Received: 21 April 2020 | Revised: 25 May 2020 | Accepted: 1 June 2020

DOI: 10.1111/mpp.12967

ORIGINAL ARTICLE

Molecular Plant Pathology | WILEY

Functional analysis of RXLR effectors from the New Zealand kauri dieback pathogen *Phytophthora agathidicida*

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Funding information
Bio-Protection Research Centre, Grant/Award Number: 3/MU 19008

Abstract

New Zealand kauri is an ancient, iconic, gymnosperm tree species that is under threat from a lethal dieback disease caused by the oomycete *Phytophthora agathidicida*. To gain insight into this pathogen, we determined whether proteinaceous effectors of *P. agathidicida* interact with the immune system of a model angiosperm, *Nicotiana*, as previously shown for *Phytophthora* pathogens of angiosperms. From the *P. agathidicida* genome, we defined and analysed a set of RXLR effectors, a class of proteins that typically have important roles in suppressing or activating the plant immune system. RXLRs were screened for their ability to activate or suppress the *Nicotiana* plant immune system using *Agrobacterium tumefaciens* transient transformation assays. Nine *P. agathidicida* RXLRs triggered cell death or suppressed plant immunity in *Nicotiana*, of which three were expressed in kauri. For the most highly expressed, *P. agathidicida* (Pa) RXLR24, candidate cognate immune receptors associated with cell death were identified in *Nicotiana benthamiana* using RNA silencing-based approaches. Our results show that RXLRs of a pathogen of gymnosperms can interact with the immune system of an angiosperm species. This study provides an important foundation for studying the molecular basis of plant–pathogen interactions in gymnosperm forest trees, including kauri.

KEYWORDS

effectors, forest pathogen, kauri dieback, NBS-LRR immune receptors, *Phytophthora agathidicida*, RXLR

1 | INTRODUCTION

New Zealand kauri (*Agathis australis*) is an ancient species in the Araucariaceae conifer family and is under threat from kauri dieback disease (Waipara *et al*., 2013; Bradshaw *et al*., 2020). New Zealand kauri forests were decimated by European settlers in the 19th century (Beever *et al*., 2009), and the few forests that remain have protected status, in keeping with their cultural and ecological importance (Wardle, 1991; Ogden, 1995; Lambert *et al*., 2018). Kauri dieback disease was first noticed in 2006 (Beever *et al*., 2009), and

now occurs throughout the geographic range of New Zealand kauri forests (Waipara *et al*., 2013; Bradshaw *et al*., 2020).

Protection of forests from pests and diseases is of paramount importance for many reasons, not least of which is their potential for mitigating climate change (Bastin *et al*., 2019). Over the last few decades, forest trees in both natural and planted forests have come under increasing threat from invasive pest and disease epidemics. A growing number of these are caused by oomycetes in the genus *Phytophthora*, such as diebacks of jarrah and alder (Hansen, 2015) and of bunya and hoop pines in the same Araucariaceae family as kauri (Shuey *et al*., 2019). Recent epidemics have been influenced by factors such as changes in climate (Woods *et al*., 2016), human-mediated movement of pathogens (Goss *et al*., 2011; Wingfield *et al*., 2015), and rapid evolution or hybridization of the pathogen (Brasier, 2000; Callaghan & Guest, 2015).

Kauri dieback is caused by a highly destructive soilborne species, *Phytophthora agathidicida* (Weir *et al*., 2015), which kills fine roots, causes collar rot, and blocks vascular tissues, ultimately killing the tree (Beever *et al*., 2009). Due to the recent emergence of kauri dieback disease, comparatively little is known about the *P. agathidicida*–kauri pathosystem and the origin of *P. agathidicida*. So far, disease management has focused on attempts to prevent its spread, chemical control with phosphite injections, and screening for resistance in the kauri population (Bradshaw *et al*., 2020). Identification of disease resistance will play an important role in the mitigation of kauri dieback disease in the long term.

Plants can resist pathogens through the recognition of pathogen virulence factors termed effectors (Cook *et al*., 2015; van der Burgh & Joosten, 2019). By understanding how effectors and their host targets interact at the molecular level, questions about key drivers of pathogen success and failure can be addressed (Ntoukakis & Gifford, 2019). Often, one of the main outputs of pathogen resistance is a localized cell death response, the hypersensitive response (HR), which occurs on recognition of specific effectors by corresponding plant immune receptors. This visual output can be used to identify effector–immune receptor interactions, as well as plant material resistant to pathogens (Rietman *et al*., 2012; Dangl *et al*., 2013; Vleeshouwers & Oliver, 2014; Van de Wouw & Idnurm, 2019).

Like other pathogens, *Phytophthora* species produce effector proteins. The main class of intracellular effectors is the RXLRs (Judelson, 2012), which target a variety of host molecules to manipulate host immunity (Wang & Jiao, 2019). Because plants have evolved to recognize pathogen effector molecules as triggers for defence, pathogens are under strong selection pressure to evade recognition and can achieve this by loss, mutation, or silencing of effectors (Qutob *et al*., 2013; Anderson *et al*., 2015; Pais *et al*., 2018; Wang *et al*., 2019). In forest health situations such as the kauri–*P. agathidicida* system, the long lifespan of the host means the pathogen has a considerable time advantage in terms of adaptability in this "arms race" with its host, although phenotypic plasticity due to processes such as epigenetic variation and somatic mutation (Bräutigam *et al*., 2013, Simberloff & Leppanen, 2019) might enable adaptability in long-lived trees and these processes deserve further investigation.

Compared to studies of plant–pathogen interactions with angiosperm crop pathogens, little is known about how pathogens of gymnosperms interact with their hosts at a molecular level (Bradshaw *et al*., 2016; Stewart *et al*., 2018). The genetic basis of disease resistance has been established for some pine diseases (Sniezko *et al*., 2014) and some species show major gene resistance (Kinloch *et al*., 2008; Sniezko *et al*., 2014). Effector candidates have been identified in fungal pathogens of gymnosperm trees that are similar in structure and function to those of angiosperm pathogens (de Wit *et al*., 2012; Raffaello & Asiegbu, 2017; Ma *et al*., 2019) and deserve further exploration in the context of forest health. Studies of effectors in forest pathogens will help to predict their adaptive potential and the dynamics of pathogen–tree coevolution in forests, and serve as tools for detection of immune receptors that could accelerate tree improvement (Keriö *et al*., 2019).

We tested the hypothesis that RXLR effectors from an oomycete that is pathogenic to a gymnosperm interact with the immune system of model angiosperm plants in a similar way to that of angiosperm pathogens. We defined a set of RXLR effectors in *P. agathidicida* and performed functional analyses to assess their roles in planta. A model-plant system was chosen due to the cultural significance and technical limitations associated with using kauri. For one of the RXLR genes that was highly up-regulated in kauri, the model-plant system was screened for candidate cognate immune receptors. To the best of our knowledge, this work is the first of its kind for any forest gymnosperm–oomycete pathosystem and provides a foundation for studies of the molecular basis of plant–pathogen interactions in forest trees, including kauri.

2 | RESULTS

2.1 | Prediction of a set of RXLR effector gene candidates in *P. agathidicida*

RXLR effectors from *Phytophthora* species can have important roles in suppressing or activating the plant immune system (Anderson *et al*., 2015). With this in mind, we used three prediction methods to identify a well-supported set of 78 RXLR effector candidates (Figure S1) from the genome of *P. agathidicida* NZFS3770, an isolate collected from Great Barrier Island, New Zealand in 2006 (Studholme *et al*., 2016). BLAST searches suggested that, of the 78 PaRXLRs, only four (PaRXLR21, PaRXLR35, PaRXLR57, and PaRXLR59) are unique to *P. agathidicida*.

The amino acid sequences of the 78 predicted RXLR effector candidates were analysed for conserved sequence motifs. Using the motif alignment search tool (MAST), 16 significantly over-represented motifs were found. In addition to the signal peptide and RXLR motif that were used, in part, for the selection of the 78 candidates, these included W, Y, and L motifs often found in RXLR effectors (Jiang *et al*., 2008), as well as sequences conserved between related RXLR effector candidates of *P. agathidicida* (Figures 1 and S2). These results suggest there are common

