Engineering bacteriocin-mediated resistance against the plant pathogen *Pseudomonas syringae*

William M. Rooney1,2,*, Rhys W. Grinter2,*,†, Annapaula Correia3,4, Julian Parkhill3,5,6, Daniel C. Walker2,* and Joel J. Milner1,*

1Plant Science Group, Institute of Molecular, Cell and Systems Biology & School of Life Sciences, University of Glasgow, Glasgow, UK
2Institute of Infection, Immunity & Inflammation, College of Medical, Veterinary & Life Sciences, University of Glasgow, Glasgow, UK
3Wellcome Sanger Institute, Wellcome Trust Genome Campus, Hinxton, UK

Summary
The plant pathogen, *Pseudomonas syringae (Ps)*, together with related *Ps* species, infects and attacks a wide range of agronomically important crops, including tomato, kiwifruit, pepper, olive and soybean, causing economic losses. Currently, chemicals and introduced resistance genes are used to protect plants against these pathogens but have limited success and may have adverse environmental impacts. Consequently, there is a pressing need to develop alternative strategies to combat bacterial disease in crops. One such strategy involves using narrow-spectrum protein antibiotics (so-called bacteriocins), which diverse bacteria use to compete against closely related species. Here, we demonstrate that one bacteriocin, putidacin L1 (PL1), can be expressed in an active form at high levels in Arabidopsis and in *Nicotiana benthamiana in planta* to provide effective resistance against diverse pathovars of *Ps*. Furthermore, we find that *Ps* strains that mutate to acquire tolerance to PL1 lose their O-antigen, exhibit reduced motility and still cannot induce disease symptoms in PL1-transgenic Arabidopsis. Our results provide proof-of-principle that the transgene-mediated expression of a bacteriocin in * planta* can provide effective disease resistance to bacterial pathogens. Thus, the expression of bacteriocins in crops might offer an effective strategy for managing bacterial disease, in the same way that the genetic modification of crops to express insecticidal proteins has proven to be an extremely successful strategy for pest management. Crucially, nearly all genera of bacteria, including many plant pathogenic species, produce bacteriocins, providing an extensive source of these antimicrobial agents.

Introduction

*Pseudomonas syringae* (*Ps*) is a Gram-negative bacterial plant pathogen. The *Ps* species complex consists of over 50 known pathovars (pv.), which are responsible for a variety of different diseases, such as spot and blight disease and bacterial speck, in a wide range of agronomically important crops, including tomato, beans and tobacco (O’Brien et al., 2011; Lamichhane et al., 2014; Lamichhane et al., 2015). Once a plant pathogen is introduced into a crop, it can spread rapidly because of the lack of genetic diversity in commercial crop varieties (Esquinás-Alcázar, 2005).

A recent example of this is the pandemic caused by *Ps* pv. *actinidiae* (*PsA*), which is currently causing great damage to the global kiwifruit industry (Vanneste, 2017). The emergence of canker disease on commercial kiwifruit (*Actinidia* spp.) varieties has been well documented since the early years of *A. delicosa* domestication in Japan in 1984 (Serizawa et al., 1989) and has subsequently spread worldwide (Takikawa et al., 1989; Scortichini, 1994), and the emergence of hypervirulent strains of *PsA* has exacerbated the problem (Balestra et al., 2009; Everett et al., 2011). For example, *Psa* was detected in 37% of New Zealand’s kiwifruit orchards, with the total cost to the industry perhaps exceeding $1.33 billion (Vanneste, 2017).

Currently, chemicals (e.g. copper salts or antibiotics) are used to protect crops from these bacterial pathogens, often with limited success. They also may have adverse environmental impacts because of off-target activity and can encourage the evolution of resistance among bacterial populations (Damalas and Eleftherohorinos, 2011; Sundin and Bender, 1993). The introduction of resistance genes, such as *EFR* in tobacco and tomato, has been successful in providing resistance against *Ps* (Lacombe et al., 2010). However, there is a distinct lack of diversity of suitable natural resistance genes that can be introduced into commercial crops. There is therefore a pressing need to develop new technologies to introduce disease resistance into economically important crops to protect them from plant pathogens like *Ps*.

The large *Ps* species complex means that individual *Ps* species are under intense selective pressure to evolve mechanisms to eliminate inter- and intra-species competition in their environmental niche. One mechanism used to eliminate competitor strains is the production of bacteriocins, which are narrow-spectrum, proteinaceous antibiotics that target and kill related bacterial species. The highly targeted, antibiotic activity of bacteriocins could potentially be exploited to provide crops with protection against specific bacterial pathogens with minimal...
impact on the wider microbial community (Riley and Wertz, 2002).

