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Novel Rosaceae plant elicitor peptides as sustainable tools to control *Xanthomonas arboricola* pv. *pruni* in *Prunus* spp.

CRISTINA RUIZ†, ANNA NADAL†, EMILIO MONTESINOS AND MARIA PLA*

Institute for Food and Agricultural Technology (INTEA), University of Girona, Campus Montilivi (EPS-1), Girona 17003, Spain

SUMMARY

Fruit crops are regarded as important health promoters and constitute a major part of global agricultural production, and Rosaceae species are of high economic impact. Their culture is threatened by bacterial diseases, whose control is based on preventative treatments using compounds of limited efficacy and negative environmental impact. One of the most economically relevant examples is the pathogen *Xanthomonas arboricola* pv. *pruni* (Xap) affecting *Prunus* spp. The plant immune response against pathogens can be triggered and amplified by plant elicitor peptides (Peps), perceived by specific receptors (PEPRs). Although they have been described in various angiosperms, scarce information is available on Rosaceae species. Here, we identified the Pep precursor (PROPEP), Pep and PEPR orthologues of 10 Rosaceae species and confirmed the presence of the Pep/PEPR system in this family. We showed the perception and elicitor activity of Rosaceae Peps using the *Prunus*–Xap pathosystem as proof-of-concept. Treatment with nanomolar doses of Peps induced the corresponding PROPEP and a set of defence-related genes in *Prunus* leaves, and enhanced resistance against Xap. Peps from the same species had the highest efficiencies. Rosaceae Peps could potentially be used to develop natural, targeted and environmentally friendly strategies to enhance the resistance of *Prunus* species against biotic attackers.

Keywords: LRR-PEPR, plant defence enhancement, plant elicitor peptide (Pep), *Prunus*, Rosaceae, *Xanthomomas arboricola*.

INTRODUCTION

Fruit consumption is part of a healthy and balanced diet, and global fruit production has seen a remarkable increase over the last decade. According to the Food and Agriculture Organization (FAO, 2013), fruit is a major agricultural crop. In temperate climate areas, pome-fruits (apples and pears) and stone-fruits (peaches, nectarines and plums) are the most important.

Several abiotic and biotic diseases limit the production of stone-fruit trees. Various *Prunus* species are important for stone-fruit production, but also as ornamental plants or in wild forests. Bacterial spot and canker of stone-fruits and almond is an economically important disease caused by *Xanthomonas arboricola* pv. *pruni* (hereafter, Xap) that affects a wide range of *Prunus* species worldwide (EPPO, 2015). It is regulated as an A2 quarantine organism by the European Union (EU) Council directive 2000/29/EC (EU, 2000) and by the European and Mediterranean Plant Protection Organization (EPPO) (EPPO, 2003). The efficacy of the current chemical control of the disease, based on the preventative treatment of trees with copper-derivative bactericides and antibiotics, is limited, and these products have a negative environmental impact and may select for resistance in the pathogen population [Baldwin and Rathmell, 1988; European Food Safety Authority (EFSA), 2014]. In addition to the implementation of a disease forecast model (Battilani *et al.*, 1999; Garçin *et al.*, 2011), the development of novel strategies to control bacterial diseases of crops, in particular Xap, based on natural substances with a low toxicity profile, is highly desirable (Rajasekaran *et al.*, 2012).

Plants perceive chemically diverse molecules originating from bacteria, fungi, viruses or herbivores (pathogen-associated molecular patterns, PAMPs) through pattern recognition receptors (PRRs), initiating a set of defence responses known as pattern-triggered immunity (PTI) (Boller and Felix, 2009; Jones and Dangl, 2006). Recently, elicitors of plant defences, such as bacterial flagellin, have emerged as a novel generation of plant protection products (Boller and Felix, 2009; Toquin *et al.*, 2011). In addition, host endogenous patterns (damage-associated molecular patterns, DAMPs) are also known to trigger a PTI-like response. One class of endogenous elicitor is the plant elicitor peptides (Peps) which trigger and amplify the innate immunity of plants against pathogens (Albert, 2013).

Peps are peptide sequences of roughly 20–23 amino acids that derive from the C-terminus of PROPEP precursor proteins (Huffaker and Ryan, 2007). The expression of a number of *PROPEP* genes has been associated with plant defence transcriptomes (Bartels *et al.*, 2013) and can be induced by herbivores, pathogens, PAMPs, wounding or ethylene (ET) and jasmonic acid (JA) hormone treatments (Huffaker and Ryan, 2007; Huffaker *et al.*, 2011, 2013). PROPEPs or mature Peps may be exported to the extracellular space or leak from disrupted cells (Ding and Chen, 2012; Yamaguchi and Huffaker, 2011), and are recognized by the extracellular leucine-rich repeat (LRR) domain of the receptor-like kinase (PEPR) from adjacent cells (Krol *et al.*, 2010; Yamaguchi

* *Correspondence*: Email: maria.pla@udg.edu
†These authors contributed equally to this work.

et al., 2010). This leads to PEPR heteromerization with BAK1 (Brassinosteroid Receptor-Associated Kinase-1), reciprocal phosphorylation and phosphorylation of BIK1 (Botrytis-Induced Kinase-1) (Liu *et al.*, 2013). PEPRs have guanylyl cyclase activity, which seems to contribute to the influx of calcium into the cytosol (Qi *et al.*, 2010) and activates the production of nitric oxide and reactive oxygen species (ROS) (Ma *et al.*, 2013). Further activation of calcium-dependent protein kinase (CDPK) and mitogen-activated protein kinase (MAPK) cascades increases the levels of ET and JA, which modulate the activity of transcription factors promoting the expression of a set of defence genes, including *PEPR*s and *PROPEP*s, resulting in the accumulation of defence proteins and metabolites [reviewed in Bartels and Boller, 2015; Yamaguchi and Huffaker, 2011].

The plant immunity Pep and PEPR system structure and function have been studied extensively in the model plant *Arabidopsis* (Bartels *et al.*, 2013; Huffaker and Ryan, 2007; Huffaker *et al.*, 2006; Klauser *et al.*, 2015; Tintor *et al.*, 2013; Yamaguchi *et al.*, 2010) and *Zea mays* (Huffaker *et al.*, 2013; Lori *et al.*, 2015). Orthologues of the *PROPEP* and *PEPR* genes have been identified in most angiosperm species (Lori *et al.*, 2015); but different numbers of *PROPEP* genes have been found in the different plant species. Up to eight *PROPEP* genes have been described in *Arabidopsis thaliana* and other Brassicaceae (Bartels *et al.*, 2013; Huffaker *et al.*, 2006), seven have been found in *Zea mays* and three in *Oryza sativa* (Huffaker *et al.*, 2011, 2013), and only between one and three in many other species (Huffaker *et al.*, 2013; Lori *et al.*, 2015; Trivilin *et al.*, 2014). Amino acid sequence comparison of Peps from different plant families shows large differences and family-specific Pep-motifs between the Brassicaceae, Solanaceae and Poaceae (Lori *et al.*, 2015). Most plant species contain one or two PEPRs, and conservation of their Pep-recognition LRR domain is lower than that of the catalytic kinase domain (Flury *et al.*, 2013; Huffaker *et al.*, 2013; Lori *et al.*, 2015; Yamaguchi *et al.*, 2006, 2010). Peps from a given plant species can only be perceived by plants from the same family (Huffaker *et al.*, 2013; Lori *et al.*, 2015), even though downstream pathways leading to PTI seem to be highly conserved among species (Lori *et al.*, 2015).

The overexpression or external application of Peps improves the resistance of the plant to pathogen infection. Overexpression of the *AtPROPEP1* precursor gene in transgenic *Arabidopsis* resulted in plants with higher expression levels of defence-related genes and enhancement of pathogen resistance (Huffaker *et al.*, 2006). Similarly, direct application of AtPep1 onto *Arabidopsis* leaves prior to inoculation with bacterial pathogens has been found to activate PTI (Huffaker *et al.*, 2006) and to increase plant resistance (Yamaguchi *et al.*, 2010), whereas local AtPep2 application induces systemic immunity (Ross *et al.*, 2014). Exogenous application of chemically synthesized ZmPep1 has been reported to protect *Z. mays* leaves against southern leaf blight and anthracnose stalk rot caused by *Cochliobolis heterostrophus* and *Colletotrichum graminicola*, respectively (Huffaker *et al.*, 2011). ZmPep3 caused a significant decrease in *Spodoptera exigua* larval mass in maize leaves (Huffaker *et al.*, 2013). Conversely, silencing of *SlPROPEP* made tomato plants more susceptible to *Pythium dissotocum* (Trivilin *et al.*, 2014). However, neither *PEPR* genes nor Peps have been studied in detail within the Rosaceae species, and no attempt has been made to use them to enhance plant resistance to biotic stress.