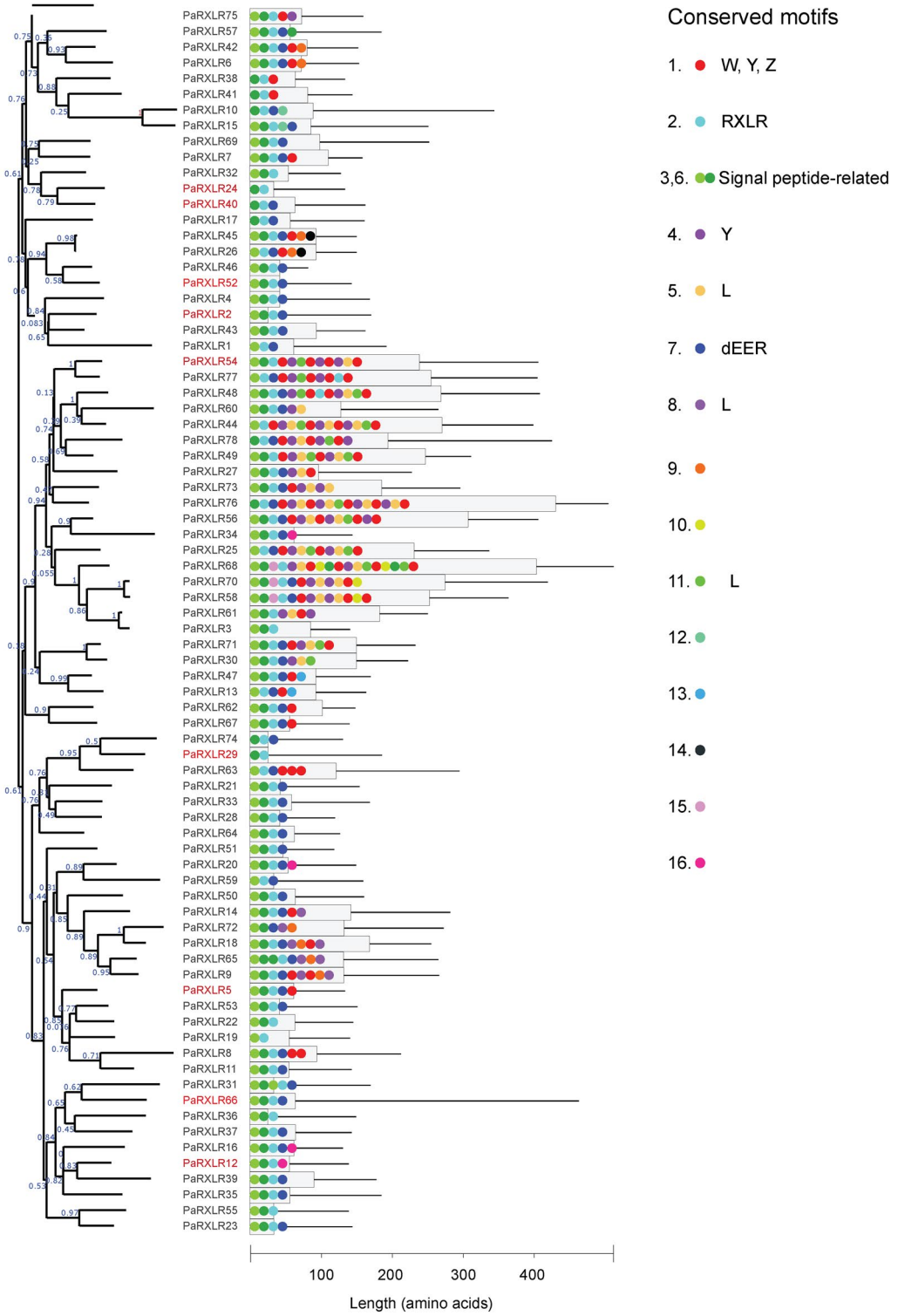

FIGURE 1 Phylogeny and domain structure of the *Phytophthora agathidicida* RXLR effector candidates. The dendrogram represents a maximum-likelihood phylogenetic tree. Numbers on the branches are approximate likelihood ratio test (aLRT) values as reported by PhyML. PaRXLRs with names in red are those that either elicited or suppressed cell death in functional assays. The histogram shows lengths of predicted proteins (thin black lines), according to the scale below, and the proportion of proteins involved in amino acid motifs that were significantly over-represented among the 78 RXLRs as predicted by MEME (grey boxes). Coloured dots represent the motifs, with the order respected but not drawn to scale. The key indicates conserved motifs, with putative functions or similarities to common RXLR motifs indicated where appropriate; the numbers correspond to those detailed in Figure S2

features in the RXLRs of *P. agathidicida* compared to those of other *Phytophthora* species.

2.2 | *P. agathidicida* isolates and RXLR effector gene candidates show low genetic diversity

We tested the hypothesis that selection for diversification of *P. agathidicida* RXLR sequences has occurred in kauri forests by examining sequence variation in isolates from throughout the kauri dieback region in the northern part of New Zealand (Table 1 and Figure 2). The numbers of single nucleotide polymorphisms (SNPs) per genome amongst 12 *P. agathidicida* isolates, relative to the 37.2 Mb genome reference strain NZFS3770 (Studholme *et al*., 2016), ranged from 29,701 to 43,737 (Table 1); this equated to sequence differences of only 0.08%–0.12% between the isolates, suggesting low genetic diversity in the population.

Amongst the 78 RXLR effector gene candidates studied in this project, only 10 had SNPs in their corresponding coding sequence, showing they are mostly identical amongst the isolates studied (Table 1). This low SNP rate was similar to that of the rest of genome; there was no significant enrichment in the proportion of RXLR effector gene candidates with SNPs compared to that of all other genes for any of the isolates studied (Tables 1, S3, and S5). Thus, we found no evidence for selection for RXLR sequence diversification among these samples.

2.3 | *P. agathidicida* RXLR effector candidates induce cell death in *Nicotiana* spp.

To gain insight into how *P. agathidicida* RXLRs interact with the plant immune system, the RXLRs were screened for the ability to trigger cell death in model angiosperm *Nicotiana* spp. Cell death is often indicative of immune system activation on recognition of an effector by a corresponding plant immune receptor, and is termed the HR (Wang *et al*., 2011). Using an *Agrobacterium tumefaciens*-mediated transient transformation assay (ATTA), eight of the *P. agathidicida* (Pa) RXLR effector candidates consistently triggered cell death in *Nicotiana tabacum*, and two of these also triggered cell death in *N. benthamiana* when tested in at least three independent experiments (Figures 3 and S3). All eight had BLASTP hits to other *Phytophthora* RXLRs, with some orthologous to functionally characterized RXLR effectors in other species based on reciprocal best BLASTP hits. Most notable among these was PaRXLR24, which is orthologous to *P. sojae* Avh238 and *P. parasitica* pPE4 (Table 2), highlighting the potential importance of these RXLRs for pathogens of both gymnosperms and angiosperms.

2.4 | PaRXLR40 can suppress RXLR-triggered immunity

Having identified RXLRs that induce cell death, we then screened for those with potential virulence functions. Some *Phytophthora* RXLR

TABLE 1 *Phytophthora agathidicida* isolates with sequenced genomes

ID	NZFS[a]	Year[b]	Location[b]	Sequence accession numbers[c]	Mbases reads	SNPs[d] per genome	Genes with SNPs	RXLRs with SNPs[e]
A	3770	2006	Great Barrier Island	SRX1116283	4,619			
B	3772	2013	Waitakeres, Huia	SRX1116282	4,057			
1	3118	2009	Waitakeres, Huia	SRX4575879	4,765	41,720	1,433	8
2	3126	2006	Maungaroa beach	SRX4575884	4,797	41,709	1,418	6
3	3128	2009	Waitakeres, Huia	SRX4575881	7,030	43,737	1,436	7
4	3616	2001	Great Barrier Island	SRX4575880	4,614	29,701	869	2
5	3687	2011	Waipoua Forest	SRX4575875	4,768	43,493	1,476	6
6	3815	2014	Coromandel	SRX4575874	4,558	42,358	1,688	7
7	3869	2014	Arapahoe	SRX4575877	4,667	42,634	1,662	7
8	3885	2014	Whenuanui, Ruawai	SRX4575876	4,799	43,146	1,673	6
9	4288	1972	Great Barrier Island	SRX4575883	3,828	41,131	950	3
10	4289	2010	Raetea	SRX4575882	4,798	42,749	1,487	6
11	4290	2010	Waipoua Forest	SRX4575885	5,122	42,789	1,483	7
12	4291	2014	Coromandel	SRX4575878	4,879	42,976	1,691	7

[a]NZFS (New Zealand Forest Service collection) number of the *P. agathidicida* isolate.

[b]Year and location (in New Zealand) of isolate collection.

[c]Accession numbers for GenBank sequence read archive Bioprojects. 3770 and 3772 are those from Studholme *et al* (2016).

[d]Total number of single nucleotide polymorphism (SNP) sites in the resequenced genomes compared to that of NZFS3770.

[e]All isolates had all 78 RXLR genes; the numbers indicate how many of those 78 had SNPs. Chi-square analysis to compare the proportions of these RXLR genes and other genes with SNPs showed no significant difference for any of the resequenced strains ($p > .6$).

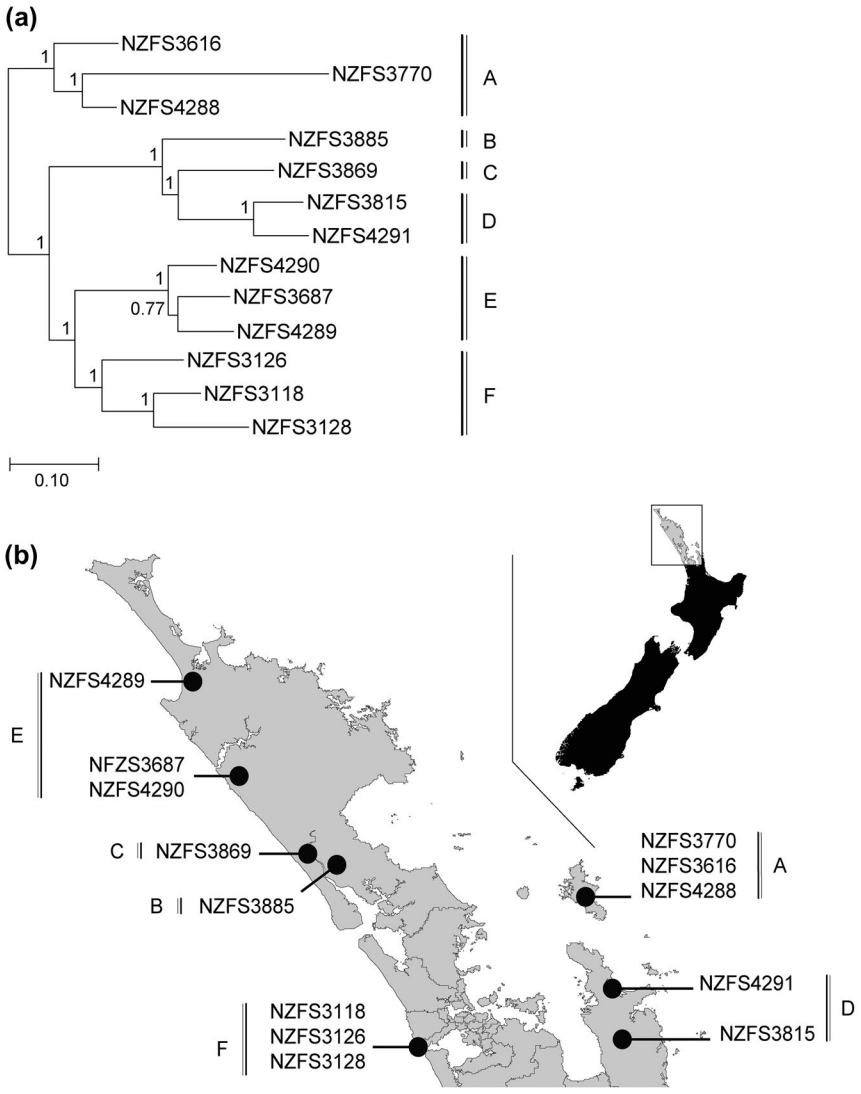

FIGURE 2 Phylogeny and location of all *Phytophthora agathidicida* samples. The phylogeny was computed using maximum likelihood on concatenated variable single nucleotide polymorphism (SNP) loci from all 12 resequenced *P. agathidicida* genomes, determined by comparison to the reference NZFS3770 genome. Numbers on the branches are approximate likelihood ratio test (aLRT). Main groupings in the phylogeny, indicated by letters, are reported on the map, which represents the top of the North Island of New Zealand.