Various prospective bacteriocins have been identified in *Pseudomonas* spp., including putidacin L1 (PL1), a 30 kDa lectin-like bacteriocin that is highly potent against *Ps. syringae*, *lachrymans* and *mosprunorum* (Parrot et al., 2003; Parrot et al., 2005). The lectin-like bacteriocins bind to D-rhamnose-containing oligosaccharides that are incorporated into lipopolysaccharide (LPS) on the bacterial surface (Ghequire et al., 2013; McCaughey et al., 2014). This binding facilitates the docking of PL1 on the cell surface and its interaction with the outer membrane insertase BamA, leading to the death of the cell via an unknown mechanism (Ghequire et al., 2018). We are not aware of any prior reports of attempts to express bacteriocins in planta as a strategy to confer resistance against plant pathogenic bacteria. Bacteriocins with activities against *E. coli*, *Salmonella* and *Pseudomonas aeruginosa* have been expressed in plants but with the objective of using these as a means of treating bacterial infections in humans (Schulz et al., 2015; Paškevičius et al., 2017; Schneider et al., 2018). The resulting successful demonstration that active bacteriocins can be expressed *in planta* suggests that PL1 could also be expressed *in planta* in an active form to protect plants against *Ps* infection.

The use of novel peptides such as antimicrobial peptides (AMPs) for defence against pathogens in agriculture is not a novel concept and there are a number of reports of AMPs being tested as a strategy for conferring pathogen resistance (De Souza Cândido et al., 2014; Holaskova et al., 2014; Ageitos et al., 2017). Interestingly, AMP activity is not always mirrored *in planta* compared with activity *in vitro*. This is mainly attributable to factors such as salt concentration, protease-based degradation and inhibition by phenolic compounds (Zeitler et al., 2013). Furthermore, AMPs have been shown to be sensitive to divalent cations common in the apoplastic fluid-like Ca²⁺ and Mg²⁺, which can drastically reduce their efficacy (De Bolle et al., 1996; Güell et al., 2011). Therefore, bacteriocins exhibit significant potential advantages over more generalized antimicrobials because of their highly targeted activity at low concentrations.

In this study, we explore this possibility and demonstrate that active PL1 can be efficiently expressed in both *Nicotiana benthamiana* and Arabidopsis. We show that the transient expression of PL1 in *N. benthamiana* and its stable expression in Arabidopsis provides quantitative and qualitative disease resistance against PL1-sensitive strains of *Ps*. Furthermore, we show that mutations associated with PL1-insensitivity/tolerance are linked to the LPS biosynthesis machinery and that *Ps* mutants with increased tolerance to PL1 are still unable to induce disease symptoms in transgenic plants. We conclude from our results that the transgenic expression of a bacteriocin in planta can provide robust disease resistance against the bacterial phytopathogen *Ps*.

**Results**

**PL1 has a narrow killing spectrum**

To determine the killing spectrum of PL1 against *Ps* pathovars, recombinant PL1-His-six was purified from *E. coli*. The killing activity of the purified protein was then assessed against a panel of 22 diverse *Ps* pathovars, including pathogens of kiwifruit, locust bean, oat, soybean, cucumber, cabbage, cherry, plum, olive, pear, maize, lilac and tomato. Of the 22 strains tested, 10 (from 6 different *Ps* pathovars) were sensitive to PL1 (Table 1 and Figure S1), including all three members of the *syringae* group. Minimum inhibitory concentrations of PL1 ranged from 0.85 nM to 1.8 µM, with several of the pathovars showing sensitivity at nanomolar concentrations. All 4 members of the tomato group were resistant to PL1. We conclude that PL1 has a very specific killing spectrum making it an ideal candidate for expression in plants.

| Pathovar   | Strain ID | Sensitive to 10 µM PL1? | MIC (nM) | Origin   | Host          |
|------------|-----------|-------------------------|----------|----------|---------------|
| *actinidiae* | NCPPB 3738 | Yes                      | 125      | Japan    | *Actinidia delici* |
| *actinidiae* | NCPPB 3739 | Yes                      | 125      | Japan    | *Actinidia delici* |
| *ciccaronei* | NCPPB 2355 | No                      | –        | Italy    | *Conataonia siliqua* |
| *coronafaciens* | LMG 5060  | No                      | –        | UK       | *Avena sativa* |
| *glycinea*   | NCPPB 2070 | Yes                     | 1070     | USA      | *Glycine max* |
| *glycinea*   | NCPPB 1245 | Yes                     | 1070     | Canada   | *Glycine max* |
| *glycinea*   | NCPPB 2895 | No                      | –        | Australia| *Glycine max* |
| *glycinea*   | NCPPB 3643 | No                      | –        | Brazil   | *Glycine max* |
| *lachrymans* | LMG 5456  | Yes                     | 22.9     | UK       | *Cucumis sativus* |
| *maculicola* | LMG 2208  | No                      | –        | UK       | *Brassica oleracea* |
| *mosprunorum* | LMG 2222  | Yes                     | 0.85     | UK       | *Pruunus avium* |
| *persicae*   | NCPPB 3687 | No                      | –        | New Zealand| *Pruunus salicina* |
| *persicae*   | NCPPB 2254 | No                      | –        | France   | *Pruunus salicina* |
| *savastoni*  | NCPPB 1506 | Yes                     | 325      | Italy    | *Olea europaea* |
| *savastoni*  | NCPPB 2327 | Yes                     | 325      | Italy    | *Olea europaea* |
| *syringae*   | LMG 5084  | Yes                     | 5.6      | UK       | *Pyrus communis* |
| *syringae*   | LMG 5082  | Yes                     | 8.3      | UK       | *Zea Mays* |
| *syringae*   | LMG 1247  | Yes                     | 1850     | UK       | *Syringa vulgaris* |
| *tomato*     | NCPPB 3160 | No                      | –        | UK       | *Solanum lycopersicium* |
| *tomato*     | NCPPB 2563 | No                      | –        | UK       | *Solanum lycopersicium* |
| *tomato*     | NCPPB 1107 | No                      | –        | UK       | *Solanum lycopersicium* |
| *tomato*     | DC3000   | No                      | –        | USA      | *Solanum lycopersicium* |
Expression of PL1 in *N. benthamiana* provides robust resistance against *Ps. syringae* LMG5084

