum of FPKM values of all the 76 RXLR effector genes detected (FPKM value larger than 1) during infection of *Arabidopsis* roots were calculated. (B) Relative *PpE4* transcript levels during different stages of *P. parasitica* infection quantitated by RT-qPCR. *Arabidopsis* roots inoculated with *P. parasitica* zoospores were harvested at different hpi. My, *P. parasitica* mycelia grown in carrot broth. The relative expression level of *PpE4* in mycelia was given a value of 1. Error bars represent standard deviation (SD) of three pooled materials.

Fig. S2 The infection process of *P. parasitica* on *N. benthamiana*. Biotrophic growth was dominant before 24 hpi, followed by rapidly switch into necrotrophic growth with large-scale cell death. *N. benthamiana* leaves infected with zoospores of strain 1121 (stably expresses hyphal cytoplasmic GFP) were observed under fluorescence microscope at 3, 6, 12, 24 and 48 hpi. The green fluorescence represents infection hyphae, the red fluorescence is the chloroplast autofluorescence of healthy leaf cells, which turns black when cell death occurs on the leaves. At 3 hpi, the cysts germinated and colonized on the epidermal cells, and extensive hyphae formed at 6 hpi. Cell death occurred at the inoculation sites at 12 hpi. Along with the spreading of abundant hyphae, cell death occurred at the

whole inoculation sites at 24 hpi. At 48 hpi, cell death occurred in large areas, with sporangia developed at the inoculation sites. CD, cell death; S, sporangia. Bars, 100 μ m.

Fig. S3 Generation of *P. parasitica* transformants expressing the E4FL-mCherry fusion protein. (A) Schematic diagram of the fusion protein constructs in vector pTH210 or pMCherryH. Expression of E4FL (full-length PpE4 with its own signal peptide) fused with mCherry at its C-terminus was driven by the constitutive *Ham34* or *Hsp70* promoter. Relative expression level of *PpE4* in vegetative mycelia (B) and in infected *N. benthamiana* leaves at 36 hpi (C) quantitated by RT-qPCR. Expression of E4FL-mCherry in E4MC4A2 is driven by the *Ham34* promoter, while in other transformants it is driven by the *Hsp70* promoter. The expression level of *PpE4* in strain 1121 was given a value of 1. Error bars represent SD of three biological replicates. (D) Accumulation of E4FL-mCherry fusion proteins in vegetative mycelia was confirmed by western blot using mCherry antibody. Protein loading is indicated by Ponceau stain (PS). Similar results were obtained from three independent experiments.

Fig. S4 The localization of PpE4-mCherry in transformant E4MC4A6 during infection. (A) Confocal image showing the accumulation of PpE4-mCherry outside the haustoria after secretion at 24 hpi. (B) The fluorescence intensities of GFP and mCherry across the haustoria were indicated by the white lines labeled “1” and “2” in (A). Identical images were obtained from more than ten haustoria in three independent biological replicates.

Fig. S5 Attenuated pathogenicity of *E4FL-mCherry*-expressing *P. parasitica* transformants. The fresh mycelial plugs of transformants (E4MC4A2, E4MC4A6, E4MC4B2 and E4MC3N4) and control strain 1121 were inoculated on the left and right sides of *N. benthamiana* leaves, respectively, and the lesion diameters were measured at 48 hpi. (A) Lesions caused by *E4FL-mCherry* expressing transformants

were significantly smaller than those caused by the 1121 strain and E4MC3N4. Error bars represent SD of 15 leaves. Asterisks denote significant differences from the control strain 1121 (two tailed *t*-test; ** $p < 0.01$, *** $p < 0.001$). (B) Representative inoculated leaves. Similar results were obtained from more than three independent experiments.

Fig. S6 Generation of *P. parasitica* *PpE4*-silencing transformants. (A) Diagram of the *PpE4* and *GFP* co-silencing hairpin structure construct. The kanamycin-resistant gene (*kanR*) was used as the linker sequence. (B) GFP signals in mycelia of five *PpE4*-silenced transformants and strain 1121. (C) Relative expression level of *PpE4* in five GFP signal-decreased transformants sampled at 24 hpi on *N. benthamiana* leaves was quantitated by RT-qPCR. Expression level of *PpE4* in strain 1121 was given a value of 1. Error bars represent SD of three biological replicates. Two independent experiments were performed with similar results.

Fig. S7 Secondary structure of the *PpE4* protein predicted by Phyre2 (<http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>).