In this study, as a result of the very limited knowledge on the PROPEP and PEPR sequences within the Rosaceae family, we characterized their Pep–PEPR system using a combination of *in silico* and sequencing approaches, with particular focus on *Prunus* species. We then explored the suitability of the new Rosaceae Peps for increasing the resistance of peach trees to the quarantine pathogen Xap.

RESULTS

Identification of PROPEP and PEPR homologues in the Rosaceae plant family

Recently, PROPEP and PEPR sequences have been identified *in silico* in most sequenced species of angiosperms, increasing the number of known sequences to 74 PROPEP and 42 PEPR (Lori *et al.*, 2015) in 33 species. Only one PROPEP (*Prunus mume* PmPROPEP5) and three PEPR sequences (*Malus domestica* MdPEPR1 and *Prunus persica* PpPEPR1a and PpPEPR1b) have been identified to date in the Rosaceae family (Lori *et al.*, 2015). Here, public databases were comprehensively searched using the described *PROPEP* and *PEPR* gene sequences to advance our knowledge of this system in Rosaceae. Nine *PROPEP* and seven *PEPR* novel sequences were identified *in silico* from *Malus domestica* (Md), *Prunus persica* (Pp), *P. mume* (Pm), *Pyrus bretschneideri* (Pb), *Fragaria* × *ananassa* (Fa) and *F. vesca* (Fv). We used primer pairs designed on the basis of the *Prunus* sequences to polymerase chain reaction (PCR) amplify and sequence two *PROPEP* and two *PEPR* orthologues from *P. avium* (Pa), *P. dulcis* (Pd), *P. domestica* (Pdo) and *P. nucipersica* (Pn). All identified *PROPEP* and *PEPR* sequences in the Rosaceae are shown in Tables S1 and S2 (see Supporting Information). The Rosaceae *PROPEP* DNA sequences were analysed *in silico* to predict gene elements. In agreement with the previously described *PROPEP* sequences, the 18 genes reported here encompassed an intron spanning 67–258 bp and two exons, with that at 3′ encoding the mature Pep. PROPEPs were 76–128 amino acids in length and were predicted to mature into PEPs corresponding to their C-terminal 21–32 amino acids (Fig. 1a).

We aligned the PROPEP amino acid sequences of the Rosaceae together with those previously reported (compiled in Lori *et al.*, 2015). To facilitate comparability, we followed the same analytical

a

sequence	length	Pep	
EVAVSFRVVRPPITRGGGGQIN	22	PaPep1	*Prunus* spp.
EVAGSLRAVRPPITTGGGGQIN	22	PdoPep1	
EVAASSRVIRPPITTGEGGQIN	22	PdPep1	
EVAGSLRAVRPPITTGGGGQIN	22	PmPep1	
EVAASSRVVRQPITTGGGGQIN	22	PnPep1	
EVAASSRVVRQPITTGGGGQIN	22	PpPep1	
YVQRITLRAARPELSTGGGAQTN	23	PaPep2	
YVQRITLRAARPEISTGSGAQTN	23	PdoPep2	
YVQRITLRAARPEISTGSGAQTN	23	PdPep2	
YVQRITLRAARPEISTGSGAQTN	23	PmPep2	
YVQRITLRAARPEISTGSGAQTN	23	PnPep2	
YVQRITLRAARPEISTGSGAQTN	23	PpPep2	
QRGIVCAENPPLSTGSGGQIN	21	MdPep3	Malaceae
DEAAEVTRIKVSTRERPGLSTGEGGKTN	28	MdPep4	
DEAAAITRIKVSARERPGLSTGKGGKTN	28	PbPep4	
EDVVVVATGTARSTRALGVKRPIGTGSGPQIN	32	FvPep5	*Fragaria*
EDVVVVATGTARSTRALGVKRPIGTGSGPQIN	32	FaPep5	
EEGVVIATGTARSTRALRMKRSIGSGSGPQIN	32	FaPep5b	

b

		Fragaria spp.			*Prunus* spp.											Malaceae		
		FvPROPEP5	FaPROPEP5	FaPROPEP5b	PaPROPEP1	PdoPROPEP1	PdPROPEP1	PmPROPEP1	PnPROPEP1	PpPROPEP1	PaPROPEP2	PdoPROPEP2	PdPROPEP2	PmPROPEP2	PnPROPEP2	PpPROPEP2	MdPROPEP3	MdPROPEP4	PbPROPPEP4
Fragaria spp.	FvPep5		98	87	15	17	18	17	17	17	12	13	13	13	13	13	15	14	13
	FaPep5	100		88	14	17	18	16	17	16	12	13	13	13	13	13	14	12	11
	FaPep5b	78	78		12	14	15	13	14	13	10	12	12	12	11	12	13	11	11
Prunus spp.	PaPep1	31	31	25		90	73	91	89	90	18	20	19	20	16	18	17	36	36
	PdoPep1	31	31	22	82		76	96	91	92	20	22	21	21	18	20	17	35	36
	PdPep1	28	28	22	77	77		76	79	79	18	21	21	21	19	20	33	22	33
	PmPep1	31	31	22	82	100	77		92	93	20	21	20	21	17	20	17	35	36
	PnPep1	31	31	22	82	82	86	82		99	19	21	20	21	17	20	16	35	36
	PpPep1	31	31	22	82	82	86	82	100		19	21	20	21	17	20	16	35	36
	PaPep2	16	16	13	39	52	39	52	39	39		87	87	87	76	86	18	20	19
	PdoPep2	22	22	19	39	52	43	52	39	39	91		97	93	83	95	17	21	21
	PdPep2	22	22	19	39	52	43	52	39	39	91	100		93	83	97	17	21	20
	PmPep2	22	22	19	39	52	43	52	39	39	91	100	100		79	93	17	20	19
	PnPep2	22	22	19	39	52	43	52	39	39	91	100	100	100		85	15	18	17
	PpPep2	22	22	19	39	52	43	52	39	39	91	100	100	100	100		17	20	20
Malaceae	MdPep3	31	31	28	29	29	29	29	29	29	25	25	25	25	25	25		15	15
	MdPep4	19	19	19	25	29	36	29	29	29	32	29	29	29	29	29	23		87
	PbPep4	16	16	22	25	29	36	29	32	32	32	29	29	29	29	29	23	86	

c

Fig. 1 Identity comparison of Rosaceae plant elicitor peptide (Pep) precursor (PROPEP) and mature Pep sequences. (a) Rosaceae Pep amino acid sequences, with different amino acids within every Pep cluster highlighted in red. (b) Full-length amino acid sequences of Rosaceae PROPEP (top half) and PEP (bottom half) sequences were compared for the percentage of identical residues in the aligned positions. Colours indicate increasing identity from high (red), through white, to low (blue). PmPROPEP1 corresponds to PmPROPEP5, described in Lori *et al.* (2015). (c) Depiction of the consensus sequences of aligned Rosaceae-specific Pep sequences using the WebLogo tool (Crooks *et al.*, 2004).

approach as in Lori *et al.* (2015). For a total of 91 sequences, Rosaceae PROPEPs did not group into any cluster together with sequences from other families. As with PROPEPs from the Brassicaceae, Fabaceae, Poaceae and Solanaceae, the Rosaceae PROPEPs formed new clusters clearly differentiated from all other analysed species (Table S3, see Supporting Information).

The alignment of all Rosaceae PROPEP sequences showed a wide range of amino acid identity, from 10% up to 99%, within this family, and similar results were obtained when comparing mature Pep sequences (Fig. 1b). Comparison of PROPEP and Pep sequences showed that they were distributed into five homology groups, each with sequences sharing more than 77% and 73% identity, respectively. Sequence identity between different homology groups was below 33% and 52%, respectively. Accordingly, PROPEP sequences were named PROPEP1–5. Two *PROPEP* sequences were identified in every Rosaceae species for which the genome sequence was available or in which it was experimentally searched for (a second Fa, Fv and Pb sequence cannot be ruled out). It should be noted that FaPep5 and FaPep5b were identified in the hybrid *Fragaria* × *ananassa*, a cross of *F. virginiana* and *F. chiloensis*, and were considered to correspond to two forms of the same Pep. There were *PROPEP1* and *PROPEP2* orthologues in all analysed *Prunus* species (Amygdaleae tribe). *PROPEP3* and *PROPEP4* were only found in *Malus* and *Pyrus* species, representing the Maleae tribe, and *PROPEP5* representatives were found only in *Fragaria* spp. (Potentilleae tribe). Phylogenetic analysis of our novel and previously compiled mature Pep sequences (Lori *et al.*, 2015) showed that Peps seem to have diverged in two phylogenetic clusters within the Rosaceae family, and all species tend to harbour a peptide belonging to each cluster (Fig. 2a).