Previously, bacteriocins that are active against human pathogens have been expressed in *N. benthamiana* leaves and in leafy green vegetables (Schulz et al., 2015; Paškevičius et al., 2017; Schneider et al., 2018). To express PL1 in planta, a construct that encodes PL1 with an N-terminal 4×-c-Myc tag was cloned into a Ti binary vector and transiently expressed in leaves of *N. benthamiana* using agroinfiltration. By 3-days post-infiltration, leaf extracts showed high levels of PL1 in western blots and high killing activity against PL1-sensitive strains in spot tests. We estimated the quantities of PL1 within the infiltrated leaves by comparing in spot tests the killing activity of leaf extracts with PL1 standards produced in *E. coli*. When correlated with killing activity, PL1 levels in planta were equivalent to 0.35% of total plant protein (~5 µM), demonstrating that active PL1 can be produced efficiently in *N. benthamiana* leaves (Figure 1a,b).

After establishing that PL1 can be expressed transiently at high levels in leaves, we challenged these leaves with *Ps. syringae* to establish whether PL1 expression produced a qualitative difference in disease symptoms. Three days post-agroinfiltration (now denoted as day 0), leaves were inoculated with *Ps. syringae* LMG5084 (a pathovar that is highly sensitive to PL1, see Table 1) or with *Pst* DC3000 (a PL1-insensitive strain). Over the next 3 days, leaves were observed for symptom development and bacterial growth was measured. The infiltration of leaves with *Agrobacterium* has been shown to induce immune responses that inhibit the growth of *Ps. syringae* in subsequent inoculation (Zipfel et al., 2006; Rico et al., 2010; Love et al., 2012). We therefore compared the growth of *Ps. syringae* in leaves that transiently express PL1 following agroinfiltration with that in leaves that transiently express green fluorescent protein (GFP), a non-bactericidal protein. In PL1-expressing leaves inoculated with *Ps. syringae* LMG5084, we observed a striking reduction in symptom severity (mild chlorosis only) compared with GFP-expressing controls inoculated with *Ps. syringae* LMG5084, which exhibited black mottling and extensive necrosis by 7 days post-infection (dpi) (Figure 1c).

When we measured bacterial load in *Ps. syringae* LMG5084-inoculated leaves that express PL1, *Ps. syringae* titres were 5-log units lower than in non-agroinfiltrated control leaves; crucially, they were also 3-log units lower than in leaves expressing GFP (Figure S3a). The process of syringe infiltration with buffer did not affect the growth of *Ps. syringae*; neither did GFP expression compared with the empty vector control (Figure S2). In PL1-expressing leaves inoculated with *Ps. syringae* LMG5084, we observed a striking reduction in symptom severity (mild chlorosis only) compared with GFP-expressing controls inoculated with *Ps. syringae* LMG5084, which exhibited black mottling and extensive necrosis by 7 days post-infection (dpi) (Figure 1c).