Fig. S8 *PpE4*-triggered cell death was not compromised in *N. benthamiana* plants with silenced expression of several genes involved in plant immune signaling. *N. benthamiana* leaves were infiltrated with pTRV2 constructs targeting *EDS1*, *NDR1*, *MEK1*, *MEK2*, *SIPK*, *MYB1*, *WRKY3*, *EDS1*, *NDR1*, *BAK1*, or *SOBIR1*; pTRV2::*GFP* was used as a control. *A. tumefaciens* carrying *PpE4* was infiltrated into the upper leaves of silenced plants at 16-20 days post infiltration (dpi). (A) Cell death photographed at 5 dpi. (B) Quantification of cell death on *N. benthamiana* leaves. Cell death degree was divided into three levels: no visible cell death, weak cell death and complete cell death. The asterisk represents a significant difference from the control (Wilcoxon rank-sum test; * $p < 0.05$). (C) Relative expression

levels of silenced genes in corresponding VIGS-treated plants determined by RT-qPCR. Error bars represent SD of three biological replicates. The experiments were repeated three times with more than ten plants for each TRV construct.

FIGURE LEGENDS

Fig. 1 The *Phytophthora parasitica* RXLR effector gene *PpE4* is highly expressed during early plant infection. Reverse transcription-quantitative PCR (RT-qPCR) was used to quantitate relative *PpE4* transcript levels during different stages of *P. parasitica* development and infection. *Nicotiana benthamiana* leaves inoculated with *P. parasitica* zoospores were harvested at different hours post inoculation (hpi). My, *P. parasitica* mycelium grown in carrot broth; Zo, zoospores; Cy, cysts; GC, germinated cysts. The relative expression level of *PpE4* in mycelia was given a value of 1. Error bars represent standard deviation (SD) of a three biological replicates.

Fig. 2 PpE4 accumulates around haustoria after secretion during *P. parasitica* infection. (A) Confocal images of mycelia cultured on 5% carrot juice agar medium. The red fluorescence distributed throughout the mycelia cytoplasm of E4MC4A2 (a transformant expressing cytoplasmic GFP and full-length PpE4 (E4FL)-mCherry), while not detected in the strain 1121 (stably expressing cytoplasmic GFP). (B) *N. benthamiana* leaves infected with E4MC4A2 and 1121 were observed under confocal microscopy at 24 hpi. The strong red fluorescence signal highly accumulated in haustoria but not in hyphae during E4MC4A2 infection, while GFP fluorescence was evenly distributed in hyphae. No red fluorescence was observed in strain 1121. (C) A magnified lateral view of haustoria showing red fluorescence focused on the outside of the haustoria base and GFP signal distributed throughout hyphae

and haustoria. (D) The fluorescence intensities of GFP and mCherry across the haustorium indicated by the white line labeled “2” in (C). Identical images were obtained from more than ten haustoria in three independent biological replicates.

Fig. 3 *PpE4*-silenced *P. parasitica* transformants exhibit reduced pathogenicity. (A) Mean lesion areas of *N. benthamiana* leaves inoculated with *PpE4*-silenced transformants and the control strain 1121 at 48 hpi. A transformant and the control strain were inoculated on opposite halves of an *N. benthamiana* leaf. Error bars represent SD of 15 leaves, and asterisks denote significant differences from control strain 1121 (two tailed *t*-test; **p* < 0.05; ***p* < 0.01; ****p* < 0.001). (B) Biomass of *P. parasitica* on *N. benthamiana* leaves determined by quantitative PCR. Bars represent *PpUBC* levels relative to *NbF-box* levels with SD of three biological replicates. Asterisks denote significant differences from control strain 1121 (two tailed *t*-test; ***p* < 0.01). (C) Representative inoculated leaves. White circles outline the water-soaked lesions. Similar results were obtained from more than three independent experiments with about 15 leaves for each experiment. (D) *PpE4* expression was restored in two transformants E4S2C4 and E4S2G5, whose virulence was not reduced. The subcultured transformants were inoculated on *N. benthamiana* leaves and sampled at 15 hpi and 24 hpi. RT-qPCR was used to determine the *PpE4* silencing level. Expression level of *PpE4* in strain 1121 sampled at 15 hpi was given a value of 1. Error bars represent SD of three biological replicates. Two independent experiments were performed with similar results.

Fig. 4 The pathogenicity of *PpE4*-silenced *P. parasitica* lines is restored by transient expression of *PpE4* in plant. *PpE4* and *GFP* were transiently expressed by agroinfiltration in *N. benthamiana* leaves one day before inoculation (OD₆₀₀, 0.01). (A) Lesions formed after the inoculation of *PpE4*-silenced lines on *PpE4*-expressing leaves were almost the same size as those formed after the inoculation of

1121 on *GFP*-expressing leaves, while lesions formed by silenced lines inoculated on *GFP*-expressing leaves were significantly smaller. Error bars represent SD of 15 leaves, and asterisks denote significant differences from control group (two tailed *t*-test; **p* < 0.05; ****p* < 0.001). (B) Biomass of *P. parasitica* on *N. benthamiana* leaves was determined by quantitative PCR. Bars represent *PpUBC* levels relative to *NbF-box* levels with SD of three biological replicates. Asterisks denote significant differences from silenced lines inoculated on *GFP*-expressing leaves (two tailed *t*-test; ****p* < 0.001). (C) Representative inoculated leaves. White circles outline the water-soaked lesions. (D) Protein accumulation detected by western blotting using anti-Flag antibodies. Protein loading is indicated by Ponceau stain (PS). Similar results were obtained from three independent experiments with more than 15 leaves inoculated for each group in each experiment.

