**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 (Ntoukasikis & 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 agathicida* 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  | NZFSa | Yearb | Locationb | Sequence accession numbersc | Mbases reads | SNPsq per genome | Genes with SNPs | RXLRs with SNPsa |
|-----|-------|-------|-----------|-----------------------------|-------------|-----------------|----------------|-----------------
| 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 |

aNZFS (New Zealand Forest Service collection) number of the *P. agathidicida* isolate.
bYear and location (in New Zealand) of isolate collection.
cAccession numbers for GenBank sequence read archive Bioprojects. 3770 and 3772 are those from Studholme et al (2016).
dTotal number of single nucleotide polymorphism (SNP) sites in the resequenced genomes compared to that of NZFS3770.
eAll 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).
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 elicitor 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 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 PaRXLR24 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...
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...
site-directed mutagenesis was used to mutate them. Mutated isoforms of these three amino acids in PaRXLR24 (Figure 6a), and replaced with serine (as found in the nonfunctional Avh238 P7076), forms of PaRXLR24, in which proline 59 or alanine 78 had been conserved in the equivalent position in the two proteins. PaRXLR24I81N mutants was not due to protein instability (Figure 6c). Cell death in N. benthamiana, whilst the 51st and 76th amino acids had minor roles (Yang et al., 2011) (Table 2 and Figure 6). The amino acids critical for cell death induction by P. sojae, Avh238 identified the equivalent amino acid at an equivalent position for cell-death activating 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-encoded 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 HP7-2 showed the most consistent and strong

### 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 | Suppressed RXLR-triggered immunity |
|-----------------|-----------------------------------------|---------|--------------|------------------------|-------------------------------|----------------------------------|
| PaRXLR2         | Phytophthora cactorum hypothetical protein | 2.0E−38 | RAW43538.1   | 53                     | Weak                          | No                               |
| PaRXLR5<sup>b</sup> | Phytophthora parasitica P1569 hypothetical protein | 6.0E−22 | ETI36999.1   | 45                     | Strong                        | No                               |
| PaRXLR12        | Phytophthora megakarya hypothetical protein | 4.0E−21 | OWZ07307.1   | 51                     | Weak                          | No                               |
| PaRXLR24<sup>c</sup> | P. parasitica pPE4<sup>4</sup> | 3.0E−48 | XP_008899734.1 | 65                     | Strong                        | Strong                           |
| PaRXLR29        | P. parasitica hypothetical protein        | 9.0E−19 | XP_008894466.1 | 38                     | Weak                          | No                               |
| PaRXLR52<sup>d</sup> | Phytophthora sojae hypothetical protein | 7.0E−56 | XP_009533161.1 | 62                     | Weak                          | No                               |
| PaRXLR54        | Phytophthora palmivora avirulence protein | 5.0E−100| POM79043.1   | 45                     | Strong                        | Strong                           |
| PaRXLR66<sup>e</sup> | P. megakarya RXLR protein | 2.0E−27 | OWZ17774.1   | 37                     | Weak                          | No                               |
| PaRXLR40        | P. palmivora RXLR protein<sup>a</sup>  | 1.0E−15 | POM65748.1   | 38                     | No                            | Yes                              |

<sup>a</sup>Reciprocal top BLAST hit.
<sup>b</sup>Ortholog of P. nicotianae Avh8 (KUG01203.1). E value 2e−21, 43.4% amino acid identity.
<sup>c</sup>Ortholog of P. sojae Avh238 (AEK81002.1). E value 8e−28, 46.4% amino acid identity.
<sup>d</sup>Ortholog of P. palmivora Avr1b-1 (POM62471.1). E value 4e−39, 60.7% amino acid identity.
<sup>e</sup>Only PaRXLR66 showed single nucleotide polymorphism variation among 14 P. agathidicida genomes.
<sup>f</sup>RXLR triggered cell death on N. tabacum (Nt) or N. benthamiana (Nb).
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 immunoblot 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).
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 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).
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
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 (H79I) 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 (Broekgaard et al., 2015). However, it is not known if...
### TABLE 3 Identification of *Nicotiana benthamiana* NBS-LRR candidates involved in recognition of PaRLXR24

| NBS-LRR Genome v. 0.4.4 (v. 1.0.1) | Hairpin screen | VIGS screen |  |
|-----------------------------------|----------------|-------------|---------|
|                                | Suppression (%) | Silencing efficiency (%) ± SD | TRV construct | Suppression (%) | Ion leakage (%) ± SD | Silencing efficiency (%) ± SD | 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<sup>h</sup> |
| 1428g0007.1 (05653g00005.1) | HP7-2 81.3 11.9 ± 19.1 | TRV-NLR6 Yes 40.8* | 58.1 ± 7.0 | R1-A-like<sup>i</sup> |
| 4955g0024.1 (03461g05022.1) | HP14-6 61.3 38.1 ± 9.9 | TRV-NLR1 No 82.4 | 65.0 ± 6.0 | R1-B-like<sup>i</sup> |
| 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<sup>i</sup> |

*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.

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

*Percentage of infiltration spots showing full or partial suppression of PaRXLR24-triggered cell death.

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

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

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

*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.

<sup>h</sup>El Kasmi et al. (2017).

<sup>i</sup>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; Brendelis 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 & Olivier, 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).
transformation assays were scored 7 days post-infiltration. A. tumefaciens death. For suppression assays, ger cell death on N. benthamiana 14 with either signal peptide PR1 The RXLR PCR products, PaRXLR24 WT and mutant plasmids, along with the Q-Gene method (Muller et al., 2002), using the geometric analysis. 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).

4.2 | A. tumefaciens-mediated transient transformation assays

RXLR effector gene candidates and PaNF1-1 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 SmaI-digested pCH41021 (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 pICH86989 (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 MgCl₂, 10 mM MES-KOH pH 5.6, 100 μM acetosyringone) and infiltrated into N. benthamiana or N. tabacum leaves at a final OD₅₀₀ 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 OD₅₀₀ 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 (1/2000), -HA (1/10000) (Santa Cruz Biotechnology) or -FLAG (1/5000) (Sigma-Aldrich) primary antibody and chicken anti-mouse IgG-HRP (1/20000) (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).

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 microtitre 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 OD600 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 (EF1α) 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 Zealand) 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 Yuying 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;00:1–18. [https://doi.org/10.1111/mpp.12967]