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As with *N. benthamiana*, although a common bacterial suspension was used to inoculate all plants, titres of bacteria recovered from PL1-expressing lines immediately following inoculation with *Ps. syringae* LMG5084 were lower than expected on the basis of titre of the original inoculum (Figure S4). Again, we assume that live bacteria, recovered from plants at 0 dpi, were being killed by PL1 released from cells during grinding. We therefore measured bacterial growth by quantifying DNA levels by qPCR as previously used with *N. benthamiana* (Figure S7). Here, we carried out infections on 14-day-old seedlings grown on agar plates because disease phenotypes are more pronounced in younger plants (Zipfel et al., 2004; Ishiga et al., 2011). We observed striking differences in symptom severity between NT...
and all three PL1-expressing transgenic lines. By 3 dpi, NT seedlings infected with either P. syringae LMG5084 or Pst DC3000 exhibited severe disease symptoms with most of the seedlings dead or dying (Figure 2c). In contrast, in all three PL1-expressing lines, nearly all the P. syringae LMG5084-infected seedlings appeared to be green and healthy (Figures 2c, S8), whereas Pst DC3000-infected seedlings showed severe symptoms similar to those in NT plants (Figures 2c, S9). To quantify disease resistance, NT Arabidopsis and the 3 PL1-expressing transgenic lines were infected by flooding plates with a suspension of bacteria and samples were taken at 0 and 3 dpi. At 3 dpi, the quantity of P. syringae LMG5084 DNA in the PL1-expressing lines was ~1.5-log units lower than in the NT control (Figure 2d); p values for PL1(1-2), PL1(2-1) and PL1(6-1) were 0.002; 0.006; 0.006, respectively, showing that the differences are highly significant. Bacterial DNA levels in PL1(1-2) seedlings infected with the PL1-insensitive line Pst DC3000 were identical to levels in NT seedlings (Figure 2e).

In conclusion, PL1 was able to provide strong qualitative and quantitative disease resistance to the PL1-sensitive strain P. syringae LMG5084 but not to the PL1-insensitive strain, Pst DC3000.

PL1-mediated resistance is not specific to P. syringae pv. syringae LMG5084

To demonstrate that the disease resistance mediated by PL1 expression in planta is not specific to a single Pst strain, we tested two additional PL1-susceptible pathovars. We first established which of the remaining nine Pst susceptibility strains could establish a compatible infection with Arabidopsis by flood-infecting NT seedlings and screening them for characteristic Pst disease symptoms. From this test, we identified Pst pv. syringae LMG5082 and pv. lachrymans LMG5456 as suitable candidates. Both strains produced much less severe symptoms in PL1-transgenic Arabidopsis compared to NT plants (Figure 3a,c; Figures S10 and S11). Also, relative to NT plants, bacterial DNA

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levels in transgenic lines were 0.8-log units lower for \( \text{PsLMG5082} \) and 1.3-log units lower for \( \text{PsLMG5456} \) (Figure 3b,d). Therefore, PL1-mediated resistance is not confined to a single strain (\( \text{PsLMG5084} \)) nor to \( \text{Ps pv. syringae} \) pathovars.

\textit{P. syringae} mutants insensitive to PL1 lack LPS and show reduced virulence in PL1 transgenic Arabidopsis

In our experiments, high levels of PL1 are produced in planta, which we predict will create a strong evolutionary pressure on \( \text{Ps} \) to acquire mutations conferring decreased sensitivity to PL1. Previous work has shown that LPS constitutes the primary receptor for the lectin-like bacteriocins and that mutations in the LPS synthesis machinery can cause resistance to this class of protein antibiotics (Ghequire et al., 2013, 2018; McCaughey et al., 2014). To assess the robustness of protection against \( \text{Ps} \) that is provided by the in planta production of PL1, we first generated spontaneously arising PL1-insensitive (Pi) mutants by growing \( \text{PsLMG5084} \) in liquid culture in rich media supplemented with 10 \( \mu \text{M} \) PL1. Surviving colonies were subcultured and eight independently arising \( \text{Ps} \) mutants that were highly tolerant to PL1 were selected for characterization. These mutants displayed only hazy zones of clearing at >10 \( \mu \text{M} \) PL1 in a spot test (Table S1). Whole-genome sequencing was then performed to identify mutations that might be responsible for PL1 tolerance. All of the PL1-tolerant lines carried mutations in genes that encode enzymes reported to be involved in LPS biosynthesis (Table S1). To investigate potential defects in LPS production, we purified and analysed LPS profiles isolated from \( \text{PsLMG5084} \) and from eight of the PL1-tolerant mutant lines. Analysis by SDS-PAGE and silver stain showed that each of these mutants lacks.

![Figure 2](https://example.com/figure2.png)