effectors suppress plant immunity to facilitate pathogen infection (Deb *et al*., 2018; Dalio *et al*., 2018). Thus, we investigated whether *P. agathidicida* RXLR effector candidates can suppress immunity triggered by an elicitin protein, INF1-1, or by effector proteins. The *P. infestans* elicitin protein INF1 triggers an HR in *N. benthamiana* (Kamoun *et al*., 1998), and this response can be suppressed by the *P. infestans* RXLR effector Avr3a (Bos *et al*., 2006). We identified three paralogs of INF1 in the *P. agathidicida* genome (*PaINF1-1*, *PaINF1-2*, and *PaINF1-3*; Figure S4a). As with *P. infestans* INF1, infiltration of *P. agathidicida* PaINF1-1 into *N. benthamiana* also induced cell death that could be suppressed by *P. infestans* Avr3a (Figure S4b), so PaINF1-1 and Avr3A were used as elicitor and suppressor controls, respectively. In the suppression assays, PaRXLR effectors were infiltrated into *N. benthamiana* leaves 24 hr before infiltration of the PaINF1-1 elicitor, but none of the PaRXLR candidate effectors tested could suppress PaINF1-1-triggered cell death (Figure S4b).

We next investigated whether any of the PaRXLRs could suppress effector-triggered cell death immunity elicited by the *P. infestans* RXLR Avr3a (Engelhardt *et al*., 2012) in the presence of its cognate potato immune receptor protein R3a (Armstrong *et al*., 2005). Out of the PaRXLR effector candidates tested, only PaRXLR40 consistently suppressed Avr3a/R3a-triggered cell death in three independent experiments (Figure 4a).

Given that PaRXLR40 suppressed cell death triggered by Avr3a/R3a, we tested whether it could also suppress cell death triggered by an effector from *P. agathidicida*, PaRXLR24. Coinfiltration of PaRXLR40 24 hr after PaRXLR24 suppressed cell death in *N. benthamiana* leaves, suggesting that PaRXLR40 can suppress PaRXLR24-induced immunity (Figure 4b).

To confirm that suppression of cell death by PaRXLR40 was not due to nonspecific inhibition of elicitor gene expression, protein immunoblots were performed to verify the presence of the elicitors and effector proteins after coinfiltration into *N. benthamiana*. As expected, the cell death elicitors Avr3a and PaRXLR24, and the PaRXLR40 suppressor, were detected in all relevant samples (Figure 4c,d). However, R3a (hemagglutinin [HA]-tagged) was unable to be detected by protein immunoblotting. Because C-terminal-tagged R3a was previously shown to be nonfunctional (Engelhardt *et al*., 2012), in this study we used R3a with a centrally located HA tag (replacing amino acids 1,167 to 1,175), which may have affected

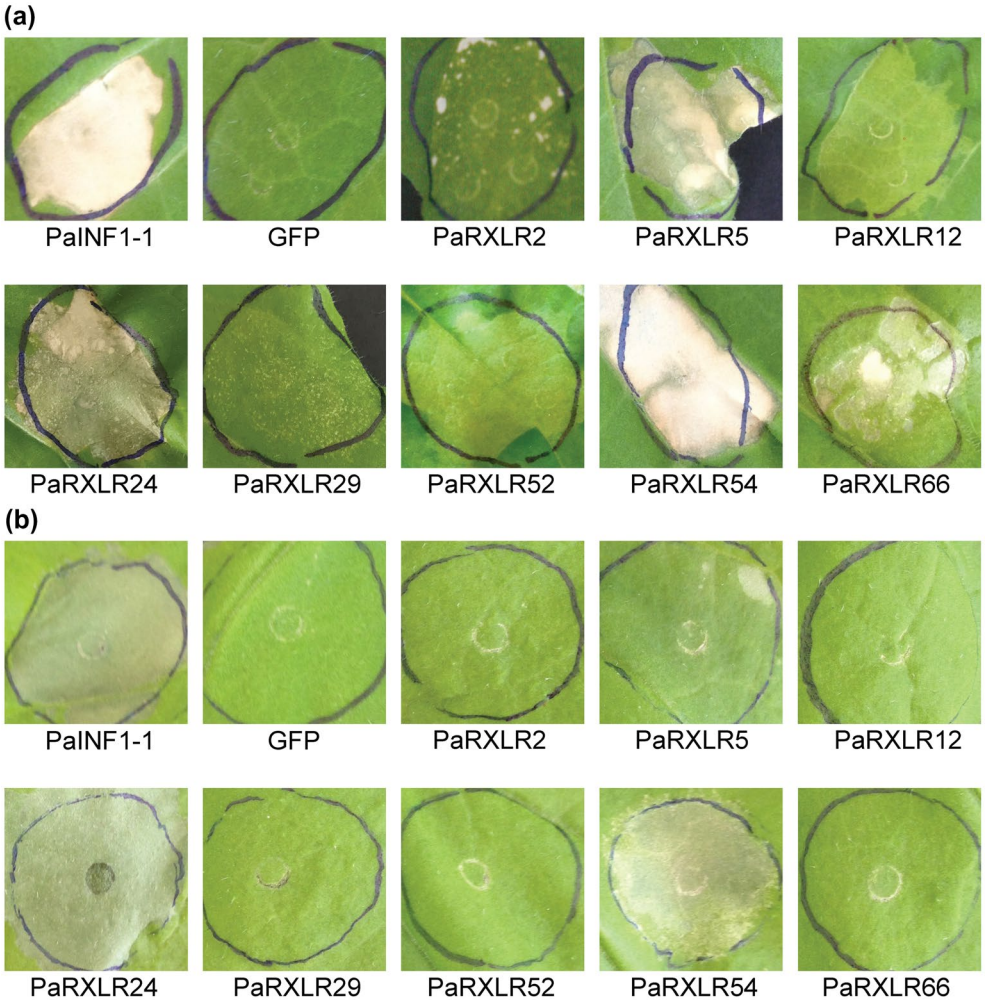

FIGURE 3 Eight RXLR effector candidates of *Phytophthora agathidicida* trigger cell death in *Nicotiana* spp. *Agrobacterium tumefaciens* GV3101 strains carrying *P. agathidicida* INF1-1 (PaINF1-1; positive control), green fluorescent protein (GFP; negative control) or *P. agathidicida* (Pa) RXLR candidates were infiltrated into 5-week-old leaves of *Nicotiana tabacum* (a) or *Nicotiana benthamiana* (b). Leaves were photographed 7 days post infiltration. Six *P. agathidicida* RXLR effector candidates triggered cell death in *N. tabacum* only, whilst two others (PaRXLR24 and PaRXLR54) triggered cell death in both *N. tabacum* and *N. benthamiana*. The experiment was repeated three times with consistent results (Figure S3).

detection of the HA tag or protein stability. However, the expression of R3a-HA in the suppression assay samples was verified by reverse transcription (RT)-PCR, with Avr3a as a positive control (Figure S5). Thus, in this study PaRXLR40 was shown to be a specific suppressor of immunity triggered by effectors, including cell death triggered by PaRXLR24, an effector with which it shares 55.1% amino acid identity (Figure S6c).

2.5 | Both PaRXLR24 and PaRXLR40 are expressed in planta

To indicate whether any of the nine *P. agathidicida* RXLR effector candidates that triggered or suppressed cell death in *Nicotiana* spp. have the potential to be functional in kauri, we determined their expression in kauri (Table S6). Roots and leaves of kauri inoculated with *P. agathidicida* were collected at intervals up to 72 hr post-inoculation and relative quantitative RT-PCR was performed on RNA from these and from in-culture samples. None of the nine *P. agathidicida* RXLR effector candidates were expressed in culture. Whilst most of the RXLRs tested showed no or low expression in planta, *PaRXLR24*, *PaRXLR40*, and *PaRXLR12* were expressed at all four time points in both leaf and root samples, although with expression remaining low until 24 hr, and with higher levels of expression in roots than leaves (Figure 5 and Table S6). Thus, PaRXLR24 and PaRXLR40, which respectively showed cell death elicitor and suppression activity in the model angiosperm *N. benthamiana*, were expressed in the kauri host.

2.6 | Identification of an amino acid required for cell death induction by PaRXLR24

PaRXLR24, the candidate effector that caused strong cell death in both *Nicotiana* species, is an ortholog of virulence factors pPE4 of

TABLE 2 Features of the nine RXLR effector candidates from *Phytophthora agathidicida* that induced or suppressed cell death in *Nicotiana* spp.