The 18 identified Peps of the Rosaceae shared a consensus sequence in the 10–13 C-terminal residues, as visualized using the WebLogo tool (Fig. 1c). The Rosaceae Pep-motif is mainly composed of amino acids with uncharged side chains. This type of residue also predominates in the Pep-motifs of Brassicaceae, Poaceae (Lori *et al.*, 2015) and Fabaceae (as built using WebLogo with the seven available sequences). The C-terminal GxGxxxN motif was fully conserved in the Rosaceae.

Every Rosaceae species analysed had a *PEPR1a* and *PEPR1b* orthologue, similar to most studied angiosperm species. On alignment of the PEPR amino acid sequences from the Rosaceae and other species (compiled in Lori *et al.*, 2015), 57 sequences, those belonging to the Rosaceae formed a specific group (Table S4, see Supporting Information).

As expected, both the kinase and LRR domains were identified *in silico* (Table S2). As shown in Table 1, the Rosaceae PEPR kinase domain identities ranged from 74% to 98%, whereas those of the substrate recognition LRR domain were somewhat lower (58%–99%). PEPRs grouped into three clusters that corresponded to PEPR1a and PEPR1b from (i) *Prunus* spp., (ii) Malaceae species, and (iii) *Fragaria* spp.

On phylogenetic analysis of the LRR domain of all available PEPR amino acid sequences (this work; Lori *et al.*, 2015), there was a clear family-specific clustering in the most studied plant families, and the Rosaceae formed a clear group (Fig. 2b).

Novel Peps enhance plant resistance against the plant-pathogenic bacterium Xap

Because treatment with Peps from the same plant family has been described to trigger plant defence responses and to significantly improve their resistance to diverse bacterial and fungal pathogens, as well as herbivores (Yamaguchi and Huffaker, 2011), we hypothesized that pretreatment of *Prunus* leaves with the corresponding *Prunus* Pep1 and Pep2 would induce the expression of *PROPEP*, *PEPR* and defence-related genes, and increase their resistance to the quarantine bacterial plant pathogen Xap. We tested this hypothesis using highly homogeneous juvenile GF-677 plants (a cross of *P. persica* × *P. dulcis* commonly used as rootstock). GF-677 plants are susceptible to Xap and the infection can be reproduced under laboratory conditions, including detached leaves. On PCR amplification from leaf genomic DNA and sequencing, sequences encoding the complete PROPEP-PEPR system from Pp and Pd were detected in GF-677, which made it a suitable experimental system.

In an initial approach, we supplied GF-677 leaves with the chemically synthesized peptides PpPep1 and PpPep2, and used reverse transcription coupled to real-time PCR to monitor the mRNA levels of the corresponding *PROPEP* after 1, 3, 12 and 24 h (Fig. S1, see Supporting Information). The expression of *PROPEP1* sustainably increased on treatment with Pep1, reaching levels up to seven-fold those of control leaves treated with water 1 day after treatment. Application of Pep2 induced *PROPEP2* expression, although 1 day after treatment mRNA levels were 10-fold below those of *PROPEP1* in samples treated with Pep1. In addition, treatment with 100 nM PpPep1 and, to a minor extent, PpPep2 transitorily increased the expression of the ET response factors *ERF-1a* and *ERF-2b*. The activation of typical pathogenesis-related genes, such as *PR4*, *PR5-TLP2* and *PR5-TLP3*, gradually increased up to at

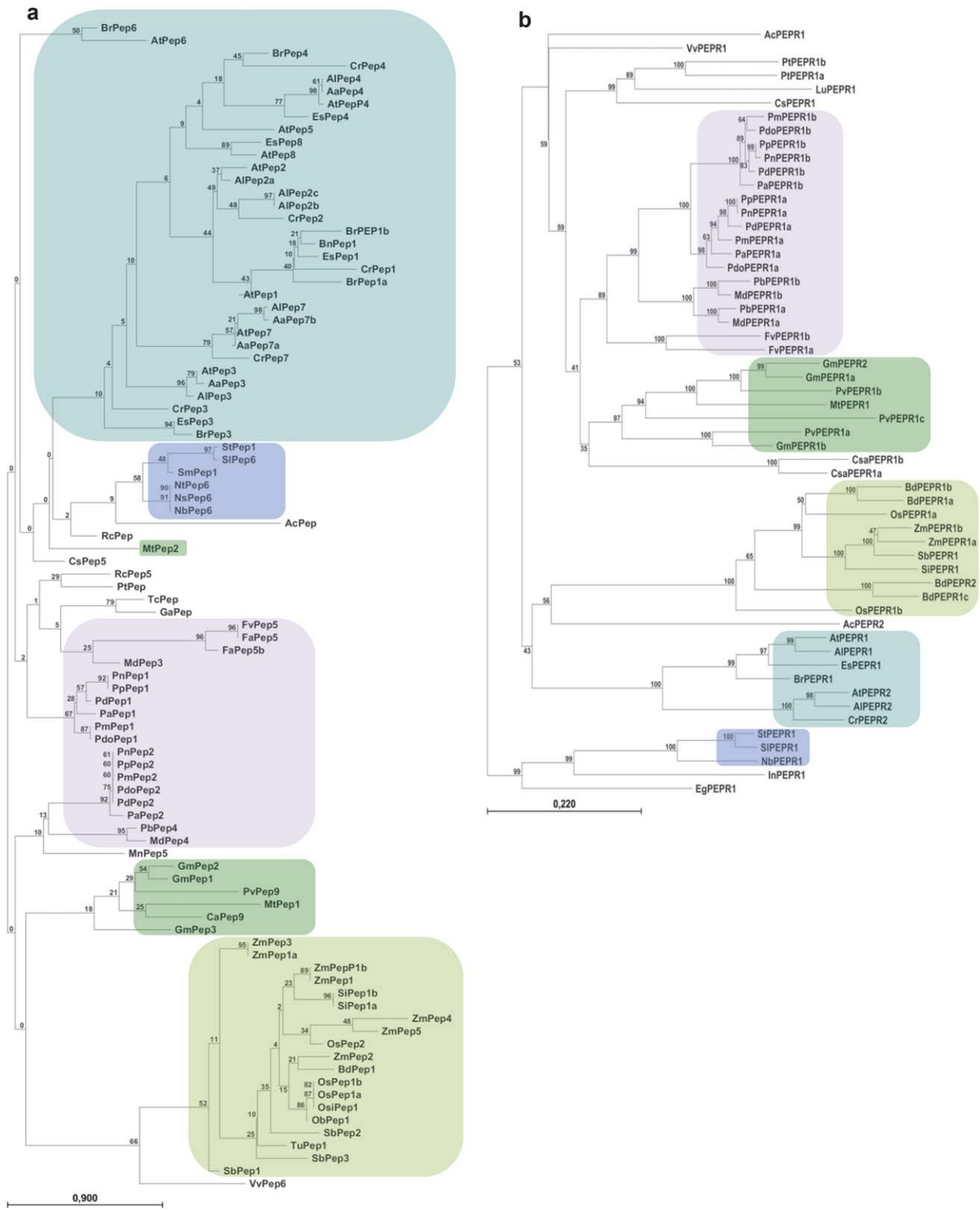

Fig. 2 Bootstrapped neighbour-joining tree of previously reported and novel angiosperm mature plant elicitor peptide (Pep) (a) and leucine-rich repeat (LRR) domain of Pep receptor (PEPR) (b) amino acid sequences, constructed using the CLC tool. Families are shaded as follows: pale blue, Brassicaceae; dark green, Fabaceae; pale green, Poaceae; purple, Rosaceae; dark blue, Solanaceae.

Table 1 Identity comparison of plant elicitor peptide receptor (PEPR) sequences. The amino acid sequences of Rosaceae PEPRs were compared for the percentage of identical residues in aligned positions in their leucine-rich repeat (LRR) (bottom half) and kinase (top half) domains using the CLC tool. Colours indicate increasing identity from low (blue) to high (red) through white. PpPEPR1a, PpPEPR1b and MdPEPR1 (here MdPEPR1a), previously described in Lori *et al.* (2015), are also shown.