Fig. 5 Heterologous expression of *PpE4* renders *N. benthamiana* and *Arabidopsis* more susceptible to *P. parasitica* infection. (A) Mean lesion areas were measured at 48 hpi. *Agrobacterium tumefaciens* strains carrying *PpE4* or *GFP* (OD₆₀₀, 0.01) were infiltrated into different sides of the same leaf one day before inoculation of strain 1121. Error bars represent SD of 15 leaves, and asterisks denote significant differences from GFP control (two tailed *t*-test; ****p* < 0.001). (B) Quantitation of *P. parasitica* biomass in infected *N. benthamiana* leaves. Bars represent *PpUBC* levels relative to *NbF-box* levels with SD of three biological replicates. Asterisks denote significant differences from the GFP control (two tailed *t*-test; ****p* < 0.001). (C) A typical leaf photographed and stained by trypan blue. White circles outline the water-soaked lesions. (D) Protein accumulation was determined at 3 dpi by western blot using anti-Flag antibody. Protein loading is indicated by PS. Similar results were obtained from three independent experiments with about 15 leaves for each experiment. (E) Disease severity index (DSI) from grade 1 to 4 was recorded at 48 hpi. Homozygous transgenic plants expressing

β -estradiol-inducible 3×*Flag-PpE4* (E4-2, E4-3, and E4-15), an empty vector pER8 transgenic plant (ER8-8), and wild-type Col-0 were injected with 10 μ M 17- β -estradiol 12 h before inoculation of strain 1121. Asterisks represent significant differences from Col-0 (Wilcoxon rank-sum test; ****p* < 0.001). (F) Biomass of *P. parasitica* on *Arabidopsis* leaves. Bars represent *PpUBC* levels relative to *AtUBC* levels with SD of five biological replicates. Asterisks denote significant differences from Col-0 (two tailed *t*-test; **p* < 0.05; ***p* < 0.01; ****p* < 0.001). (G) Disease symptoms of representative leaves. Trypan blue stain was used to highlight the infection hyphae in colonized leaves. (H) Verification of *PpE4* expression 12 h after injection of 10 μ M 17- β -estradiol using semi-quantitative PCR. Similar results were obtained from three independent experiments with about 25 leaves for each experiment.

Fig. 6 Analysis of cell death triggered by *PpE4*. (A) Cell death phenotype induced by *PpE4* in Solanaceae plants. *A. tumefaciens* carrying *PpE4* (OD₆₀₀, 0.4) was infiltrated into the leaves of *N. benthamiana*, *N. nesophila*, *N. glutinosa*, *N. tabacum* cv. Florida 301, *Solanum lycopersicum*, and *S. tuberosum*. Photographs were taken at 5 days post infiltration (dpi) for the *Nicotiana* species and 8 dpi for the *Solanum* species. Red circles represent the *PpE4*-expressing areas, and white dotted circles represent the *GFP*-expressing areas. (B) Cell death symptoms triggered by *PpE4* in *Arabidopsis*. Leaves of transgenic *Arabidopsis* plants harboring pER8::3×*Flag-PpE4* or the empty vector and Col-0 were injected with 10 μ M 17- β -estradiol. Photographs were taken after 5 days. (C) Schematic diagrams of protein secondary structure of *PpE4* deletion mutants. (D) Cell death symptoms in *N. benthamiana* leaves expressing *PpE4* deletion mutants. Photographs were taken at 5 dpi. (E) Western blot detection of *PpE4* deletion proteins using anti-Flag antibody. The red asterisk indicates protein band of correct size. Protein loading is indicated by PS. Similar results were obtained from three independent experiments.

Fig. 7 *HSP90*, *NPK* and *SGT1* are involved in *PpE4*-induced cell death. (A) Representative images of *PpE4*-induced cell death on silenced *N. benthamiana* leaves at 5 dpi. *PpE4* was transiently expressed in the upper leaves of silenced plants at 16-20 dpi of TRV constructs. (B) Quantification of cell death on *N. benthamiana* leaves scored at 5 dpi. Cell death degree was divided into three levels: no cell death, weak cell death and complete cell death. Asterisks indicate significant differences from *GFP*-silenced plants (Wilcoxon rank-sum test; *** $p < 0.001$). (C) Relative expression levels of *HSP90*, *NPK* and *SGT1* transcripts in corresponding VIGS-treated plants determined by RT-qPCR. Error bars represent SD of three biological replicates. (D) Detection of *PpE4* protein accumulation in silenced leaves using the anti-Flag antibody. Protein loading is indicated by PS. Similar results were obtained from more than three independent experiments with ten plants for each TRV construct.