Name	Best BLAST hit	E-value	GenBank ID	% amino acid identity	Cell death-triggering activity[f]		Suppressed RXLR-triggered immunity[g]
					Nt	Nb	
PaRXLR2	*Phytophthora cactorum* hypothetical protein[a]	2.0E−38	RAW43538.1	53	Weak	No	No
PaRXLR5[b]	*Phytophthora parasitica* P1569 hypothetical protein[a]	6.0E−22	ETI36999.1	45	Strong	No	No
PaRXLR12	*Phytophthora megakarya* hypothetical protein	4.0E−21	OWZ07307.1	41	Weak	No	No
PaRXLR24[c]	*P. parasitica* pPE4[a]	3.0E−48	XP_008889734.1	65	Strong	Strong	No
PaRXLR29	*P. parasitica* hypothetical protein	9.0E−19	XP_008894466.1	38	Weak	No	No
PaRXLR52[d]	*Phytophthora sojae* hypothetical protein[a]	7.0E−56	XP_009533161.1	62	Weak	No	No
PaRXLR54	*Phytophthora palmivora* avirulence protein	5.0E−100	POM79043.1	45	Strong	Strong	No
PaRXLR66[e]	*P. megakarya* RXLR protein	2.0E−27	OWZ17774.1	37	Weak	No	No
PaRXLR40	*P. palmivora* RXLR protein[a]	1.0E−15	POM65748.1	38	No	No	Yes

[a]Reciprocal top BLAST hit.
[b]Ortholog of *P. nicotianae* Avh8 (KUG01203.1). E value 2e−21, 43.4% amino acid identity.
[c]Ortholog of *P. sojae* Avh238 (AEK81002.1). E value 8e−28, 46.4% amino acid identity.
[d]Ortholog of *P. palmivora* Avr1b-1 (POM62647.1). E value 4e−39, 60.7% amino acid identity.
[e]Only PaRXLR66 showed single nucleotide polymorphism variation among 14 *P. agathidicida* genomes.
[f]RXLR triggered cell death on *N. tabacum* (Nt) or *N. benthamiana* (Nb).
[g]RXLR suppressed Avr3a-R3a and PaRXLR24-triggered cell death on *N. benthamiana*.

P. parasitica (Huang *et al*., 2019) and Avh238 of *P. sojae* (Wang *et al*., 2011) (Table 2 and Figure 6). The amino acids critical for cell death induction by *P. sojae* Avh238 have been identified (Yang *et al*., 2017) and we tested the hypothesis that equivalent amino acids show a similar function in PaRXLR24. The 79th amino acid of Avh238 (histidine) was shown to be critical for its cell death-inducing activity, whilst the 51st and 76th amino acids had minor roles (Yang *et al*., 2017). Alignment of the predicted amino acid sequence of PaRXLR24 with those of isoforms of *P. sojae* Avh238 identified the equivalent positions of these three amino acids in PaRXLR24 (Figure 6a), and site-directed mutagenesis was used to mutate them. Mutated isoforms of PaRXLR24, in which proline 59 or alanine 78 had been replaced with serine (as found in the nonfunctional Avh238 ^{P7076}), did not affect its ability to trigger cell death. However, mutation of PaRXLR24 isoleucine 81, either alone or in combination with the other mutations, showed reduced cell death compared to the wild type (WT) (Figure 6b). PaRXLR24 WT and mutant proteins were detected in extracts from infiltrated *N. benthamiana* leaves by immunoblotting, suggesting that the loss of cell death-inducing ability by PaRXLR24^{I81N} mutants was not due to protein instability (Figure 6c). Thus, the ability of both PaRXLR24 and *P. sojae* Avh238 to induce cell death in *N. benthamiana* is dependent on an amino acid that occurs in the equivalent position in the two proteins.

One of the Avh238 isoforms of *P. sojae*, Avh238 ^{P7076}, which is unable to able to elicit cell death, was shown to suppress INF1-triggered defence (Wang *et al*., 2011). To determine if PaRXLR24 has the ability to suppress cell death, a function that could normally be masked because of its own cell death induction activity, the single (I81N) and triple (I81N-P59S-A78S) cell death-deficient mutants of PaRXLR24 were tested. Neither of these mutants were able to suppress either PaINF1-1- or Avr3a/R3a-triggered cell death (Figure S6). Thus, while PaRXLR24 and *P. sojae* Avh238 are similar in requiring a specific amino acid at an equivalent position for cell-death activity, they differ in respect of their ability to suppress INF1-triggered defence.

2.7 | Potential NBS-LRR receptors for PaRXLR24 identified in *N. benthamiana*

Next, we sought to identify potential plant targets for PaRXLR24. Plant nucleotide-binding site and leucine-rich repeat (NBS-LRR) immune receptors can recognize RXLR effectors from *Phytophthora* pathogens (Lee & Yeom, 2015), thus a hairpin library for silencing of NBS-LRR-encoding genes (Brendolise *et al*., 2017) was used to identify potential *N. benthamiana* NBS-LRR receptors that recognize PaRXLR24. Two pooled hairpin constructs (HP7 and HP14) that each target six or eight NBS-LRRs, out of a total of 48 pools targeting 345 NBS-LRRs, were able to suppress PaRXLR24-triggered cell death (Figure S7). Then, hairpin constructs targeting individual NBS-LRRs from the HP7 and HP14 pools showed that HP7-1, HP7-2, HP14-6, and HP14-8 gave the most effective suppression of PaRXLR24-triggered cell death (Figure 7). Whilst silencing efficiencies of these hairpins showed none fully suppressed their NBS-LRR target (Table S7), hairpin HP7-1 suppressed PaRXLR24-triggered cell death in 75% of the trials (Figure 7) and was predicted to target an NBS-LRR with similarity to an RPM1-like immune receptor (El Kasmi *et al*., 2017) (Tables 3 and S7). Hairpin HP7-2 showed the most consistent and strong

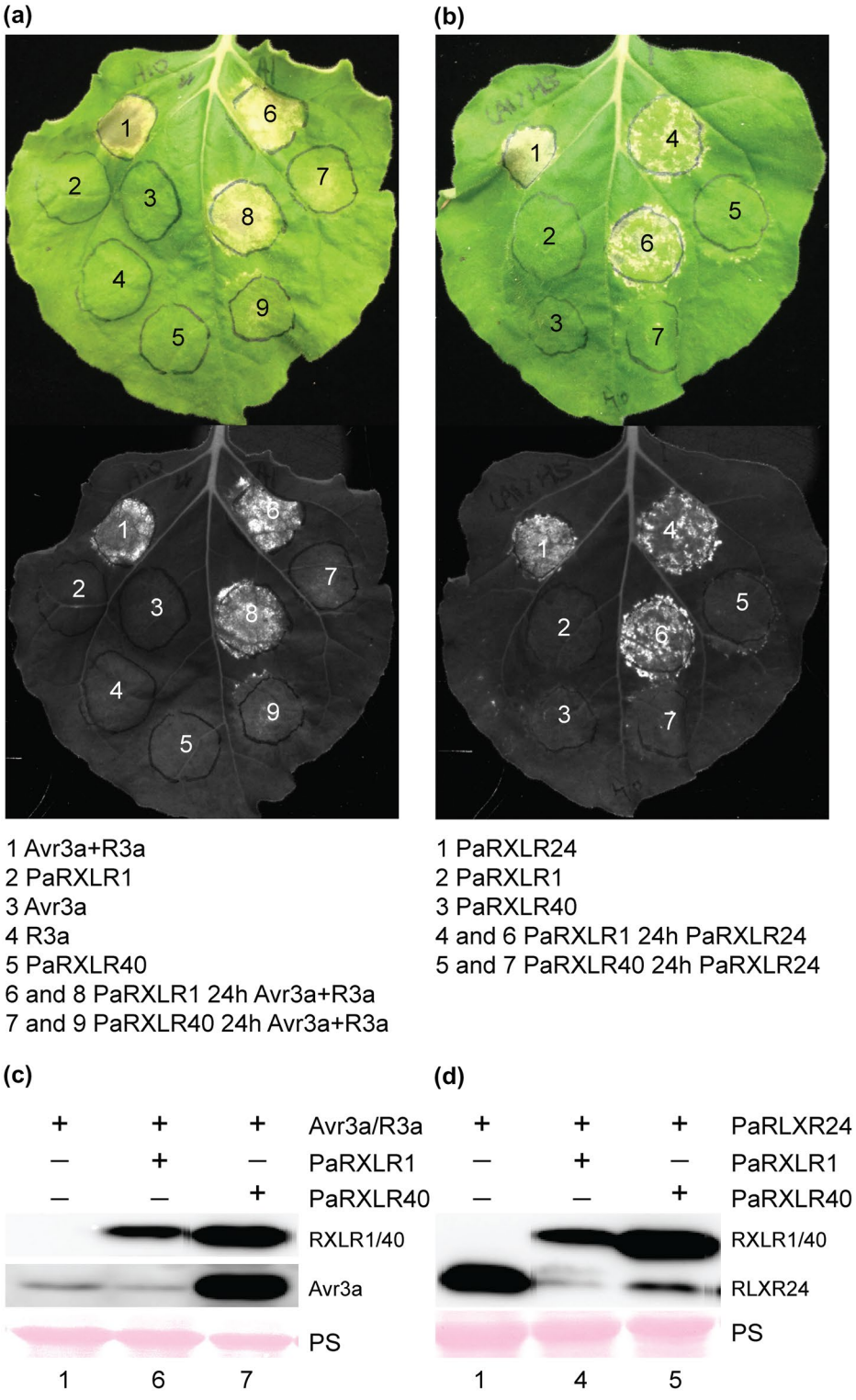

FIGURE 4 Suppression of RXLR-triggered immunity by PaRXLR40. Suppression of (a) Avr3a and R3a-triggered cell death and (b) PaRXLR24-triggered cell death by PaRXLR40 on 5-week-old *Nicotiana benthamiana*. Avr3a, R3a, and PaRXLR were labelled with GFP, HA, and FLAG tags, respectively. *Agrobacterium tumefaciens* carrying cell death elicitors were infiltrated 24 hr after infiltration of *Phytophthora agathidicida* RXLR effector candidate PaRXLR40, or the negative suppression control, PaRXLR1. Photographs with visible light (top) and UV (bottom) were taken 7 days post-infiltration of cell death elicitors. Suppression is shown by lack of cell death at infiltration spots (a) 7 and 9 and (b) 5 and 7. The experiment was repeated three times with consistent results. (c) and (d) Protein immunoblots of total proteins extracted from *N. benthamiana* leaves collected 3 days post-infiltration confirmed the presence of elicitors and PaRXLR effector candidates. Representative protein loading is shown by Ponceau staining (PS).