		Kinase	*Fragaria* spp.		*Prunus* spp.											Malaceae		
		LRR	FvPEPR1a	FvPEPR1b	PaPEPR1a	PdoPEPR1a	PdPEPR1a	PmPEPR1a	PnPEPR1a	PpPEPR1a	PaPEPR1b	PdoPEPR1b	PdPEPR1b	PmPEPR1b	PnPEPR1b	PpPEPR1b	MdPEPR1a	MdPEPR1b	PbPEPR1a
Fragaria spp.	FvPEPR1a			90	79	75	80	80	80	80	75	76	75	76	76	76	80	79	80
	FvPEPR1b		76		79	74	79	79	80	80	76	77	75	77	76	76	81	79	80
Prunus spp.	PaPEPR1a		62	62		88	94	95	95	95	85	87	85	87	87	87	86	87	86
	PdoPEPR1a		61	61	95		88	88	89	89	83	85	84	85	86	86	82	82	82
	PdPEPR1a		61	61	93	92		96	97	97	86	87	86	87	88	88	86	87	87
	PmPEPR1a		62	62	95	94	95		97	97	87	88	87	90	89	89	86	87	87
	PnPEPR1a		62	62	94	93	97	96		100	87	88	87	88	89	89	87	88	88
	PpPEPR1a		62	62	94	93	97	96	100		87	88	87	88	89	89	87	88	88
	PaPEPR1b		59	59	87	87	86	87	86	86		96	94	95	96	96	82	82	82
	PdoPEPR1b		59	59	86	87	85	86	85	85	96		96	98	98	98	83	83	84
	PdPEPR1b		59	59	86	87	85	86	85	85	96	97		95	98	98	81	81	82
	PmPEPR1b		58	58	85	86	84	85	84	84	94	96	96		96	96	83	83	84
	PnPEPR1b		59	59	86	87	85	86	85	85	95	96	98	95		100	83	83	83
	PpPEPR1b		59	59	86	88	86	86	86	86	96	97	98	95	99		83	83	83
Malaceae	MdPEPR1a		58	59	75	76	74	76	75	75	72	72	72	71	72	72		91	96
	MdPEPR1b		58	59	74	75	74	76	75	75	73	73	73	72	73	73	91		92
	PbPEPR1a		58	58	75	75	74	75	75	75	72	71	71	70	72	72	96	90	

least 24 h after peptide treatment (Table 2). This demonstrated that the PROPEP/PEPR system is working.

In an *ex vivo* approach, leaves from intact plants were treated with water or the chemically synthesized peptides PpPep1, PdPep1 or PpPep2 (with amino acid sequence identical to PdPep2), 1 day before inoculation with different concentrations of Xap. Three weeks after infection, leaves with no peptide pretreatment had the typical symptoms of bacterial spot infection. On inoculation with 10^6 colony-forming units (cfu)/mL Xap, chlorotic and necrotic lesions appeared at the inoculation site and chlorosis was spread along the central nerve in about one-third of the infected leaves. Inoculation of 10^7 cfu/mL Xap resulted in blade weakening at the necrosis site and general chlorosis in the whole leaf area. In contrast, pretreatment with 1000, 100 or 10 nM of PpPep1, PdPep1 or PpPep2 prior to inoculation with 10^6 and 10^7 cfu/mL Xap resulted in leaves with a completely normal phenotype, with the exception of residual chlorosis at the lowest tested PpPep1 concentration (Fig. 3a). One-way analysis of variance (ANOVA) of the intensity of symptoms demonstrated that these Pep treatments efficiently protected plant leaves from Xap infection (Fig. 3b). Xap levels were quantified 3 weeks after infection using quantitative PCR. Infected leaves pretreated with PpPep1, PdPep1 or PpPep2 had lower Xap DNA relative to non-pretreated leaves (Table S5, see Supporting Information). The one-way ANOVA *P* value between different treatments was 0.000 and the Tukey-b *post hoc* test (with $\alpha = 0.01$) separated control and Pep-treated samples. Leaves pretreated with 1 nM PpPep1, PdPep1 or PpPep2 were phenotypically indistinguishable from control leaves with the same Xap inoculum, but no peptide pretreatment (Fig. 3b). Shorter pretreatments (3 h)

Table 2 Expression of a selection of defence-related genes in response to treatment with PpPep1 and PpPep2.

	Treatment (h)											
	PpPep1				PpPep2				Xap			
Target gene	1	3	12	24	1	3	12	24	1	3	12	24
ERF-1a	9.7	2.1	1.5	1.1	2.8	1.6	1.1	1.4	7.3	1.7	0.9	0.9
ERF-2b	2.6	1.5	1.9	1.6	2.1	2.0	1.1	1.0	2.8	0.7	1.6	1.0
PR4	1.1	1.2	4.3	6.8	1.7	1.5	2.2	3.0	nd	nd	nd	2.3
PR5-TLP2	1.5	1.2	3.1	18.9	1.2	0.8	2.5	4.5	nd	nd	nd	7.6
PR5-TLP3	1.6	0.6	1.5	6.4	1.0	0.5	1.3	1.9	nd	nd	nd	0.9

Leaves of juvenile GF-667 plants were excised and treated with water, 100 nM PpPep1 or 100 nM PpPep2 for 1, 3, 12 or 24 h in a humid chamber. Gene expression was assessed by reverse transcription coupled to real-time polymerase chain reaction (PCR) using *TEF2* to normalize the expression values. For each treatment, normalized expression values were divided by those in the corresponding water control to calculate the relative fold change in expression of each target gene. The results show the means of two independent experiments; fold values ≥ 2 had relative standard deviation (RSD) $< 20\%$. The colour intensity is proportional to the fold change in expression values.
nd, not determined.

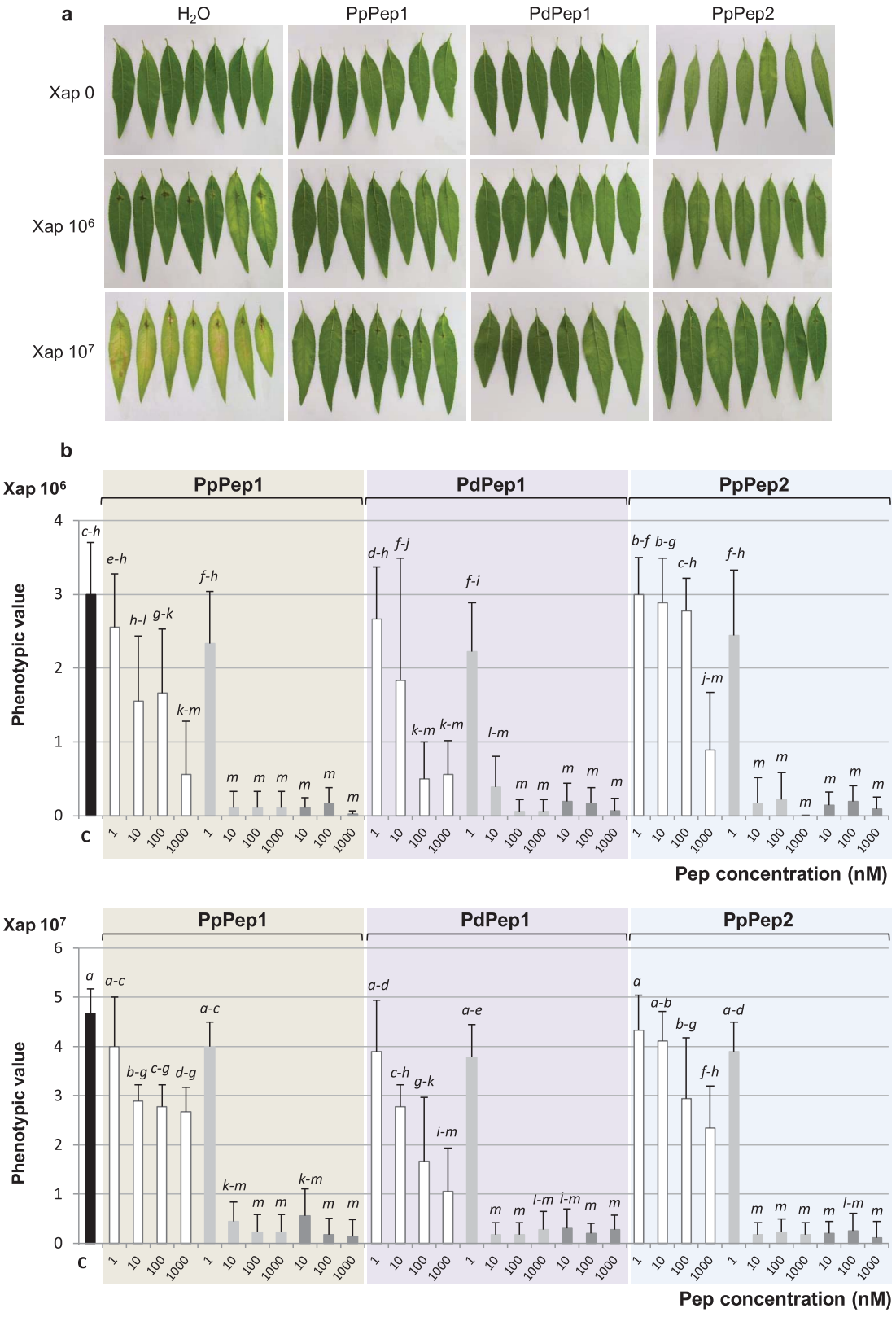
a
H2O
PpPep1
PdPep1
PpPep2
Xap 0
Xap 10^6
Xap 10^7
b
Xap 10^6
PpPep1
PdPep1
PpPep2
Phenotypic value
Pep concentration (nM)
Xap 10^7
PpPep1
PdPep1
PpPep2
Phenotypic value
Pep concentration (nM)

