



CONTROL OF FIRE BLIGHT INFECTIONS WITH SYNTHETIC PEPTIDES THAT ELICIT PLANT DEFENSE RESPONSES

E. Badosa¹, L. Montesinos¹, C. Camó², L. Ruz¹, J. Cabrefiga¹, J. Francés¹,
B. Gascón¹, M. Planas², L. Feliu² and E. Montesinos¹

¹Institute of Food and Agricultural Technology-CIDSAV-XaRTA, University of Girona, Campus Montilivi, 17003 Girona, Spain

²LIPPSO, Department of Chemistry, University of Girona, Campus Montilivi, 17003 Girona, Spain

SUMMARY

Antimicrobial peptides (AMPs) are reliable compounds to develop strategies for integrated plant disease management. These peptides can act directly against the pathogens and/or induce plant defense responses. In this work, 33 sequences including linear and cyclic peptides, linear and cyclic peptidotriazoles, and cyclic lipopeptides were tested for induction of plant defense. A first screening was carried out with BY2 tobacco cell cultures by determining the alkalization and production of hydrogen peroxide. Peptides BP13, BP100, BP143 and BPC200W were slightly positive in these assays and were tested by RT-qPCR for expression of 10 defense-related genes in tomato, which are representative of the jasmonic acid, salicylic acid and ethylene pathways. BP13 and BPC200W induced overexpression of several genes that are also overexpressed by reference plant defense peptide elicitors. Plant assays with potted pear plants in the greenhouse confirmed the effect of peptides BP13 and BPC200W in controlling *Erwinia amylovora* infections, putatively through plant defense induction.

Keywords: *Erwinia amylovora*, antimicrobial peptides, induced resistance, fire blight control.

INTRODUCTION

Fire blight caused by *Erwinia amylovora* is one of the major diseases of pomefruits and ornamentals of the family Rosaceae (Agrios, 2005; Johnson and Stockwell, 1998). Its control is mainly based on copper compounds and certain antibiotics depending on the specific regulations of each country (Aćimović *et al.*, 2015; McGhee and

Sundin, 2010). Biological control is a promising approach, for which several bacterial and fungal strains are commercially available (Johnson and Temple, 2013). Antimicrobial peptides, either natural or synthetic, have also been proposed as novel compounds for preparing biopesticides for fire blight control (Badosa *et al.*, 2007; Montesinos and Bardají, 2008). Most of these products have been developed for targeting directly the pathogen, although there are other compounds (e.g. harpins, bion, fosetyl-Al, etc.) that act as plant defense elicitors and inducers of resistance to infections (IR) (Bektas and Eulgem, 2014; Choi *et al.*, 2013). This IR is a new approach in the strategy against fire blight.

Two types of IR have been described in plants: (i) systemic acquired resistance (SAR), basically induced by pathogens, that can be local (LAR, in the tissue surrounding the site of initial infection) or systemic (SAR, also in the distal, uninfected plant parts); (ii) induced systemic resistance (ISR) caused by the colonization of plant roots by selected rhizobacteria in the below- and above-ground parts of the plants (Albert, 2013; Conrath, 2009; Henry *et al.*, 2011; Pieterse *et al.*, 2012). IR can be triggered by elicitor molecules. In the case of SAR, elicitors include the so-called pathogen-associated molecular patterns (PAMPs), corresponding to microbial molecules, and the damage-associated molecular patterns (DAMPs), that are endogenous signal compounds from the host plant. During SAR, there is an accumulation of salicylic acid (SA) and several genes encoding pathogenesis related proteins (PRs) are expressed, some presenting antimicrobial activity. In the defense response, the host perceives the elicitors, followed by a signal transmission and, consequently, defenses are induced (Henry *et al.*, 2011). As to ISR, it has mainly been described in plant growth-promoting rhizobacteria (PGPRs) and the elicitors are known as microbe-associated molecular patterns (MAMPs). This response is associated to jasmonic acid (JA) and ethylene (E), but not to salicylic acid (SA) or PRs production. Examples of MAMPs are flagellins, lipopolysaccharides, siderophores, pyoverdines, pyocyanines, secondary metabolites (i.e. 2,4-diacetylphloroglucinol), products without a microbial origin (i.e. laminarin, chitosan), and synthetic compounds (i.e. 2,6-dichloroisonicotinic acid, its methyl ester and 1,2,3-benzothiadiazole-7-carbothioic acid S-methyl ester).

Table 1. Antibacterial activity (MIC) and cytotoxicity of selected peptides.