**Figure 2** Growth of PL1-susceptible but not of PL1-resistant strains is attenuated in transgenic Arabidopsis plants that constitutively express PL1. a, PL1 can be expressed to high levels in Arabidopsis, as visualized by western blot analysis. PL1 levels in Arabidopsis whole seedlings obtained from 3 independent PL1-susceptible but not of PL1-resistant strains is attenuated in transgenic Arabidopsis plants that constitutively express PL1. a, PL1 can be expressed to high levels in Arabidopsis, as visualized by western blot analysis. PL1 levels in Arabidopsis whole seedlings obtained from 3 independent myc-tagged PL1 transgenic lines (1-2, 2-1 and 6-1). The corresponding Ponceau S staining of the large RuBisCO subunit (RbcL) was used as a loading control. A positive control (NbPL1) from \( \text{N. benthamiana} \) and a negative control of a non-transgenic Arabidopsis (NT) are also included on this blot. b, Serial dilutions of whole-protein extracts obtained from each Arabidopsis PL1 transgenic line seedlings were spotted onto lawns of LMG5084 to estimate the percentage of PL1 activity. c, PL1-expressing seedlings showed qualitative resistance to \( \text{PsLMG5084} \) but not to \( \text{PstDC3000} \). 14-day-old Arabidopsis PL-expressing (PL1(1-2), PL1(2-1) and PL1(6-1)), and non-transgenic (NT) seedlings were flooded with either \( \text{PsLMG5084} \) or \( \text{PstDC3000} \) for 1 min, and symptoms were left to develop over 3 days. d, e, PL1-expressing seedlings showed quantitative resistance to (d) \( \text{PsLMG5084} \) but not to (e) \( \text{PstDC3000} \). Leaves were collected 0 and 3-day post-infection, and genomic DNA was extracted from the leaves to estimate the bacterial load, as measured by qPCR, relative to plant tissue. Error bars represent the standard deviation of 3 independent replicates. c-myc PL1-expressing seedlings showed qualitatively better growth in PL1 transgenic Arabidopsis plants than in non-transgenic Arabidopsis plants (Figure 3b,d). Therefore, we predict that PL1-mediated resistance is not confined to a single strain (\( \text{PsLMG5084} \)) nor to \( \text{Ps pv. syringae} \) pathovars. Whole-genome sequencing was then performed to identify mutations that might be responsible for PL1 tolerance. All of the PL1-tolerant lines carried mutations in genes that encode enzymes reported to be involved in LPS biosynthesis (Table S1). To investigate potential defects in LPS production, we purified and analysed LPS profiles isolated from \( \text{PsLMG5084} \) and from eight of the PL1-tolerant mutant lines. Analysis by SDS-PAGE and silver stain showed that each of these mutants lacks.
The outer membrane O-antigen that is produced by the parental *Ps* LMG5084 strain (WT; Figure 4a). In addition, each of these mutants showed defects in motility, as measured in swimming assays and increased sensitivity to reactive oxygen species, as determined by exposure to 1% H₂O₂ (Figure 4b,c; Figures S12 and S13). Interestingly, when inoculated with the PL1-tolerant mutants, NT Arabidopsis plants still developed severe symptoms similar to WT *Ps* LMG5084, but the transgenic PL1-producing lines retained a healthy appearance suggesting that the latter retain effective resistance even to PL1-tolerant *Ps* mutants (Figure 4d; Figure S14).

**Discussion**

In this study, we set out to investigate whether the expression of bacteriocins in planta could be used as a strategy to confer resistance against bacterial infection. Here, we have established a proof-of-principle for bacteriocin-mediated resistance against a key genus of plant pathogenic bacteria in two different model plant species. We are therefore optimistic that the concept of bacteriocin-mediated crop protection is viable. Encouragingly, where bacteriocins have previously been assessed by the US FDA for safety as antibacterials for use in humans, they have been classified as ‘Generally Regarded as Safe’ (Schulz et al., 2015). Furthermore, bacteriocins are narrow-spectrum antimicrobial agents and they should therefore selectively target only specific plant pathogenic bacterial species and not affect the many commensal/mutually beneficial bacteria that persist in the plant rhizosphere; however, this requires further investigation (MenDES et al., 2013). The combination of highly specific target range and negligible impact on benign species has been a crucial factor in the extensive worldwide adoption of BT-insecticidal GM crops (KoCH et al., 2015) and we see parallels with the use of bacteriocins for protection against bacterial infections.

The use of novel peptides for defence against pathogens in agriculture is not a novel concept. Over 900 synthetic and natural AMPs have been characterized in the literature with a broad spectrum of effects including defence against pathogens, and there are several reports of transgene-mediated overexpression being tested as strategy for conferring resistance to infection by plant pathogenic bacteria (De Souza Cândido et al., 2014; HolASKova et al., 2014; Ageitos et al., 2017). For example, Hao et al. (2017) reported that expression of the AMP D2A21 conferred resistance against *Ps* pv. tabaci in transgenic tobacco, as evidenced by disease symptoms, although importantly the bacterial titres were not significantly reduced (Hao et al., 2017). Transgenic expression of Bombinbin (Zakharchenko et al., 2018) and LFChimera (Chahardoli et al., 2018) also conferred a degree of broad-spectrum resistance against bacterial pathogens, but again, this was based primarily on symptom expression rather than having a molecular basis.
Figure 4  PL1-tolerant *P. syringae* mutants have mutations in LPS biosynthesis genes and cannot overcome PL1-mediated resistance in Arabidopsis. a, LPS extracted from PL1-tolerant *Ps* lacks O-antigen (PL1-tolerant mutants are labelled Pi1 to Pi-8). LPS of Pi1-8 were visualised by SDS-PAGE and silver staining. b, PL1-tolerant *Ps* show increased sensitivity to hydrogen peroxide. Filter paper soaked with 1 % H$_2$O$_2$ was placed onto lawns of wild type (WT) and mutant *Ps* and incubated overnight. c, PL1-tolerant *Ps* display defective motility. The swimming motility of WT and mutant *Ps* was assessed after 4 days of culture in 0.3% hrp-derepressing media. d, PL1-insensitive *Ps* can produce disease symptoms in non-transgenic plants, but not in PL1-transgenic plants. 14-day-old non-transgenic (NT) or PL1 transgenic (PL1) seedlings were flooded with the mutant bacteria, and symptoms were observed after 3 days. The images shown are representative of the results obtained from three replicate experiments. [Colour figure can be viewed at wileyonlinelibrary.com]