Fig. 3 Elicitor activity of PpPep1, PdPep1 and PpPep2 peptides in protection of GF-677 against *Xanthomonas arboricola* pv. *pruni* (Xap). Leaves of juvenile GF-667 plants were excised, pretreated with 1000, 100, 10 and 1 nM PpPep1, PdPep1 or PpPep2 for 3 h, 1 or 2 days before inoculation with 50 μL of 10^6 or 10^7 colony-forming units (cfu)/mL of exponentially growing Xap. Infection was allowed to proceed for 3 weeks under controlled conditions (16-/8-h light/dark photoperiod, 25 ± 1 °C) in a humid chamber. The severity of infection was measured on an arbitrary scale from '0' (normal phenotype) to '5' (severe necrotic lesions at the inoculation site and the spread of chlorosis throughout the leaf). (a) Examples illustrating the phenotypic effects of PpPep1, PdPep1 and PpPep2 pretreatments. One-day pretreatments with 100 nM peptides are shown, together with control leaves: (i) pretreated with water to show normal infection; and (ii) pretreated with water or 100 nM peptides and not Xap-inoculated. (b) Means and standard deviations (SDs) of the phenotypic evaluation of Xap infection in leaves pretreated with water (C, black bars) and increasing concentrations of PpPep 1, PdPep1 or PpPep2 for 3 h (white bars), 1 day (light grey bars) and 2 days (dark grey bars) prior to Xap inoculation at 10^6 and 10^7 cfu/mL concentration. Letters indicate statistically significant differences [one-way analysis of variance (ANOVA), Tukey-b *post hoc* test with $\alpha < 0.01$]. Note that PdPep2 and PpPep2 have identical sequences.

resulted in leaves with somewhat reduced disease symptoms relative to infected control leaves (Fig. 3b), although these differences tended to be statistically significant only at high Pep concentrations and full protection was not achieved. In contrast, a period of 2 days between Pep treatment and Xap infection did not alter the protection effect observed with 24-h pretreatments (Fig. 3b). As a further control, PpPep1, PdPep1 and PpPep2 were applied with no subsequent Xap inoculation; at the end of the experiment, these leaves were phenotypically indistinguishable from those treated with water (Fig. 3a). In an attempt to demonstrate that peptide pretreatment did not protect GF-677 leaves through direct antibacterial activity, we finally assessed the capacity of PpPep1, PdPep1 and PpPep2 to inhibit the growth of the same Xap strain in an *in vitro* test (Fig. 4a). No interference with normal Xap growth was observed at 10, 100 or even 1000 nM Pep (i.e. 100-fold above the active dose) up to 3 days after inoculation. Similarly, Xap death curves showed a lack of any bactericidal effect by PpPep1, PdPep1 and PpPep2 over a 6-h time course (Fig. 4b). The synthetic antibacterial peptide BP100 was used as a positive control (Badosa *et al.*, 2007).

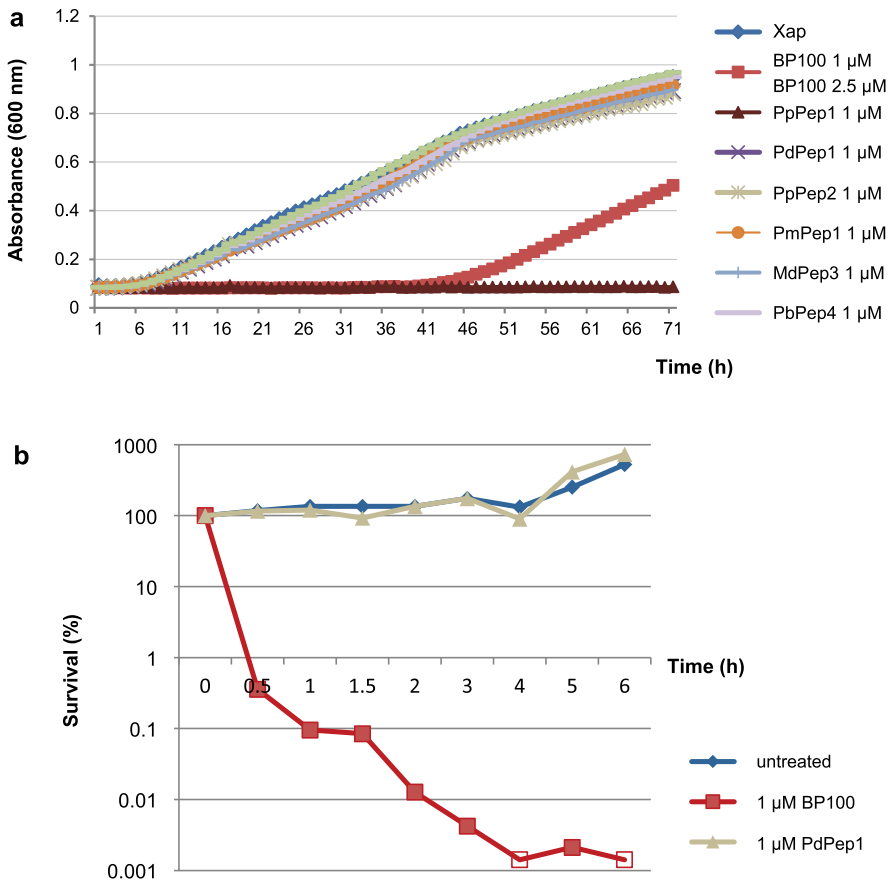

Fig. 4 *In vitro* assessment of the antibacterial activity of PpPep1, PdPep1, PpPep2, PmPep1, MdPep3 and PbPep4 against *Xanthomonas arboricola* pv. *pruni* (Xap). (a) *In vitro* growth inhibition test at 1000, 100, 10 and 0 nM final plant elicitor peptide (Pep) concentrations, using BP100 (2.5 and 1 μM) as positive control. Representative examples of growth curves are shown. (b) Kinetics of bactericidal activity on mid-logarithmic phase Xap cultures in the presence of 1 μM PdPep1 taken as an example, using 1 μM BP100 as control. Viable cells were counted at different time intervals. Empty squares correspond to survival values below the limit of detection (LOD) of 55 colony-forming units (cfu)/mL.

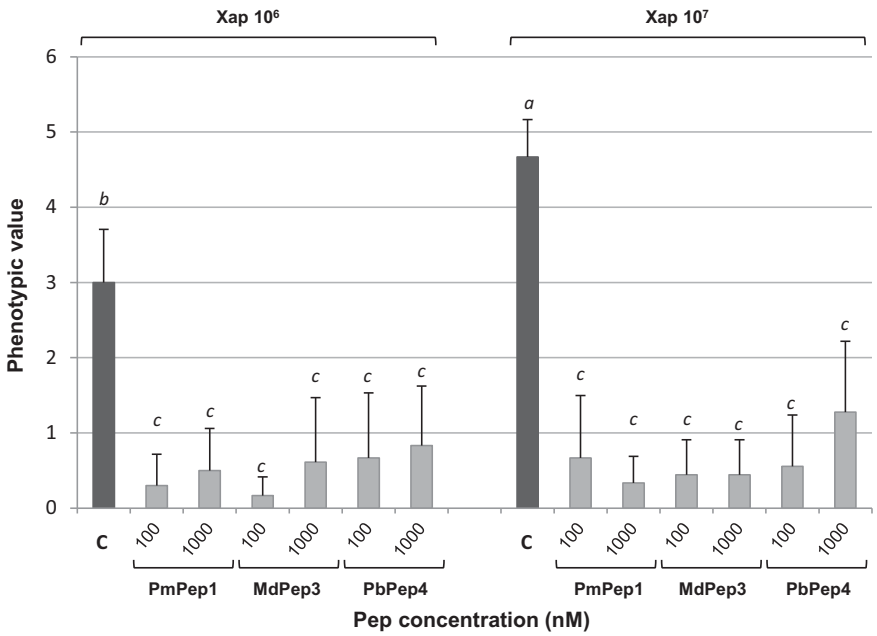

Fig. 5 Inter-species compatibility of the elicitor activity of Rosaceae plant elicitor peptides (Peps) in the protection of GF-677 against *Xanthomonas arboricola* pv. *pruni* (Xap). Leaves of juvenile GF-667 plants were excised, pretreated with 1000 and 100 nM PmPep1, MdPep3 or PbPep4 and inoculated with 50 μL of 10^6 or 10^7 colony-forming units (cfu)/mL of exponentially growing Xap after 1 day of incubation with the peptides. Infection was allowed to proceed for 3 weeks under controlled conditions (16-/8-h light/dark photoperiod, 25 ± 1 °C) in a humid chamber. The severity of infection was measured on an arbitrary scale from '0' (normal phenotype) to '5' (severe necrotic lesions at the inoculation site and the spread of chlorosis throughout the leaf). Means and standard deviations (SDs) of the phenotypic evaluation of Xap infection in leaves pretreated with water (C, black bars) and increasing concentrations of PmPep1, MdPep3 or PbPep4 (grey bars) prior to Xap inoculation at 10^6 and 10^7 cfu/mL concentration. Letters indicate statistically significant differences [one-way analysis of variance (ANOVA), Tukey-b *post hoc* test with $\alpha < 0.01$].