Peptide		MIC (μ M)			Hemolysis (%)
Code	Sequence	<i>Pss</i>	<i>Xav</i>	<i>Ea</i>	150 μ M
<i>Linear peptidotriazoles</i>					
BP249	KKLA(Tr-Ahx)KKILKYL-NH ₂	12.5-25	25-50	>50	0
<i>Linear peptides derived from the CECMEL11 library</i>					
BP13	FKLFKKILKVL-NH ₂	12-25	50-100	25-50	15 \pm 4
BP14	YKLFKKILKVL-NH ₂	12-25	50-100	25-50	30 \pm 3
BP15	KKLFKKILKVL-NH ₂	2.5-5	12.5-15	5-7.5	16 \pm 3
BP16	KKLFKKILKVL-NH ₂	>100	>100	>100	0
BP134	KKLFKKILKYL-OH	7.5-10	10-20	7.5-10	8 \pm 3
BP143	KKLfKKILKYL-NH ₂	2.5-5	5-7.5	2.5-5	5 \pm 0.8
BP195	SKKLFKKILKYLAKGPAKKLFFK ILKYL-OH	7.5-10	10-20	10-20	73 \pm 5
BP270	KKLHKKILKVL-NH ₂	12.5-25	>50	>50	0
BP271	Ac-LKLHKKILKVL-NH ₂	12.5-25	>50	>50	0
BP272	Ac-HKLHKKILKVL-NH ₂	12.5-25	>50	>50	0
BP273	Ac-FKLHKKILKVL-NH ₂	12.5-25	25-50	>50	0
BP285	FKLFKKILKHL-NH ₂	>25	>50	>25	26 \pm 0.8
<i>Cyclic peptidotriazoles</i>					
BPC512	c(KKLKA(Tr-Nle)FKKLQ)	25-50	<3.1	>50	2 \pm 0.1
BPC698	c(KKLKK(CO-Tr-Nle)FKKLQ)	25-50	3.1-6.2	>50	3 \pm 0.5
<i>Cyclic peptides derived from iturins</i>					
BPC660	c(QPnS-dap(C ₆ H ₁₁ O)-Nyn)	>50	>50	>50	2 \pm 1
BPC664	c(QPnS-dap(C ₁₂ H ₂₃ O)-Nyn)	>50	>50	>50	3 \pm 4
<i>Cyclic peptides derived from the CYCLO10 library</i>					
BPC194	c(LKKKKKKFLQ)	3.1-6.2	3.1-6.2	6.2-12.5	3 \pm 0.6
BPC148W	c(KKLLWKKKQ)	12.5-25	12.5-25	>100	3 \pm 1
BPC200W	c(LLLKWKKLQ)	>50	3.1-6.2	75-100	7 \pm 0.5
<i>Cyclic lipopeptides</i>					
BPC500	c(KKLKK(CO-C ₃ H ₇)FKKLQ)	6.2-12.5	<3.1	12.5-25	28 \pm 5
BPC596	c(KKLKK(LK-CO- <i>n</i> -C ₃ H ₁₁)FKKLQ)	6.2-12.5	<3.1	12.5-25	93 \pm 5
BPC634	c(KKLKk(CO- <i>iso</i> -C ₅ H ₁₁)fKKLQ)	12.5-25	25-50	>50	7 \pm 1
BPC638	c(KKLKK(CO- <i>iso</i> -C ₅ H ₁₁)HKKLQ)	6.2-12.5	25-50	25-50	6 \pm 2
BPC676	c(KKLKK(LK-CO- <i>n</i> -C ₃ H ₁₁)HKKLQ)	12.5-25	6.2-12.5	25-50	3 \pm 0.3
BPC684	c(k(CO- <i>n</i> -C ₃ H ₁₁)KLKfFKKLQ)	12.5-25	6.2-12.5	25-50	8 \pm 1
BPC686	c(K(CO- <i>n</i> -C ₃ H ₁₁)KLKfFKKLQ)	12.5-25	6.2-12.5	25-50	10 \pm 0.2
BPC688	c(K(CO- <i>n</i> -C ₃ H ₁₁)KLKHKKLQ)	25-50	25-50	>50	1 \pm 2
BPC702	c(KKLKk(CO-C ₃ H ₇)FKKLQ)	6.2-12.5	6.2-12.5	25-50	1 \pm 0.1
BPC714	c(KK(CO-C ₃ H ₇)LKKfFKKLQ)	25-50	12.5-25	25-50	3 \pm 1
BPC724	c(KKLKk(LK-CO-C ₃ H ₇)FKKLQ)	12.5-25	6.2-12.5	25-50	3 \pm 2
BPC726	c(KKLKK(LK-CO-C ₃ H ₇)fKKLQ)	12.5-25	6.2-12.5	25-50	0 \pm 1
BPC728	c(KKLKK(LK-CO-C ₃ H ₇)HKKLQ)	12.5-25	6.2-12.5	>50	0
<i>Flagellins</i>					
flg15	RINSAKDDAAGLQIA-OH	>100	>100	>100	0
flg21	RLSTGSRINSAKDDAAGLQIA-OH	>100	>100	>100	0

Xav, *Xanthomonas axonopodis* pv. *vesicatoria*; *Pss*, *Pseudomonas syringae* pv. *syringae*; *Ea*, *Erwinia amylovora*. Percent hemolysis at 150 μ M plus confidence interval ($\alpha = 0.05$).