than a reduction in bacterial growth *in planta*. In contrast, the resistance conferred by expression of PL1 appears to be more robust and crucially more targeted against a narrow spectrum of specific bacterial pathogens. Moreover, the impact of AMPs on the root microbiome is still poorly understood; thus, the transgenic expression of biological agents like bacteriocins is advantageous because of their narrow target spectrum. Currently, there has been one study (involving wild tobacco plants expressing Mc-AMP1 from *Mesembryanthemum crystallinum*) that suggested expression of the peptide had a marginal effect on the root-associated microbiota (Weinhold et al., 2018).

To future-proof the use of bacteriocins in agriculture, the expression of heterologous cocktails of bacteriocin proteins would help to overcome the development of bacteriocin insensitivity and ensure the eradication of plant pathogenic bacteria (Wulff et al., 2011). In this study, we show that lectin-like bacteriocins represent promising candidates for transgenic expression. However, genome mining has identified additional classifications of bacteriocins in plant pathogenic bacteria, including talocins, colicin-M like bacteriocins and nuclelease bacteriocins (Grinter et al., 2012a). An important consideration when expressing bacteriocins *in planta* is the effect they might have on plant growth and development. Although we did not observe any obvious phenotypic effects of PL1-expression, we will need to address this more thoroughly in the future. However, from our initial observations, we see no phenotypic differences between the transgenic and the NT plants used in this study. A key question for future studies to address is therefore whether the expression of PL1, or of other bacteriocins, negatively affects crop yield/quality.

A further consideration is the natural selection of bacterial populations that develop insensitivity to a bacteriocin; however, this can come with a fitness cost. For example, non-pathogenic strains of Agrobacterium that express agrocin 84 can suppress the formation of crown gall by pathogenic strains in the field (Kerr and Htay, 1974; Ellis et al., 1979). Our results suggest that for the PL1-tolerant mutants tested here, which were isolated *in vitro*, the levels of PL1 exposure in the transgenic lines remained sufficient to offer robust resistance to infection, although perhaps surprisingly, these mutant lines retained virulence in NT plants. Possibly, the high bacterial titres in the flood inoculation method used in these experiments (much greater than titres that would normally be encountered in natural infections) are masking any fitness costs of a rough LPS phenotype such as the ability to survive out with the plant (epiphytic fitness) or infect in a biologically relevant manner. For example, mutations resulting in the loss of o-antigen in *Ps* pv. *syringae* 61 cause a reduction in virulence in bean pods when inoculated with a toothpick (Deng et al., 2010). Moreover, although LPS mutations associated with PL1 tolerance should diminish the ability of PL1 to dock to the bacterial cells, they will not affect the interaction with BamA which is the primary biological target. This could explain why the transgenic plants retain resistance to the PL1 tolerant mutants, particularly given the high levels of bacteriocin present.

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Bacteriocin production is not exclusive to the genus *Pseudomonas* and so this strategy should in principle be applicable to a wide variety of important phytopathogens, such as *Xanthomonas* spp. (which cause potato soft rot and blackleg; Roh *et al.* 2015) and *Pectobacterium* and *Dickeya* spp. (which causes the greening and blight of rice and banana; Pham *et al.* 2011; Czajkowski *et al.* 2013).

We propose that bacteriocin-mediated resistance in plants represents a technology that can be utilized to control bacterial pathogens in agronomically important crops. Critically, plant-bacterial ecosystems are dynamic and complex, therefore, we expect that their great genomic diversity will promote bacteriocin evolution and hence provide a very large, exploitable resource for future applications.

**Experimental procedures**

**Bacterial strains**

*Ps* isolates (Table 1) were obtained from the National Collection of Plant Pathogenic Bacteria and the Belgian Coordinated Collections of Microorganisms (LMG). *Ps* strains were cultured at 28°C in Kings broth B (KB) media, 20 g/L peptone, 1.5 g/L K₂PO₄, 1.5 g/L MgSO₄ and 10 mL/L glycerol (pH 7.5).

**Motility experiments**

For the motility swimming assay, an overnight liquid culture of *Ps* was stabbed into 0.3 % hrp-derepressing minimal media (10 mM sucrose, 50 mM potassium phosphate buffer, 7.6 mM (NH₄)₂SO₄, 1.7 mM MgCl₂, 1.7 mM NaCl, pH 5.7) agar and incubated at 28°C for 4 days (Huynh *et al.*, 1989; Deng *et al.*, 2010).