Thus, PpPep1, PdPep1 and PdPep2/PpPep2 pretreatment protected GF-677 leaves from Xap attack in a dose-dependent manner, and was mostly effective after at least 1 day of application before pathogen inoculation.

Inter-species compatibility of Peps within the Rosaceae family

Peps from a given plant species have been shown to elicit defences not only in their original plant species, but also in those of the same family. This has been observed in the Brassicaceae, Poaceae and Solanaceae (Huffaker *et al.*, 2013; Lori *et al.*, 2015). Pep and PEPR recognition sequences are postulated to have co-evolved, leading to family-specific Pep motifs and a lack of compatibility between plants from different families.

We assessed the possible compatibility between Rosaceae plant species in a detached leaf infection assay, using a peptide from a different *Prunus* species, *P. mume* (PmPep1), and two peptides from other Rosaceae species not belonging to the Amygdaleae tribe, *M. domestica* and *Pyrus bretschneideri* (MdPep3 and PbPep4). None of these Peps showed antibacterial activity against Xap, as demonstrated in *in vitro* growth inhibition and cell death tests (Fig. 4a). GF-677 leaves were pretreated with 1000 and 100 nM PmPep1, MdPep3 or PbPep4 for 1 day and infected with 10^6 and 10^7 cfu/mL Xap. After 3 weeks, Xap symptoms were visually estimated using the described phenotypic scale. Leaves of plants pretreated with any peptide showed highly reduced infection symptoms relative to control leaves infected with the same Xap inocula (one-way ANOVA, $P < 0.01$, Fig. 5). This demonstrated the compatibility of the tested Peps within the Rosaceae.

DISCUSSION

The PROPEP/PEPR system has been studied extensively in *Arabidopsis* and maize, and its role in the enhancement of resistance against microbial pathogens through the induction and amplification of innate immunity has been described in these model species (Huffaker *et al.*, 2006, 2011; Liu *et al.*, 2013). *PROPEP* orthologues have been identified in a range of angiosperm species and they show extensive sequence diversity (Lori *et al.*, 2015). In spite of their economic relevance, scarce information is available to date on PROPEP and PEPR sequences and function in Rosaceae species. The objective of this study was to identify and characterize the PROPEP/PEPR system in one of the most widely commercialized species of this plant family, and to assess the capacity of the new Peps to enhance resistance to pathogens; we used the economically relevant pathosystem Xap–*Prunus* spp. as proof-of-concept.

In a combined approach of *in silico* genome database search and experimental sequencing (when *in silico* data were not available), *PROPEP* and *PEPR* sequences were identified in Rosaceae species: almond (Pd), apple (Md), apricot (Pm), cherry (Pa), nectarine (Pn), peach (Pp), pear (Pb), plum (Pdo) and strawberry (Fa and Fv). By enlarging the range of species known to contain these sequences, we further confirmed the extensive presence of the PROPEP/PEPR system within the angiosperms, putting the Rosaceae family amongst the most well-known in terms of the number of reported sequences, alongside the Brassicaceae and Poaceae. On alignment of mature Pep sequences from the Rosaceae and other angiosperms, and the corresponding PEPR-LRR recognition domains, the Rosaceae sequences grouped into phylogenetic clusters separate from other plant families, as has been reported for the Poaceae, Brassicaceae, Fabaceae and Solanaceae (Lori *et al.*, 2015).

Although we cannot completely exclude the existence of additional, more distant, Pep sequences, the analysis of 10 Rosaceae species indicated that they each have a limited number of Peps. Only one to three Peps have been reported in most studied species from other plant families (Huffaker *et al.*, 2013; Lori *et al.*, 2015; Trivilin *et al.*, 2014), with the remarkable exceptions of *A. thaliana* (eight Peps; Bartels *et al.*, 2013; Huffaker *et al.*, 2006) and *Z. mays* (five Peps; Huffaker *et al.*, 2011, 2013). Functions for most *Arabidopsis* and maize Peps are still lacking and the extent of redundancy between individual peptides is not well understood. Different *PROPEP* genes have distinct expression patterns that are regulated in a tissue- and treatment-specific manner; factors such as microbe-associated molecular patterns (MAMPs), hormones, pathogens or the same Peps have been described to enhance the expression of certain *PROPEPs* (Bartels and Boller, 2015; and references therein). In spite of the number of Peps in *Arabidopsis*, AtPEPR1 and AtPEPR2 are the only receptors capable of interacting with AtPeps (Flury *et al.*, 2013; Krol *et al.*, 2010; Yamaguchi *et al.*, 2010). Two receptors have been described in most species, *Glycine max* being amongst the few exceptions having three PEPRs (Lori *et al.*, 2015). Similar to PEPRs from many other species, such as maize and rice, the two sequences in every Rosaceae species analysed showed strong homology to AtPEPR1, and corresponded to PEPR1a and PEPR1b. This indicates that the PROPEP/PEPR system in the Rosaceae most probably has two LRR-receptor-like kinases and two PROPEPs, giving rise to two mature Peps.

Not only was the Rosaceae PROPEP/PEPR system predicted from genomic sequencing, it also proved to be functional. In *Arabidopsis* and *Z. mays*, Pep perception triggers the transcription of the corresponding *PROPEP* in a positive feedback loop (Huffaker *et al.*, 2006). Exogenous application of chemically synthesized PpPep1, PdPep1 (data not shown) or PpPep2 (which is identical to PdPep2) induced the expression of the corresponding *PROPEP* gene in *P. persica* × *P. dulcis* leaves, demonstrating the perception of the peptides. Among the events following Pep perception are rapid ET production and the up-regulation of defence genes (Bartels and Boller, 2015; and references therein). Pp/PdPep1 and Pp/PdPep2 activate plant defence mechanisms, as demonstrated by the transcriptional induction of a selection of ET-responsive factors in the short term, and pathogenesis-related proteins in longer treatments. The same genes were activated in *A. thaliana* and *Z. mays* by perception of AtPep1 and ZmPep1, respectively (Huffaker *et al.*, 2011; Liu *et al.*, 2013). Consistent with a role of *P. persica* and *P. dulcis* Pep1 and Pep2 as endogenous elicitors, *ERF-1a*, *ERF-2b*, *PR4* and *PR5-TLP2* defence-related genes were also induced, at comparable levels and in a similar time course, in response to challenge with Xap (Table 2; see also Sherif *et al.*, 2012a,b).

We additionally proved that the novel elicitor peptides protected the corresponding Rosaceae species against the bacterial pathogen Xap in an *ex vivo* assay. Pretreatment of *P. persica* × *P. dulcis* leaves with chemically synthesized Peps derived from either *P. persica* or *P. dulcis* enhanced their resistance to the bacterial pathogen Xap. This was evident up to at least 3 weeks after bacterial challenge at the phenotypic level. At this time point, Pep pretreatment also resulted in lower Xap contents relative to control leaves (Table S5). Taking into account that Peps do not have antibacterial activity, the Pep-triggered enhancement of plant defences would affect the proliferation of the pathogen. Major resistance was achieved 1 and 2 days after Pep treatment, which correlated with the induction of most tested defence genes. Three hours after Pep treatment the resistance appeared to be less induced and only the ET-responsive subset of defence genes was up-regulated.

Enhancement of resistance against Xap occurred in a dose-dependent manner. Doses as low as 10 nM Peps induced almost complete protection against infection with very high doses of pathogen. Optimal Pep working concentrations were set at 10–100 nM, lower concentrations exhibiting only partial protection. This result opens up the possibility of using Peps as phytosanitary products because these concentrations are considered to be very low doses compared with the 100-fold higher concentrations needed for other synthetic peptides which directly target the pathogen (e.g. BP100 linear undecapeptides; Badosa *et al.*, 2007; Güell *et al.*, 2011). In addition to the lower economic cost of the expensive chemical synthesis, low working doses would minimize putative unexpected phytotoxic effects, such as chlorosis (Gully *et al.*, 2015), which was not observed in our experimental conditions up to 1 μM. In consequence, PpPep1, PdPep1 and PpPep2 (which is identical to PdPep2) seem to be suitable candidates for the development of plant protection products to assist in the control of infection of peach and almond trees by the quarantine pathogen Xap.