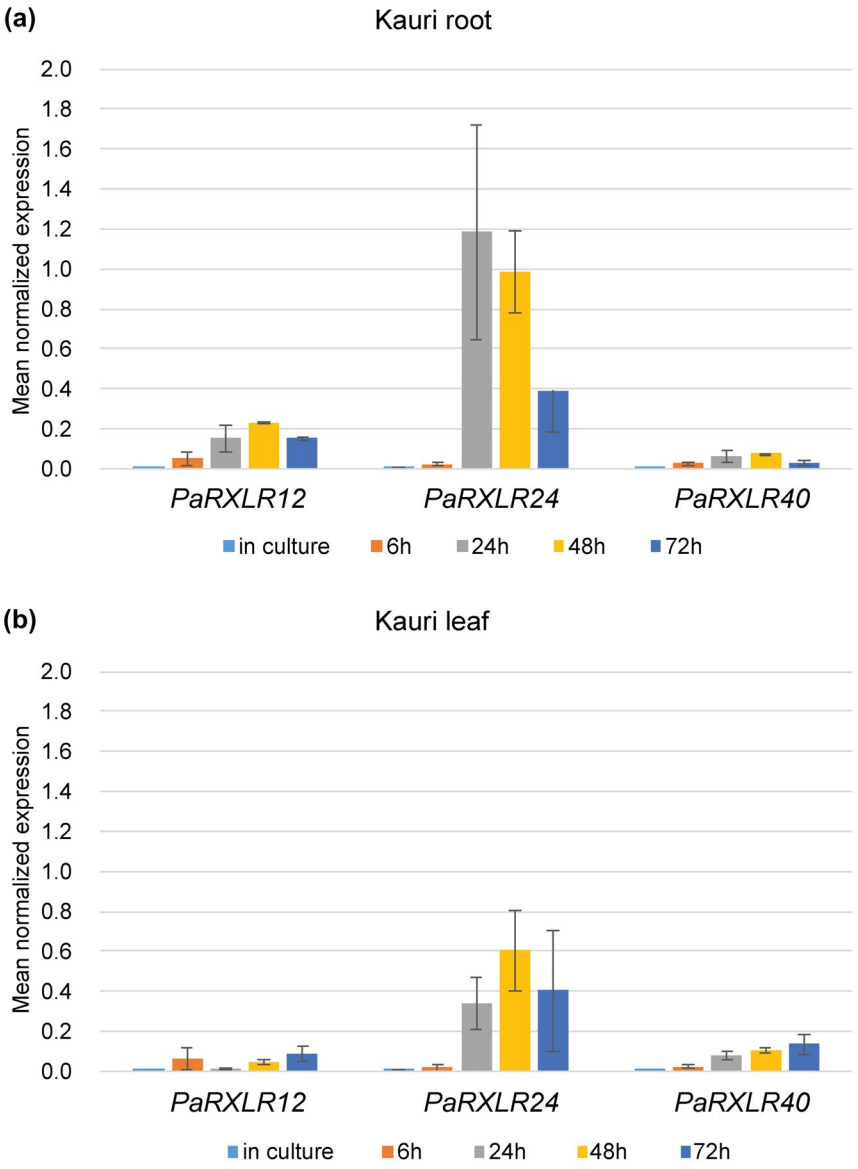

FIGURE 5 *PaRXLR24* and *PaRXLR40* are expressed in kauri tissue. Gene expression was analysed in (a) roots and (b) leaves of kauri inoculated with *Phytophthora agathidicida* mycelium for the nine RXLRs that either triggered cell death or suppressed effector triggered defence in *Nicotiana* spp. Expression of PaRXLR genes in vitro (mycelium) and in planta (6, 24, 48, and 72 hr post-inoculation) was normalized to the geometric mean of three *P. agathidicida* housekeeping genes, *β-tubulin*, *actin*, and *elongation factor 2*. The normalized means are shown with standard error bars. Only three (PaRXLR12, PaRXLR24, and PaRXLR40) were expressed in kauri (full results in Table S6).

suppression of PaRXLR24-triggered cell death (in 81% of infiltration spots) and appears to target an R1-like NBS-LRR (Ballvora *et al*., 2002) (Figure 7, and Tables 3 and S7). From the HP14 hairpin pool, HP14-6 and HP14-8 both suppressed PaRXLR24-triggered cell death in about 60% of infiltration spots and their predicted *N. benthamiana* NBS-LRR targets were similar to putative late blight resistance protein R1-like proteins of *Nicotiana* species (Figure 7, and Tables 3 and S7).

To further assess whether the candidate NBS-LRRs are required for recognition of PaRXLR24, those targeted by hairpins HP7-1, HP7-2, HP14-6, and HP14-8 were silenced by virus-induced gene silencing (VIGS) in *N. benthamiana* (Velásquez *et al*., 2009). Because the hairpin construct library was designed using NBS-LRRs predicted from an older annotated genome of *N. benthamiana*, Niben.genome.v. 0.4.4 (Bombarely *et al*., 2012; Brendolise *et al*., 2017), the VIGS constructs were targeted to equivalent updated gene models in the Niben v. 1.0.1 genome (Grosse-Holz *et al*., 2018) (Tables 3 and S7). The NBS-LRR targeted by hairpin HP7-1 had two hits in the Niben v. 1.0.1 genome (Table S7); these shared 98.8% nucleotide identity and are adjacent to each other on the same contig. Similarly, the NBS-LRR targeted by hairpin HP14-8 also had two hits in the Niben v. 1.0.1 genome (Table S7), with those hits sharing 82.9% nucleotide identity and being located on different contigs in the genome assembly. In total six VIGS constructs (TRV-NLR1 to -NLR6) were designed to silence the four candidates (Tables 3 and S7).

The VIGS-silenced plants showed no difference in phenotype compared to TRV-GFP-silenced control plants (Figure S8), as expected. *N. benthamiana* plants with TRV-NLR3, -NLR5, and -NLR6, corresponding to NBS-LRRs silenced by hairpin HP7-1 and HP7-2, showed suppression of PaRXLR24-induced cell death (Figure S8). This was supported by the observation that PaRXLR24-infiltrated spots showed significant reduction in ion leakage in VIGS-silenced plants compared to GFP-silenced plants (Tables 3 and S7). Although NBS-LRRs silenced by HP14-6 and HP14-8 showed suppression of PaRXLR24-triggered cell death (Figure 7), those results were not confirmed in VIGS assays, where PaRXLR24-infiltrated spots showed the same levels of cell death and ion leakage in VIGS-silenced plants