**LPS extraction**

A 2 mL bacterial culture with an OD₆₀₀ = 1 was pelleted by centrifugation and washed in 10 mM MgCl₂ (to remove any trailing media). LPS was then extracted using an LPS extraction kit (iNTRON Biotechnology, Gyeonggi, South Korea). The LPS pellet was resuspended in 50 µL of 10 mM Tris, pH 8. To ensure complete solubilization of the LPS pellet, the sample was boiled at 95 °C for 2 min and was further treated with 3 µg/µL of proteinase K at 50 °C for 30 min to obtain highly pure LPS from bacterial cells.

**Plant growth conditions**

*Nicotiana benthamiana* plants were grown using long-day conditions (16 h light/8 h dark at 26 and 18 °C, respectively) at 60% humidity and at a light level of 80 µmol/m²/s. Arabidopsis plants were grown in short-day conditions at light level of 80 µmol/m²/s, consisting of 9 h light/15 h dark (at 22 and 18 °C, respectively) at 60/70% humidity.

**Gene cloning**

To express PL1 in *E. coli*, the PL1-encoding sequence (with no stop codon) was amplified using standard PCR reactions, a high fidelity Phusion Taq polymerase enzyme (New England Biolabs, Hitchin, UK) and appropriate templates, followed by cloning into *Ndel*-*Xhol* sites in the pET21 vector (McCaughey *et al.*, 2014). For constitutive transgene-mediated expression in *planta*, the PL1 coding sequence was fused to an N-terminal 4xMyc tag and cloned into the *KpnI* site of pJOS30, a derivative of pBIN19 (Cecchini *et al.*, 1997). A Ti plasmid vector that expresses GFP (Haseloff *et al.*, 1997) was used as a control for transient assays in *N. benthamiana* (Cecchini *et al.*, 1997). These plasmids are denoted pJOP1 and p35S-GFP, respectively. Plasmids used in this study were linearized by digestion using the appropriate restriction enzymes (New England Biolabs, Hitchin, UK). All DNA constructs were verified by sequencing (Source Bioscience, Oxford, UK).

**Expression and purification of PL1**

Expression and purification were carried out according to McCaughey *et al.* (2014). Briefly, the pET21 plasmid containing PL1 was transformed into BL21 DE3 pLysS cells (Agilent, Edinburgh, UK). PL1 expression was induced at mid-log phase by supplementing the media with 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and the cells were grown at 22°C for 20 h and harvested by centrifugation. The cells were lysed using an MSE Soniprep 150 (Wolf Laboratories, York, UK) and the cell-free lysate was applied to a 5 mL HisTrap HP column (GE Healthcare, Amersham, UK), and PL1 was eluted using a 5–500 mM imidazole gradient. The remaining contaminants were removed by gel filtration chromatography on a Superdex 75 26/600 column (GE Healthcare, Amersham, UK). PL1 was concentrated using a centrifugal concentrator (Vivaspin 20, Epsom, UK) with a 5 kDa molecular weight cut-off and stored at −80°C.

**Soft agar overlay susceptibility assays**

Soft agar overlay spot assays were performed using the method of Frye *et al.* (1984). Fifty microlitres of test strain culture at mid-log was inoculated in 0.8 % soft agar and poured over a KB agar plate, as appropriate. 5 µL of undiluted and serially diluted bacteriocin solution/plant protein extract was spotted onto the plates and incubated for 20 h at 28 °C, after which time the plates were inspected for zones of bacterial growth inhibition.

**Transgene expression in planta**

Transgene expression of the T-DNA constructs in *planta* was achieved by transforming the constructs into *Agrobacterium tumefaciens* (strain GV3101). *N. benthamiana* leaves were infiltrated with GV3101 containing the appropriate vector (Kapila *et al.*, 1997). For Arabidopsis transformations, plants were floral dipped according to Zhang *et al.* (2006). PL1 expression was detected using western blots. Protein was extracted by macerating frozen leaf tissue in 20 mM Tris-HCl, 200 mM NaCl, pH 7.5, supplemented with Complete™, EDTA-free Protease Inhibitor Cocktail (Roche, West Sussex, UK), and the protein concentration of the supernatant was determined by Bradford assay (BioRad, Perth, UK). Ten micrograms of protein extract was separated on 16% SDS-PAGE and transferred onto a PVDF membrane. Proteins were detected using an anti-c-myc monoclonal antibody (sc-40; Santa Cruz Biotech, Texas) and an anti-mouse HRP conjugate antibody (W4021; Promega, Southampton, UK). Ten micrograms of protein extract was separated on 16% SDS-PAGE and transferred onto a PVDF membrane. Proteins were detected using an anti-c-myc monoclonal antibody (sc-40; Santa Cruz Biotech, Texas) and an anti-mouse HRP conjugate antibody (W4021; Promega, Southampton, UK).
bacteria supplemented with 0.025% Silwet L-77 (Lehle Seeds, Texas). Plates containing transgenic seedlings were supplemented with 15 µg/mL of hygromycin B (Sigma-Aldrich, Gillingham, UK).