The perception of these elicitors induces the synthesis of jasmonic acid and its methyl ester (MeJA) from membrane lipids (alfa linolenic acid). Although SAR and ISR are distinct pathways, there is a cross talk between both of them (Pieterse *et al.*, 2012).

PAMPs and MAMPs are recognized by certain receptors named pattern recognition receptors (PRRs), that usually contain a leucine-rich external domain (leucine rich repeats, LRR), and an internal serine/threonine/kinase domain (receptor-like kinases, RLKs, or receptor-like protein, RLPs, without the kinase domain). In the case of DAMPs, these endogenous compounds are delivered by

the action of pathogen-induced enzymes or by wounds, and are recognized by peptide receptors (PEPRs) (Yamaguchi and Huffaker, 2011; Guillaume *et al.*, 2012).

The usefulness of plant resistance inducers in fire blight management has been demonstrated, in some cases with a significant reduction of blossom and shoot blight symptoms, by treatments with potassium phosphite and acibenzolar-*S*-methyl (Aćimović *et al.*, 2015). However, these were not effective in other cases (Ngugi and Schupp, 2009; Sundin *et al.*, 2009).

Within the last 10 years, we have reported families of peptides active against *E. amylovora*, which include linear

and cyclic peptides, linear and cyclic peptidotriazoles, as well as cyclic lipopeptides (Badosa *et al.*, 2007; Ferre *et al.*, 2006; Güell *et al.*, 2011, 2012, 2013, 2017; Monroc *et al.*, 2006a, 2006b; Vilà *et al.*, 2013, 2016). In the present work we explored the possibility that some peptides may have a defense elicitation activity. The objectives were: (i) select a collection of synthetic peptides from the different reported families based on their distinct antibacterial and hemolytic activities; (ii) analyse the capacity of selected sequences to induce extracellular pH changes and hydrogen peroxide production in tobacco cell cultures; (iii) evaluate the capacity of selected peptides to express defense-related genes in tomato plants, and (iv) assay the best peptide elicitor candidates for *in planta* infection inhibition.

MATERIALS AND METHODS

Peptides. Peptides were selected from different families reported by our group (Table 1) and were synthesized as described elsewhere (Badosa *et al.*, 2007; Güell *et al.*, 2011, 2012, 2013, 2017; Monroc *et al.*, 2006a, 2006b; Vilà *et al.*, 2013, 2016). They included 13 linear and 20 cyclic peptides. Cyclic peptides BPC148W, BPC200W, BPC660 and BPC664 have not been previously described. Flagellin derivatives flg15 and flg21, and KKLFKKILKYL-NH₂ (BP100) were used as control in some *in vitro* and *in planta* experiments, respectively. The minimum inhibitory concentration (MIC) against *Pseudomonas syringae* pv. *syringae* (Pss), *Xanthomonas axonopodis* pv. *vesicatoria* (Xav) and *Erwinia amylovora* (Ea) as well as their hemolytic activity at 150 µM are shown in Table 1. These activities were evaluated as described elsewhere (Güell *et al.*, 2011).

Determination of extracellular pH changes and hydrogen peroxide-production in tobacco cell cultures. Tobacco cells (*Nicotiana tabacum* cv. Brighth Yellow-2) were cultivated in Murashige and Skoog medium (Duchefa Biochemie B.V., The Netherlands) containing 4.3 g l⁻¹ at pH 5.8, completed with sucrose at 30 g l⁻¹, KH₂PO₄ at 0.2 g l⁻¹, myo-inositol at 0.1 g l⁻¹, thiamine at 1.0 mg l⁻¹ and 2,4-dichlorophenoxyacetic acid 0.2 mg l⁻¹. Cells were grown at 24°C in the dark on a rotary shaker at 120 rpm and subcultured weekly by the addition of 10 ml of cells to 100 ml of fresh liquid medium. Experiments were performed using cells in exponential growth phase after 5 to 6 days of culture.

Extracellular pH variation of the peptides (Table 1) was monitored with a glass pH microelectrode (Thermo Fisher Scientific, Spain). Briefly, tobacco cells in exponential phase were diluted 1:3 in fresh MS medium and 1.5 ml of the cell suspension were distributed in 24 well plate and let to stabilize during 30 min. Then, 6 µl of each peptide were added to a final concentration of 20 µM in the well. pH was measured every 5 min during 25 min. A positive control with flagellin and a negative

control with water were also included. The experiment was repeated twice.