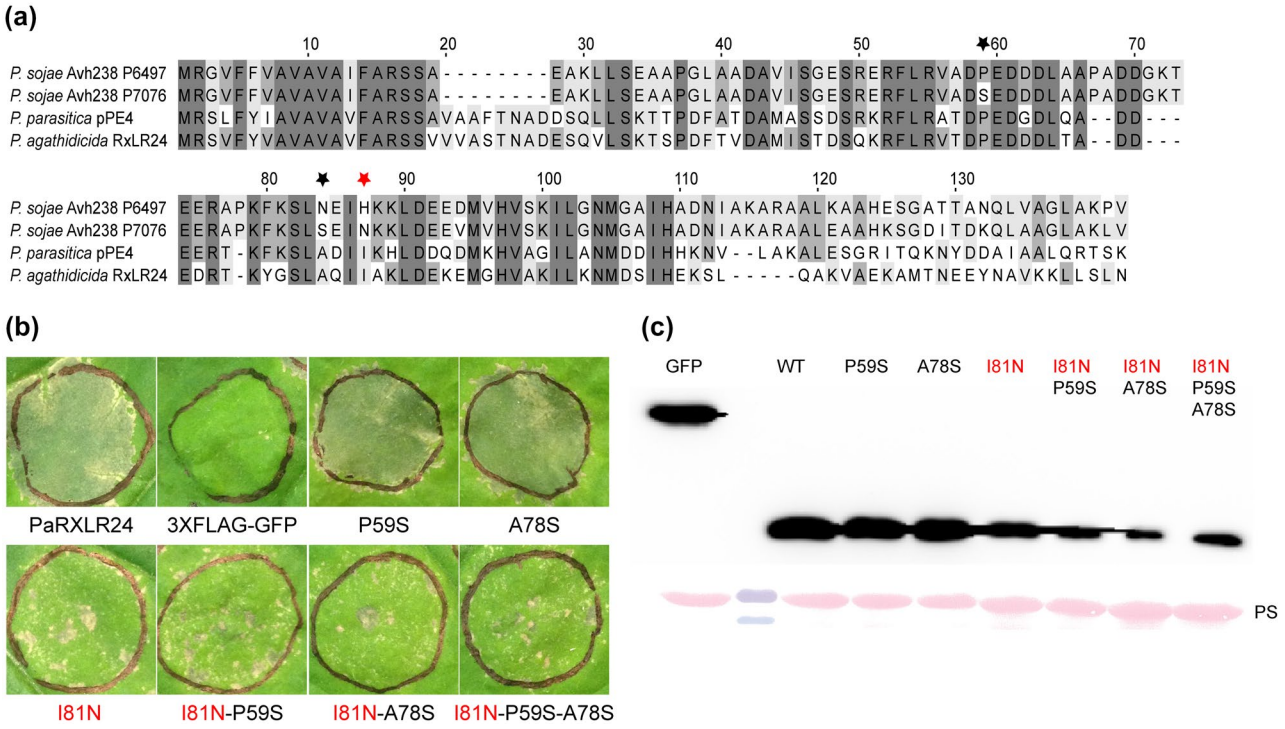

FIGURE 6 Identification of amino acids required for PaRXLR24 cell death-triggering activity. (a) Amino acid alignment of *Phytophthora sojae* Avh238 (two alleles), *Phytophthora parasitica* pPE4, and PaRXLR24. The red star indicates the position of the 79th amino acid (histidine) in the active P6497 allele of Avh238 required for cell death-inducing activity (Yang *et al*., 2017), and the corresponding 81st amino acid (isoleucine) in PaRXLR24. Black stars indicate positions of amino acids shown to affect cell death-inducing activity in Avh238 and corresponding amino acids in PaRXLR24. (b) Agroinfiltration of PaRXLR24 wild-type and mutants on 5-week-old *Nicotiana benthamiana* leaves. The experiment was repeated three times with consistent results. 3 × FLAG-GFP was used as negative control. The PaRXLR24^{P59S} and PaRXLR24^{A78S} single (black star) mutants showed similar levels of cell death as the wild type. PaRXLR^{I81N} single (red star), double and triple mutants showed reduced cell death compared to the wild type. Photographs were taken 7 days post-infiltration. (c) Western blotting confirmed the stability of wild-type (WT) and mutant PaRXLR24 proteins in total leaf extracts.

as in GFP-silenced plants (Figure S8, Tables 3 and S7). Together the VIGS and hairpin silencing results suggest that NBS-LRRs silenced by hairpins HP7-1 and HP7-2 may be involved in PaRXLR24 recognition in *N. benthamiana*. Thus, an RXLR from a *Phytophthora* species that is pathogenic to a gymnosperm can be recognized by immune receptors from a model angiosperm plant.

3 | DISCUSSION

3.1 | Identification and functional analysis of *P. agathidicida* RXLR effector candidates

There are currently few studies of molecular plant–microbe interactions involving gymnosperm tree pathogens, despite their immense importance for forest health. *Phytophthora* species are particularly notorious pathogens of forest gymnosperms (Hansen, 2015; Shuey *et al*., 2019; Bradshaw *et al*., 2020) and there is an urgent need to understand how they interact with plants in order to develop new methods of disease control. To help address this knowledge gap, we identified 78 RXLR effector candidates from the kauri dieback pathogen *P. agathidicida*. Eight of the PaRXLRs tested elicited cell death in *Nicotiana* spp.; this proportion of cell-death eliciting RXLRs is similar to those found in studies with *P. sojae* (11/169) (Wang *et al*., 2011) and *Plasmopara viticola* (10/83) (Liu *et al*., 2018). None of the PaRXLRs tested were able to suppress immunity elicited by *P. agathidicida* INF1-1. This is in contrast to other studies in which 23 of 49 *P. sojae* RXLRs and 52 of 78 *P. viticola* RXLRs could suppress INF-triggered cell death (Wang *et al*., 2011; Liu *et al*., 2018).

3.2 | *P. agathidicida* RXLR genes showed low genetic diversity and only some were expressed

In our study, genome analysis of 13 isolates of *P. agathidicida* from across the geographic range of kauri in New Zealand showed a lower level of nucleotide diversity (99.9% identical) based on pairwise SNP analysis. Among 78 PaRXLRs, only 10 showed polymorphism; of those only one was shown to elicit cell death in *N. benthamiana* but was not expressed in kauri. This low level of RXLR diversity was similar to the overall genome diversity, suggesting lack of enrichment for RXLR polymorphisms. This finding is concordant with an asexually

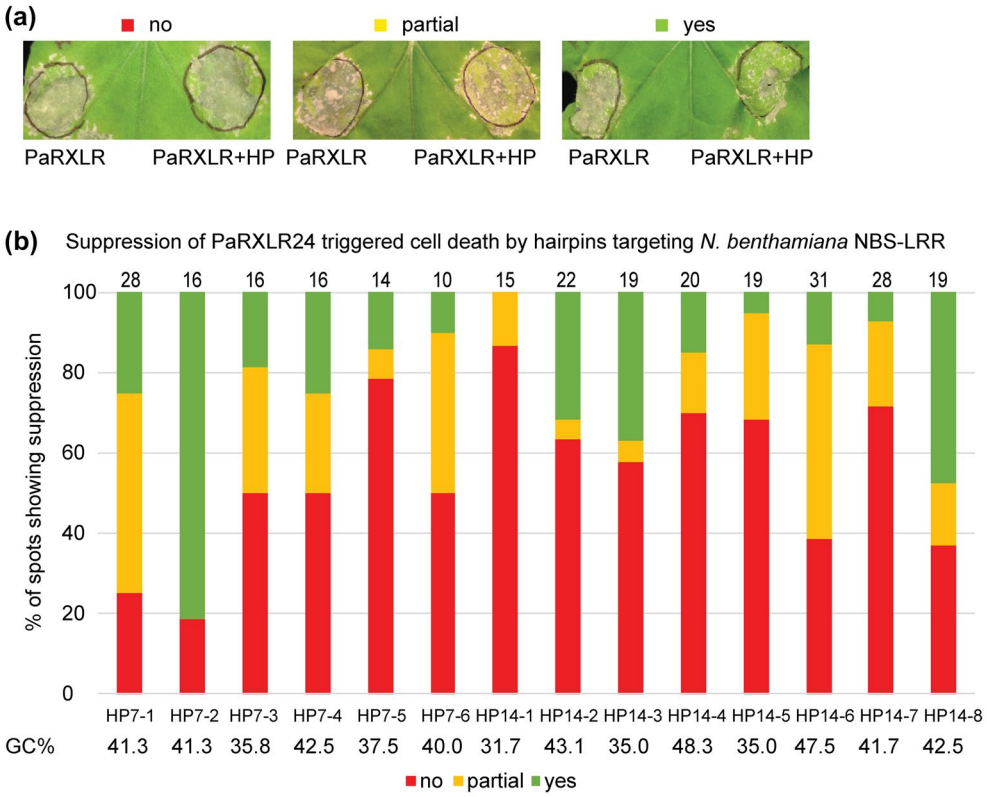

FIGURE 7 Identification of NBS-LRRs required for PaRXLR24-triggered cell death. *Agrobacterium*-mediated screening was used to identify NBS-LRRs required for PaRXLR24-triggered cell death in 5-week-old *Nicotiana benthamiana*. Hairpin constructs (HP) that targeted individual NBS-LRRs were infiltrated 48 hr before PaRXLR24. Photographs were taken 7 days after PaRXLR24 infiltration. (a) Leaves showing examples of suppression scoring, with no suppression (red), partial suppression (yellow), and strong suppression (green) of PaRXLR24-triggered cell death by hairpin constructs. (b) Percentages of infiltration spots showing different levels of suppression by individual hairpin constructs that silenced NBS-LRRs from hairpin pools 7 and 14. Numbers of infiltration sites counted are shown above each bar. NBS-LRRs targeted by hairpin constructs HP7-1, HP7-2, HP14-6, and HP14-8 suppressed PaRXLR24-triggered cell death more than 50% of the time

reproducing population that is not endemic to New Zealand. Asexual reproduction is common among *Phytophthora* pathogens, and asexual lineages have been shown to cause epidemics (Pais *et al*., 2018).

Of the nine PaRXLRs that either elicited or suppressed cell death in *N. benthamiana*, only three were expressed in kauri. Whilst expression levels were generally lower in leaves than roots, consistent with *P. agathidicida* being a root pathogen, there were similar patterns of expression in the two tissue types, with PaRXLR24 most highly expressed in both. *P. agathidicida* has been shown to cause lesions on kauri leaves (Herewini *et al*., 2018) and our expression results indicate that some aspects of plant–pathogen interactions may be consistent across tissues. The observation that most of the PaRXLRs tested were not expressed in kauri was not surprising. Not all RXLR genes are expressed in planta, with lack of expression being one mechanism to evade recognition by cognate immune receptors (Gilroy *et al*., 2011; Pais *et al*., 2018).

Studies with other *Phytophthora* species have shown that the timing of in planta RXLR gene expression is important during infection (Wang *et al*., 2011; Cooke *et al*., 2012; Yin *et al*., 2017). In our study, the expression of PaRXLR40 peaked later than PaRXLR24 in both kauri root and leaf. These results, combined with our finding that PaRXLR40 is also able to suppress PaRXLR24 or Avr3a/R3a-triggered cell death, suggest that PaRXLR40 may suppress downstream defence responses triggered by PaRXLR24.