**Bacterial titre assay using qPCR**

DNA was extracted from infected *N. benthamiana* leaves using a DNAzol kit (Thermo Fisher Scientific, Paisley, UK) according to the manufacturer’s protocol. To extract DNA from infected Arabidopsis seedlings, plant tissue was frozen in liquid nitrogen and the DNA was extracted using FastDNA™ SPIN Kit for Soil (MP Biomedicals, California). Bacterial and plant DNA levels were quantified using qPCR, essentially as described by Love et al. (2007). qPCR was performed in an Applied Biosystems StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Paisley, UK), using Fast SYBR™ Master Mix (Thermo Fisher Scientific, Paisley, UK) and 0.16 µM of primers. Bacterial DNA levels in planta were determined using primers specific for the *Ps* *oprF* gene (Ross and Somssich, 2016) and were normalized against the *Ps oprF* gene in Arabidopsis or the 18S rRNA genes in *ACT2* (Somssich, 2016) and were normalized against the *Ps oprF* gene in Arabidopsis or the 18S rRNA genes in *ACT2* (Somssich, 2016). Statistical analysis used in this study was performed with Minitab 17 statistical software using one-way ANOVA followed by Tukey’s multiple comparison test or a Dunnett’s test.

**Statistical analysis**

Statistical analysis used in this study was performed with Minitab 17 statistical software using one-way ANOVA followed by Tukey’s multiple comparison test or a Dunnett’s test.

**Isolation and whole-genome sequencing and analysis of PL1-insensitive *Ps* strains**

Fifty microlitre aliquots of a *Ps* LMG5084 overnight culture were pelleted at 3000 g for 10 min and re-suspended in 1 mL of 10 µM PL1. The bacteria were then incubated at 28 °C for 4 h, plated out on KB plates, and incubated overnight. DNA was extracted from wild type *Ps* LMG5084 and its PL1-insensitive mutants using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich). Libraries were prepared with the NEBNext Ultra II library kit, according to the manufacturer’s instructions, and sequenced on the Illumina HiSeq x platform to obtain 150 bp paired-end reads with an average depth of 40-fold. Raw sequences from this study have been deposited in the European Nucleotide Archive (ENA) under the accession numbers detailed in Table S3. *Ps* LMG5084 wild type was assembled using Velvet v 1.2 with multiple assemblies generated using VelvetOptimser v 2.2.5 (Zerbino and Birney, 2008; Zerbino, 2010; Page et al., 2016). The assembly with the best N50 was subjected to assembly improvement. Contigs were ordered using Mauve v 2.4.0, scaffolded with SSPACE v 2.0-1 and gaps filled with GapFiller v 1.11-1 (Rissman et al., 2009; Boetzer et al., 2011; Boetzer and Pirovano, 2012). The assembly was then annotated using Prokka v 1.5 and Roary v 3.11.2 based on the reference *Ps* LMG5084 wild type sample (Santisetti et al., 2012). The assembly was then annotated using Prokka v 1.5 and Roary v 3.11.2 based on the reference *Ps* LMG5084 wild type sample (Santisetti et al., 2012).

**Accession Numbers**

| Sanger ID     | Sample     | ENA accession number          |
|--------------|------------|------------------------------|
| 4526ST0Y7070045 | *Ps* LMG5084 Wild type | SAMEA104233059               |
| 4526ST0Y7070060 | *Ps* LMG5084 Pi1       | SAMEA104233065               |
| 4526ST0Y7070076 | *Ps* LMG5084 Pi2       | SAMEA104233071               |
| 4526ST0Y7070084 | *Ps* LMG5084 Pi3       | SAMEA104233074               |
| 4526ST0Y7070092 | *Ps* LMG5084 Pi4       | SAMEA104233077               |
| 4526ST0Y7070100 | *Ps* LMG5084 Pi5       | SAMEA104233080               |
| 4526ST0Y7070108 | *Ps* LMG5084 Pi6       | SAMEA104233083               |
| 4526ST0Y7070116 | *Ps* LMG5084 Pi7       | SAMEA104233086               |
| 4526ST0Y7070037 | *Ps* LMG5084 Pi8       | SAMEA104233055               |

ENA accession numbers for sequenced samples

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**Conflicts of Interest**

A PCT patent (PCT/EP2018/057826) was submitted on behalf of the University of Glasgow in March 2018 to the European patent office.

**Author Contributions**

WMR and RWG carried out the experimental work and contributed to the writing of the manuscript. AC and JP contributed to the genomic sequencing of *P. syringae* pathovars and mutants. DCW and JIM directed the project and contributed to the writing of the manuscript.

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