The Rosaceae Pep1 and Pep2 sequences were highly conserved in all six commercially significant *Prunus* species studied. All six Pep2 sequences were identical, with the exception being two

conservative amino acid substitutions in *P. avium*. Pep1 sequences were in the range of 100% to 77% identical, with mostly conservative amino acid substitutions in the N-terminal region, and notably PdPep1 had G17E, which placed an amino acid with an acidic side chain within the otherwise uncharged C-terminal region. As expected, *P. mume* Pep1, which is different from both PpPep1 and PdPep1, was active in enhancing the protection of *P. persica* × *P. dulcis* leaves from Xap. Rosaceae species belonging to other tribes showed more divergent Peps, with identity values of 13%–36% compared with Pep1 and Pep2. This is similar to the overall similarity within the Brassicaceae (down to 22% on comparison of, for example, BrPep3 and AlPep7), but clearly below that of the Poaceae Peps (above 40%). We demonstrated that MdPep3 and PbPep4 were also perceived by *Prunus* spp. leaves. Although there was clear interspecies compatibility of Peps within the Rosaceae family, optimal activity was achieved with those from the most closely related species. As described in other plant families, within the Rosaceae, the sequence identity of the PEPR-LRR ligand-recognition domain was distinctly lower than that of the PEPR-kinase domain, a sequence constrained by the catalytic function (down to 58% and 74%, respectively). The interspecies compatibility of Peps and PEPRs within this family is in agreement with the reported high plasticity of Pep and PEPR-LRR sequences with impact on the Pep/PEPR-LRR interaction efficiency (Lori *et al.*, 2015).

The C-terminal amino acids of Rosaceae Peps showed the highest conservation among Pep1–5 sequences, suggesting that they define a motif involved in recognition by the receptor. Family-specific Pep consensus sequences were identified in the Brassicaceae, Solanaceae and Poaceae, which proved to be the basis of the interaction with the receptor (Lori *et al.*, 2015). The recently resolved crystal structure of AtPEPR1-LRR with the mature AtPep1 showed that the peptide adopts an extended conformation and the 10 C-terminal residues of the peptide interact with PEPR1-LRR (Tang *et al.*, 2015). Within this sequence, S^2, G^4 and N^{10} (see amino acid positions in Fig. 6) are critical for Pep activity (Pearce *et al.*, 2008), whereas additional residues are required for a full strength response (Roux *et al.*, 2011; Tang *et al.*, 2015). Comparison of the 10 C-terminal amino acids of all Pep sequences identified within the different plant families showed limited conservation (Fig. 6). The Pep motif from Rosaceae species clearly differs from that of other family-specific motifs, further supporting the hypothesis that each plant family has evolved its own, rather distinct, Pep motif. Co-evolution of Peps and the LRR domains of the PEPRs has been described, leading to distinct motifs and inter-family incompatibility (Lori *et al.*, 2015).

Because the PEP/PEPR system activates multiple defence pathways, rather than a single metabolite, Huffaker *et al.* (2007) hypothesized that it might provide a strategy to increase the plant resistance of, in particular, valuable crops against pathogen attack. The Rosaceae species, and particularly those of the *Prunus*

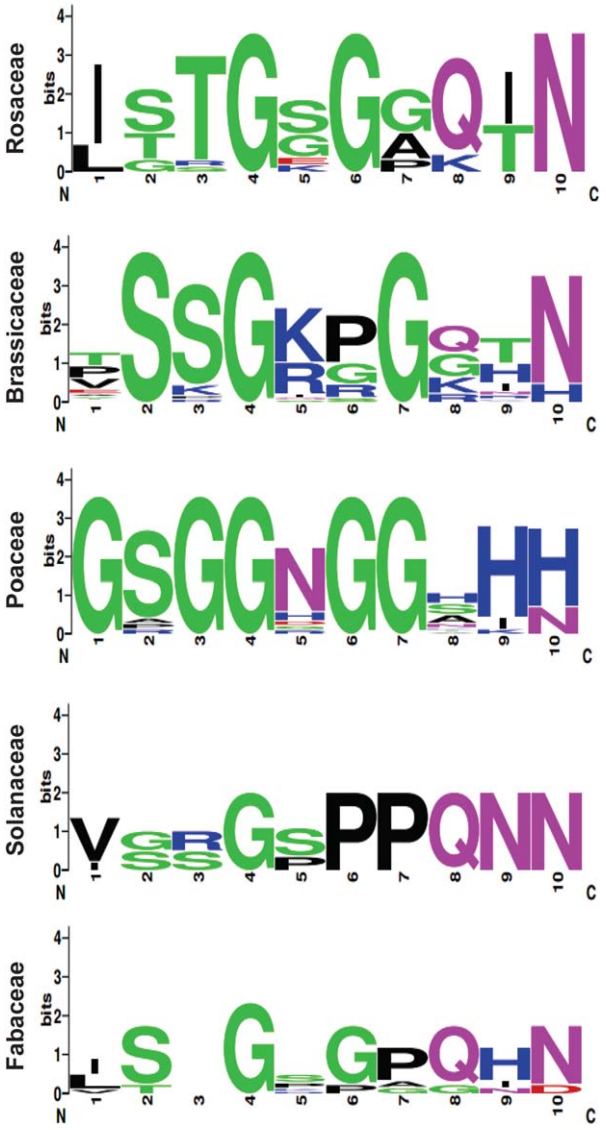

Fig. 6 Comparison of the consensus sequences of aligned Rosaceae-, Brassicaceae-, Poaceae-, Solanaceae- and Fabaceae-specific plant elicitor peptide (Pep) sequences using the WebLogo tool (Crooks *et al.*, 2004).

genus, are amongst the fruit crops with the highest economic impact. Here, we have identified the Pep/PEPR system in this plant family and have demonstrated that the Rosaceae Peps elicit the improvement of peach and almond resistance to the bacterial pathogen Xap. The natural origin of Peps, the low active doses needed to induce resistance and the inter-family incompatibility described make Rosaceae Peps sustainable candidates to boost plant resistance when disease-predictive models indicate high probability of pathogen attack, so complementing control strategies. For the development of Pep-based phytosanitary products, further investigation of the dynamics of the induced resistance *in planta* is needed to establish the treatment schedules. Because of

their mode of action, the Rosaceae Peps would most probably be suitable to control other relevant bacterial diseases, such as those caused by *Pseudomonas syringae* pathovars, in *Prunus* species.

EXPERIMENTAL PROCEDURES

Plant and bacterial materials

GF-677, a cross of *P. persica* × *P. dulcis* (peach tree and almond tree) which has become the most-used rootstock in peach production in Europe in recent decades, was employed in most experiments. A continuous supply of highly homogeneous juvenile plants (height, 10 cm; five leaves), produced by *in vitro* technology, was provided in individual small pots by a professional grower (Agromillora Iberia S.A., Barcelona, Spain). They were allowed to acclimatize for 1 week at 21 °C with a 16-h/8-h light/dark photoperiod in the glasshouse prior to further experiments. Leaves from the commercial *P. persica nectarine* var. Armking, *P. dulcis* var. Guara, *P. avium* var. Picota and *P. domestica* var. Santa Rosa were obtained from professional growers (Soljardí S.L., Jafre, Spain) and used for DNA analysis.

The plant-pathogenic bacterium Xap strain CFBP 5563 (Collection Française de Bactéries Phytopathogènes, Angers, France) (Boudon *et al.*, 2005) was cultured on yeast–peptone–glucose (YPGA) medium at 25 °C for the infection assay. After 24 h, bacterial colonies were scraped off the surface, suspended in sterile water and adjusted to 10^8 cfu/mL for immediate use in PEP activity assays. Stock cultures of the strain were maintained at −80 °C in YPGA broth with 20% glycerol.

Bioinformatics

Novel Rosaceae *PROPEP* and *PEPR* sequences were identified *in silico* with the National Center for Biotechnology Information (NCBI) BLASTN tool using previously reported *PROPEP* and *PEPR* DNA sequences belonging to the Brassicaceae, Solanaceae, Fabaceae and Poaceae families. Recently, one *PROPEP* and three *PEPR* Rosaceae sequences have been reported and used to search for novel sequences within this family. In a complementary approach, the KEGG tool (Kanehisa and Goto, 2000) was used to identify *PROPEP* and *PEPR* orthologues in the Rosaceae.

We used the GeneMark tool (Besemer and Borodovsky, 2005) for intron prediction and ExPASy (Gasteiger *et al.*, 2003) for sequence translation. CLC Main workbench 6.9.1 (CLC bio, Aarhus, Denmark) was used for: (i) DNA and protein alignment; (ii) identification of the PEPR kinase and LRR domains by scanning Pfam domains; and (iii) building of identity graphs and phylogenetic trees through neighbour joining with the Kimura protein distance measure and 1000 bootstraps. PEP consensus sequences were visualized with the WebLogo tool (Crooks *et al.*, 2004).