3.3 | PaRXLR24 as an ortholog of *P. sojae* Avh238

Because of its high expression in kauri and its strong cell-death eliciting function in *N. benthamiana*, PaRXLR24 was compared to the orthologous *P. sojae* Avh238 in more detail. Site-directed mutagenesis of PaRXLR24 identified that isoleucine 81 is important for PaRXLR24-triggered cell death. An equivalent mutant version of its ortholog *P. sojae* Avh238 ($H79^{th}$) also lost the ability to trigger cell death but revealed a cryptic virulence function as it was able to suppress INF1-triggered defence. Suppression assays with cell-death negative mutants of PaRXLR24 suggested that PaRXLR24 cannot suppress INF1-triggered defence, and therefore does not appear to show the same virulence function as Avh238. *P. sojae* Avh238 interacts with, and destabilizes, type 2 1-aminocyclopropane-1-carboxylic acid synthase (ACS), which interrupts ethylene biosynthesis that is required for resistance against *P. sojae* in soybean (Yang *et al*., 2017). Ethylene is an important hormone in plant defence against pathogens (Broekgaarden *et al*., 2015). However, it is not known if

TABLE 3 Identification of *Nicotiana benthamiana* NBS-LRR candidates involved in recognition of PaRLXR24

	Hairpin screen			VIGS screen				
NBS-LRR Genome v. 0.4.4 (v. 1.0.1)[a]	Hairpin[b]	Suppression (%)[c]	Silencing efficiency (% ± SD)[d]	TRV construct[g]	Suppression (%)[f]	Ion leakage (%)[g]	Silencing efficiency (% ± SD)[d]	NBS-LRR type
8754g0022.1 (05566g02009.1; 05566g02008.1)	HP7-1	75.0	33.7 ± 10.7	TRV-NLR3	Yes	51.3*	82.5 ± 5.0	RPM1-like[h]
				TRV-NLR5	Yes	60.1*	79.6 ± 3.0	
1428g0007.1 (05653g00005.1)	HP7-2	81.3	11.9 ± 19.1	TRV-NLR6	Yes	40.8*	58.1 ± 7.0	R1-A-like[i]
4955g0024.1 (03461g05022.1)	HP14-6	61.3	38.1 ± 9.9	TRV-NLR1	No	82.4	65.0 ± 6.0	R1-B-like[i]
5032g0004.1 (03924g01008.1; 15476g01014.1)	HP14-8	63.2	38.1 ± 15.1	TRV-NLR2	No	83.9	77.1 ± 4.0	R1-B-like
				TRV-NLR4	No	71.7	88.7 ± 3.0	

[a]Top NBS-LRR candidates predicted from the *N. benthamiana* genome v. 0.4.4 (NbS0000 numbers) and equivalent gene models from the genome v. 1.0.1 in parentheses (Niben101Scf numbers). Two of the v. 0.4.4 candidates each had two matches in the *N. benthamiana* v. 1.0.1 genome.

[b]Hairpin construct used to silence *N. benthamiana* NBS-LRR, designed using *N. benthamiana* genome v. 0.4.4 NBS-LRR gene models.

[c]Percentage of infiltration spots showing full or partial suppression of PaRXLR24-triggered cell death.

[d]Silencing efficiency of NBS-LRR candidates determined by reverse transcriptionPCR. 100% is complete loss of NBS-LRR expression.

[e]Virus-induced gene silencing (VIGS) construct used to silence *N. benthamiana* NBS-LRR, designed using *N. benthamiana* genome v. 1.0.1.

[f]Infiltration spots showing suppression of PaRXLR24-triggered cell death on VIGS-silenced *N. benthamiana* plants (see Figure S8).

[g]Ion leakage of PaRXLR24-infiltrated spots in TRV-NLR1-6 silenced plants, shown as % conductivity compared to boiled leaf samples. Asterisks (*) indicate PaRXLR24 values significantly different in TRV-NLR-silenced plant versus GFP-silenced plant while values for GFP-infiltrated control sites on the same plants were not significant.

[h]El Kasmi *et al*. (2017).

[i]Ballvora *et al*. (2002).

ethylene is involved in defence against *P. agathidicida* in kauri and whether PaRXLR24 shares the same host target as Avh238.

3.4 | Potential immune receptor targets were found in *N. benthamiana*

In this study, NBS-LRRs were identified from the angiosperm model-plant *N. benthamiana* that specifically recognized RXLR effectors from *P. agathidicida*, which is pathogenic to a gymnosperm. It was previously shown that NBS-LRR receptors from distantly related species can confer disease resistance, such as a maize NBS-LRR enhancing resistance to a bacterial pathogen in *Arabidopsis* and rice plant hosts (Xu *et al*., 2018), indicating highly conserved mechanisms of plant defence. Our work supports the premise that these mechanisms may be very broadly conserved at the molecular level between gymnosperm and angiosperm systems.

Both of the top candidate NBS-LRRs that recognized PaRXLR24 showed similarity to characterized immune receptors. The NBS-LRR silenced by HP7-2 showed similarity to late-blight resistance protein R1 (Ballvora *et al*., 2002), which is encoded by the major R1 resistance gene cluster in potato (Kuang *et al*., 2005). R1 is involved in defence against *P. infestans* Avr1, an RXLR effector that directly interacts with host exocyst component Sec5, potentially disrupting the host vesicle trafficking system required for defence (Du *et al*., 2015). The other candidate NBS-LRR, silenced by HP7-1, showed similarity to *Arabidopsis* immune receptor RPM1 (El Kasmi *et al*., 2017). RPM1 guards RIN4, a conserved plant immunity signalling hub and a strong activator of plant defence (Toruño *et al*., 2019). Phosphorylation of RIN4 in the presence of pathogen effectors such as *Pseudomonas syringae* type III effectors AvrRpm1 and AvrB leads to activation of RPM1-mediated downstream signal transduction and plant defence response (Toruño *et al*., 2019).

The hairpin-based RNA silencing method used to identify NBS-LRRs had some limitations. Inaccurate annotation of the *N. benthamiana* gene models may have led to over- or underestimation of functional NBS-LRRs. Indeed, different numbers of targets were identified in the two versions of the *N. benthamiana* genome. Furthermore, due to similarities between *N. benthamiana* NBS-LRR gene family sequences, off-target silencing could occur (Guo *et al*., 2016; Brendolise *et al*., 2017). It is possible that the initial positive screening results with the R1-like NBS-LRRs targeted by HP14-6 and HP14-8 may have been off-targets related to the stronger R1-like HP7-2 NBS-LRR candidate. Confirmation of the NBS-LRRs candidates in independent experiments using VIGS lent strong support for potential roles of NBS-LRRs targeted by HP7-1 and/or HP7-2 in recognition of PaRXLR24 in *N. benthamiana*, but no support for those targeted by HP14-6 and HP14-8.

3.5 | Implications for kauri dieback

There are few studies of molecular plant–microbe interactions involving forest trees, particularly gymnosperms. Indications from this work are that *P. agathidicida* may use similar molecular tools as other *Phytophthora* species that are principally angiosperm pathogens and it is feasible that immune receptors identified in model-plants may enable the development of genetic markers for resistance in kauri. At the time of writing, the kauri genome sequence was not available. Meanwhile there is more that needs to be learned about the responses of kauri tissue to the effector proteins themselves and their effects on the ability of *P. agathidicida* to cause disease. There are also early indications that *P. agathidicida* may be able to colonize other gymnosperm hosts as well as some angiosperms, including Myrtaceae (Bradshaw *et al*., 2020).

In the event that immune receptors that recognize specific PaRXLRs can be identified in kauri, the implications for the continued health of such a long-lived forest tree need consideration. Studies of short-rotation crop pathogens and an increasing number of tree species have warned of the breakdown of major gene resistance due to rapidly evolving pathogens (Kinloch *et al*., 2008; Stam & McDonald, 2018). In agricultural crops, durability of resistance can be increased by pyramiding immune receptors and by selecting those that recognize effectors with important virulence functions that may incur a fitness cost if lost or mutated (Vleeshouwers & Oliver, 2014; Moscou & Van Esse, 2017). In forest trees the basis of resistance is very broad, including qualitative as well as quantitative genetic resistance (Ennos, 2015; Fraser *et al*., 2016), along with a complexity of biotic and abiotic environmental factors that can influence plant health in forests (Sniezko, 2006; Feau & Hamelin, 2017; Sniezko & Koch, 2017; Bradshaw *et al*., 2020). The long-lived nature of trees also means that understanding the evolutionary ecology of the forest is critical to ensure durable resistance (Ennos, 2015). Thus, a wholistic approach to tree health involving all aspects from genetic resistance to population diversity to the dynamic microbiome is needed (Desprez-Loustau *et al*., 2016; Feau & Hamelin, 2017; Moscou & Van Esse, 2017; Sniezko & Koch, 2017; Bradshaw *et al*., 2020). A deeper knowledge of the biology underlying plant–pathogen interactions that influence resistance and susceptibility will help illuminate the path forward.

3.6 | Conclusions

Our work reveals how a *Phytophthora* pathogen of a gymnosperm tree species interacts with plants at the molecular level in ways consistent with those of angiosperm pathosystems and provides a foundation for studying the molecular basis of plant–pathogen interactions in gymnosperm trees. Notably, candidate immune receptors identified using this approach might ultimately provide molecular markers for resistance breeding in forest trees.

4 | EXPERIMENTAL PROCEDURES

4.1 | RXLR gene identification, motif and orthology predictions

RXLR effector gene candidates were predicted from the genome sequence of *P. agathidicida* strain NZFS3770 (Studholme *et al*., 2016)

(GenBank: GCA_001314445.1). Gene models were computed using Augustus v. 2.5.5 (Stanke & Morgenstern, 2005), from which 78 RXLR effector gene candidates were identified by combining three prediction methods (Bhattacharjee *et al*., 2006; Whisson *et al*., 2007; Win *et al*., 2007) (Figure S1), using HMMER v. 3.0 (Finn *et al*., 2011) and SignalP v. 3.0 for signal peptide prediction (Bendtsen *et al*., 2004). Predicted nucleotide and amino acid sequences for these RXLRs are in Table S1 and on GenBank (accession numbers MT503101–MT503178). Maximum-likelihood phylogenetic analysis was done as previously described (Ozturk *et al*., 2019). Over-represented amino acid motifs in the 78 RXLR effector candidates were identified using default parameter values in the MEME suite motif alignment search tool (MAST) (Bailey *et al*., 2009).

4.2 | *A. tumefaciens*-mediated transient transformation assays

RXLR effector gene candidates and PaINF1-1 were PCR-amplified from genomic (g)DNA of *P. agathidicida* isolate NZFS3616 (the primers used are listed in Table S2). Single, double, and triple mutant versions of PaRXLR24 were made with a QuickChange II site-directed mutagenesis kit (Agilent) using wild-type (WT) PaRXLR24 template cloned into *Sma*I-digested pICH41021 (Yanisch-Perron *et al*., 1985). The RXLR PCR products, PaRXLR24 WT and mutant plasmids, along with either signal peptide PR1α (apoplastic) or N-3 × FLAG tag (cytoplasmic) (Integrated DNA Technologies), were used as entry modules for Golden Gate assembly (Engler *et al*., 2008) into the *Agrobacterium* expression vector pICH86988 (Weber *et al*., 2011).

Verified plasmid constructs were transformed into *A. tumefaciens* GV3101 (Holsters *et al*., 1980). Three of the PaRXLRs could not be cloned so only 75 were screened for their ability to induce cell death. For these cell death screening assays, overnight cultures of transformed *A. tumefaciens* GV3101 were resuspended in buffer (10 mM $MgCl_2$, 10 mM MES-KOH pH 5.6, 100 μM acetosyringone) and infiltrated into *N. benthamiana* or *N. tabacum* leaves at a final OD_{600} of 1.0 (Ma *et al*., 2012). The 73 PaRXLRs that did not trigger cell death on *N. benthamiana* were tested for suppression of cell death. For suppression assays, *A. tumefaciens* carrying cell-death elicitor genes were infiltrated 24 hr after *P. agathidicida* RXLR effectors at OD_{600} of 0.4 for all constructs (Wang *et al*., 2011). Symptoms were scored 7 days post-infiltration.

4.3 | Protein immunoblotting and RT-PCR

To verify protein production in suppression assays, *N. benthamiana* leaves were infiltrated as described for suppression screening, harvested after 3 days, then snap-frozen in liquid nitrogen. Total proteins were extracted using GTEN protein extraction buffer (Choi *et al*., 2018). Twenty microlitres of total protein extract was separated by SDS-PAGE (10%–12% polyacrylamide). Gel electrophoresis was performed at 110 V for 2–3 hr in running buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% SDS) (Laemmli, 1970). Proteins were transferred to PVDF membrane (Sigma-Aldrich) in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% (vol/vol) methanol) overnight at 30 V. Proteins were probed with mouse anti-GFP (1/200), -HA (1/1000) (Santa Cruz Biotechnology) or -FLAG (1/5000) (Sigma-Aldrich) primary antibody and chicken anti-mouse IgG-HRP (1/20,000) (Santa Cruz Biotechnology) secondary antibody. Membranes were treated with chemiluminescent substrate (SuperSignal West Dura Extended Duration; Thermo Fisher Scientific) and protein bands detected using a C600 Gel Imaging System (Azure Biosystem).

Because R3a-HA2 could not be detected on a western blot, gene expression was verified by RT-PCR. RNA was extracted using a Spectrum Plant Total RNA Kit (Sigma-Aldrich) and cDNA synthesised using random primers (QuantiTect Reverse Transcription Kit, Qiagen). Gene-specific primers were used to amplify R3a-HA2 and GFP-Avr3a from cDNA samples using Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific).

4.4 | Expression of *P. agathidicida* RXLR genes in planta

Quantitative RT-PCR was used to determine expression of PaRXLRs in kauri tissue. Using *P. agathidicida* isolate 3813 (Herewini *et al*., 2018), grown in carrot broth and V8 juice (Horner & Hough, 2014; Herewini *et al*., 2018), small pieces of mycelium were placed onto fine root tips of 8-month-old susceptible kauri seedlings (HTHF-2017-MW8-G). For leaves, a small surface wound was made 0.5 cm from the base of each leaf prior to inoculation. The roots were then sealed between wet paper towels in a plastic cassette and incubated at 17°C with 14.5 hr light:9.5 hr dark. Leaf and root samples were collected from three infected seedlings at 6, 24, 48, and 72 hr post-inoculation, with one leaf or two root tips from each seedling (up to 2 cm from infection point). Samples were snap-frozen in liquid nitrogen and stored at −80°C. All kauri plant material was respectfully destroyed at the completion of the experimental work.

RNA was extracted from the samples and cDNA synthesised as above. One microlitre of 2-fold diluted cDNA was mixed with 5 μl of 2 × SensiFAST SYBR No-ROX (Bioline) and 0.5 mM forward and reverse primer in a total volume of 10 μl. Two technical replicates for each of three biological replicates were subject to RT-PCR, using 40 cycles of 95°C for 5 s, 60°C for 10 s, and 72°C for 20 s. Relative expression of *P. agathidicida* RXLR gene candidates was calculated with the Q-Gene method (Muller *et al*., 2002), using the geometric mean of three *P. agathidicida* housekeeping genes, *β-tubulin*, *actin*, and *translation elongation factor 2*, as reference (Table S1).

4.5 | Genome sequencing and SNP analysis of 12 *P. agathidicida* isolates

Twelve isolates of *P. agathidicida* (Table 1) were grown in clarified carrot broth (Herewini *et al*., 2018) for 7 days at 17°C, with gDNA

extracted from freeze-dried mycelium (Moller *et al*., 1992). *P. agathidicida* gDNA was sequenced on an Illumina HiSeq 2500 at the Australian Genome Research Facility, using Illumina gDNA shotgun library preparation and HiSeq HT chemistry with 125 bp paired-end reads (Illumina).

Raw DNA sequence data were processed with fastq-mcf to remove primer and sequencing adapter sequences (Aronesty, 2011), then quality trimmed to a Phred score of >20 using SolexaQA v. 3.1.4 (Cox *et al*., 2010). Data quality was analysed using FastQC v. 0.11.5 (Bioinformatics, 2015). Sequence data are available from the Sequence Read Archive (SRA): BioProject PRJNA486676.

Paired-end reads were mapped to the NZFS3770 reference genome (Studholme *et al*., 2016) with Bowtie 2 v. 2.2.6 (Langmead & Salzberg, 2012). SNPs in the 12 genomes were compared to the reference genome using FreeBayes v. 1.1.0-46 (Garrison & Marth, 2012), with ploidy set to 2 (diploid). The FreeBayes VCF files were annotated based on *P. agathidicida* NZFS3770 gene models using SnpEff v. 4.3t, with default parameters and quality filtering at $Q > 30$ (Cingolani *et al*., 2012). Numbers of SNPs in coding sequences were determined using bedtools (Quinlan & Hall, 2010) (Table S3). Homozygous SNPs were extracted from VCF files and an alignment built by concatenating all 5,851 SNPs. A phylogeny was built using the poppr R package (Kamvar *et al*., 2014).

4.6 | Screening for *N. benthamiana* receptors

Screening for *N. benthamiana* NBS-LRR receptors involved in PaRXLR24 recognition was performed by RNA silencing (Brendolise *et al*., 2017). *A. tumefaciens* GV3101 cells carrying the constructs were infiltrated into 5-week-old *N. benthamiana* leaves with final OD_{600} of 0.2 for hairpin constructs and 0.4 for PaRXLR24. The 48 sets of pooled hairpins (Brendolise *et al*., 2017) were used in the first round of screening. Hairpins targeting individual NBS-LRR genes from two positive pools (Table S4) were then used in the final screening. Symptoms were assessed 7 days post-infiltration. Top individual NBS-LRR candidates were assessed further using VIGS (Table S4), carried out as described previously (Wang *et al*., 2018), with silencing constructs developed using PCR primers (Table S2). Cell death was quantified by ion leakage as described previously (Jing *et al*., 2016). Silencing efficiencies of individual hairpins were determined using quantitative RT-PCR, with RNA and cDNA prepared as above. NBS-LRR gene expression in hairpin-infiltrated *N. benthamiana* leaves is shown as a percentage of that in noninfiltrated leaves ($n = 3$) with expression of elongation factor 1-α (*EF1a*) used for normalization.

ACKNOWLEDGMENTS

The work was supported by the Tertiary Education Commission of New Zealand via Bio-Protection Research Centre grants, along with in-kind support through the Scion Healthy Trees, Healthy Future programme (MBIE, C04X1305). Kauri germplasm was provided with permission from Taoho Patuawa on behalf of the Te Roroa Iwi Trust. J.W. was supported by the Gatsby Charitable foundation. Thanks to Dr Cyril Brendolise (Plant & Food Research, New Zaland) for provision of hairpin constructs, Professor Paul Birch and Dr Piers Hemsley (University of Dundee, UK) for GFP-Avr3a and R3a-HA2 constructs, Professor Jonathan Jones and Mark Youles (The Sainsbury Laboratory, UK) for plasmids pICH41021 and pICH86988, and Wenyue Zheng and Yuyin Wang (Nanjing Agricultural University, China) for assistance with VIGS screening.

DATA AVAILABILITY STATEMENT

Sequence data of RXLR effector gene candidates from *P. agathidicida* strain NZFS3770 are available from GenBank at https://www.ncbi.nlm.nih.gov/ as genome assembly GCA_001314445.1 and accession numbers MT503101–MT503178. Sequence data of 12 isolates of *P. agathidicida* are available from the Sequence Read Archive (SRA) https://www.ncbi.nlm.nih.gov/bioproject/ as PRJNA486676.

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SUPPORTING INFORMATION

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

How to cite this article: Guo Y, Dupont P-Y, Mesarich CH, et al. Functional analysis of RXLR effectors from the New Zealand kauri dieback pathogen *Phytophthora agathidicida*. *Molecular Plant Pathology*. 2020;21:1131–1148. https://doi.org/10.1111/mpp.12967