Ex vivo peptide elicitor assays

PpPep1, PdPep1, PmPep1, PpPep2 (which is identical to PdPep2 and PmPep2), MdPep3 and PbPep4 (Fig. 1a) were chemically synthesized (purity above 95%) and their identity was confirmed by Matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) (Caslo ApS, Lyngby, Denmark). Peptides were dissolved in double-distilled water to a stock concentration of 1 mM, and serial dilutions were prepared at 1000, 100, 10 and 1 nM for elicitor assays.

Leaves were excised from juvenile GF-677 plants and surface sterilized by immersion in 0.4% sodium hypochlorite, followed by rising in double-distilled water. Nine leaves, each from a different plant, were used for each treatment. Peps (1000, 100, 10, 1 or 0 nM) were sprayed onto the leaf abaxial surface and the leaves were placed onto humid filter paper in transparent boxes, with incubation in a culture chamber (25 ± 1 °C with a photoperiod of 16 h light/8 h dark under fluorescent Sylvania Cool White lamps, London, UK). After 3-h or 1-day pretreatment with Peps, a 0.3-mm cut was made across the main vein of every leaf with a scalpel, and 50 μL of a Xap suspension at the corresponding concentration (10^6, 10^7 or 0 cfu/mL) was inoculated at the wound site. Inoculated leaves were incubated for 3 weeks in the above-described conditions, and disease progression was determined using an arbitrary scale: 0, normal healthy leaves; 1, some local necrosis at the inoculation site; 2, strong necrosis at the inoculation site; 3, strong necrosis and chlorosis around the inoculation site; 4, strong necrosis and chlorosis spread along the central portion of the leaves; and 5, strong necrosis and fully chlorotic leaves. Detached leaf assays were performed at least twice, with different batches of plants. Infection intensity values were statistically analysed using one-way ANOVA and Tukey-b *post hoc* test with $\alpha < 0.01$.

Nucleic acid extraction, PCR amplification and sequencing

Genomic DNA from 100 mg of juvenile plant leaves was extracted using the commercial NucleoSpin® Plant II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. It was quantified by UV absorption at 260 nm in a NanoDrop ND1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), and the optical density (OD) 260/280- and 260/230-nm absorption ratios were used to confirm the purity of the DNA samples.

For the characterization of novel sequences, *PROPEP* and *PEPR* genomic sequences were amplified using primers designed at their 5′ and 3′ non-coding flanking regions (Table S6, see Supporting Information). PCR assays were performed in a final volume of 50 μL of 1 × reaction buffer with 1.5 mM Mg^{2+}, 300 nM of each primer (Sigma, Mannheim, Germany), 200 μM deoxynucleoside triphosphates (dNTPs) and 2.5 U/μL Expand High Fidelity DNA polymerase (Roche Diagnostics Corporation GmbH, Mannheim, Germany). The reaction conditions were as follows: 2 min at 94 °C; 10 cycles of 15 s at 94 °C, 30 s at the appropriate annealing temperature (Table S6) and 1 min at 72 °C; 20 cycles of 15 s at 94 °C, 30 s at the same annealing temperature and 1 min, plus an additional 5 s for each successive cycle, at 72 °C; and a final extension of 7 min at 72 °C. Elongation of amplicons above 2000 bp in length was performed at 68 °C for 2 min. PCR products were cloned using the pSpark DNA cloning system (Canvax, Córdoba, Spain) and then sequenced (Macrogen Europa, Amsterdam, the Netherlands).

To confirm the presence of both peach and almond tree PROPEP and PEPR sequences in GF-677 plants, genomic DNA was amplified using primers designed at the regions with the highest possible divergence (Table S6) in the same reaction conditions. The PCR products were subsequently confirmed by sequencing with the same primers.

Total RNA was extracted from plant leaves using a protocol based on the Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and remaining DNA was digested with DNAse I (Ambion, Grand Island, NY,

USA). Reverse transcription coupled to real-time PCR (RT-qPCR) was carried out as described previously (Ruiz *et al.*, 2016) using the primer pairs described in Table S6. *TEF2* was used for normalization (Tong *et al.*, 2009). PROPEP real-time PCR assays targeted common sequences in *P. persica* and *P. dulcis*.

Xap quantification was coupled to *ex vivo* peptide elicitor assays. All leaves from any given treatment were jointly homogenized with 100 mL of buffered peptone water for 90 s in a Stomacher Lab-Blender (Masicator, IUL Instruments, Barcelona, Spain). Twenty-five millilitres were used for DNA extraction employing a cetyl trimethyl ammonium bromide (CTAB)-based protocol. Real-time PCR was carried out with Xap-specific primers (Table S6) as described previously (Palacio-Bielsa *et al.*, 2011).

Antimicrobial activity

The peptide solutions (1 mM) were filter sterilized through a 0.22-μm pore filter and serial dilutions were prepared. Twenty microlitre aliquots of Xap strain CFBP 5563 bacterial suspensions (10^6 cfu/mL) were mixed in a microtitre plate with 160 μL of Luria Bertani broth (LB) and 20 μL of the adequate peptide dilutions to achieve 1000, 100, 10 and 0 nM final concentrations. They were incubated at 25 °C for 72 h, and ODs at 600 nm were recorded hourly after 20 s of shaking. Three replicates were carried out per peptide type and concentration, with two experimental replicates. The synthetic antibacterial peptide BP100 (2.5 and 1 μM) was used as an additional control, as described previously (Badosa *et al.*, 2007)

Using the same experimental set-up, 20-μL aliquots of samples with 1 μM PdPep1 and BP100 were serially diluted and drop plated on LB agar, 0, 30, 60, 90 and 120 min, and 3, 4, 5 and 6 h, after inoculation. After drop absorption, the plates were incubated at 25 °C for 2 days and Xap viable cells were counted in drops with 3–30 colonies. The total cfu count was averaged over at least three drops at the countable dilution.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Expression of *PROPEP1* (green bars) and *PROPEP2* (orange bars) genes in response to treatment with PpPep1 and PpPep2. Leaves of juvenile GF-667 plants were excised and treated with water (light green and light orange bars), 100 nM PpPep1 (dark green bars) or 100 nM PpPep2 (dark orange bars) for 0, 1, 3, 12 or 24 h in a humid chamber. *PROPEP1* and *PROPEP2* gene expression was assessed by reverse transcription coupled to real-time polymerase chain reaction (PCR) using *TEF2* to normalize the expression values. The means and standard deviations (SDs) of two independent experiments are shown.

Table S1 DNA and protein sequences of the identified Rosaceae plant elicitor peptide (Pep) precursors (PROPEPs), indicating the mature Pep sequences. Intron sequences are shown in lower case.

Table S2 DNA and protein sequences of the identified Rosaceae plant elicitor peptide receptors (PEPRs), indicating the leucine-rich repeat (LRR) and kinase domains. Intron sequences are shown in lower case.

Table S3 Identity comparison of plant elicitor peptide (Pep) precursor (PROPEP) sequences. The amino acid sequences of a total of 91 PROPEPs from the Rosaceae and other species (Lori *et al.*, 2015) were compared for the number (*y*-axis) and percentage (*x*-axis) of identical residues in the aligned positions. Colours indicate increasing identity from low (blue) to high (red) through white. PmPROPEP1 corresponds to PmPROPEP5, described in Lori *et al.* (2015).

Table S4 Identity comparison of plant elicitor peptide receptor (PEPR) sequences. The amino acid sequences of a total of 57 PEPRs from the Rosaceae and other species (Lori *et al.*, 2015) were compared for the number (*y*-axis) and percentage (*x*-axis) of identical residues in the aligned positions. Colours indicate increasing identity from low (blue) to high (red) through white. MdPEPR1a corresponds to MdPEPR1, described in Lori *et al.* (2015).

Table S5 *Xanthomonas arboricola* pv. *pruni* (Xap) contents in *Prunus* leaves pretreated with plant elicitor peptides (Peps) relative to control leaves pretreated with water. Leaves of juvenile GF-667 plants were excised, pretreated with water or 100 nM PpPep1, PdPep1, PpPep2, MdPep3 or PbPep4 for 1 day before inoculation with 50 μL of 10^7 colony-forming units (cfu)/mL of exponentially growing Xap. Infection was allowed to proceed for 3 weeks under controlled conditions (16-h/8-h light/dark photoperiod, 25 ± 1 °C) in a humid chamber. Xap contents were measured by real-time polymerase chain reaction (PCR) and those corresponding to peptide-pretreated samples were compared with the control. The means and standard deviations (SDs) of two independent experiments, each with nine leaves per treatment, are shown.

Table S6 Primers used in this work, with their use and optimal reaction conditions.