H₂O₂ production from the peptides (Table 1) was assessed using the Oxiselect hydrogen peroxide assay kit (Cell Biolabs, USA). First of all, tobacco cells in exponential phase were filtered on a Whatman filter and resuspended in HEPES solution (mannitol at 31.8 g l⁻¹, HEPES at 480 mg l⁻¹, K₂SO₄ at 90 mg l⁻¹ and CaCl₂ at 73 mg l⁻¹, adjusted at pH 7) to obtain a final concentration of approximately 0.2 g of cell ml⁻¹. Then, 1 ml of the cell suspension was distributed in 24 well plate and 4 µl of each peptide were added to a final concentration of 20 µM in the well. Cells were maintained in a rotatory shaker for 15 min and subsequently cells were let to sediment. Then, 50 µl of the supernatant were transferred into an individual µl plate well and 50 µl of ADHP/HRP working solution (ADHP at 100 µM and HRP at 0.2 U ml⁻¹ in 1X assay buffer) were added to each well. The well content was mixed thoroughly and incubated for 30 min at room temperature protected from the light. After incubation, the plate was read with a fluorescence microplate reader equipped with excitation (530-570 nm) and emission (590-600 nm) systems. In order to quantify the production of H₂O₂ a standard curve containing known concentrations of H₂O₂ (25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.19, 0.09 and 0 µM) was prepared. Each sample was assayed in triplicate.

Effect of peptide treatment on defense gene expression of tomato plants. Seeds of tomato cv. Rio Grande were sown in hydroponic seed plugs (rockwool) and grown under controlled greenhouse conditions (25 ± 2°C, 16h light / 15 ± 2°C, 8h dark, and 60% RH). Two-week-old seedlings (two cotyledons) were transplanted into Rockwool plugs (7.5 × 7.5 × 6.5 cm, Gordan Iberica, Spain). The experimental design consisted of three replicates of six plants per treatment. After two weeks, tomato leaves were sprayed with an aqueous solutions of peptides BP13, BP100, BP143 and BPC200W, at 125 µM, salicylic and jasmonic acid at 2.5 mM (Sigma-Aldrich, USA), until the runoff point. For the ethylene treatment, plants were exposed to ethylene generated in a sealed chamber containing ethephon (1 mM) (Nufarm España, Spain) amended with disodium hydrogen phosphate buffer (2.5 mM) (Zhang and Wen, 2010). Water was used as a not treated control. Leaf samples were collected 24h after the treatments and processed to extract RNA to be used for obtaining the corresponding complementary DNA (cDNA). Plant material was ground to a fine powder in liquid nitrogen with the Tissuelyzer II system (Qiagen, USA). Total RNA was extracted from leaves using TriZol (Invitrogen, USA) according to manufacturer's instructions, was solubilized in RNase-free water and subjected to DNase treatment (Ambion Turbo DNA-free™, Life Technologies, USA) to remove contaminant DNA. In each step, the RNA was quantified using Nanodrop N-2000 spectrophotometer

(Thermo Fisher Scientific, USA), and its integrity verified by denaturing agarose gel electrophoresis. First-strand of cDNA was generated from extracted RNA using reverse transcriptase (High Capacity cDNA Reverse Transcription Kit, Invitrogen, USA) according to manufacturer's instructions.

To test defense gene induction in the treated tomato plants, a qPCR assay was performed. Quantitative PCR was carried out in a fluorometric thermal cycler (7300 Real-Time PCR System, Applied Biosystems, USA) using a Mix SYBRGreen PCR Master Mix (Applied Biosystems, USA). The total reaction volume of this PCR reaction was 20 μ l and the reaction mixture were 1 μ l of each primer set at the adequate concentration, 10 μ l of MixSyber Green, 6 μ l of distilled water and 2 μ l of cDNA. Specific oligonucleotides according to the literature, and other designed in the present study were used for the quantification of the expression of the target genes involved in plant defense (Table 2). For each gene system, the concentration of the primer pair was optimized to prevent non-specific reactions or artefacts that could jeopardize the result. Melting curve analysis was performed after each amplification to confirm the specificity of reaction. A constitutive gene (*actin* gene) was used as reference control, and the following genes implicated in plant defense response were analyzed: Phenylalanine ammonia lyase (*PAL*), Pathogenesis-related protein-1 (*PR1*), Chitinase A (*Chi3*, *PR3*), Acidic β -1,3 endoglucanase (*GLuA*, *PR2*), Basic β -1,3 endoglucanase (*GluB*), Lipoxygenase (*LOX*), Protein inhibitor II (*PinII*), Heat shock protein (*S13*), Dehydrin (*TAS*) and a Na⁺/H⁺ antiporter (*NHX1*). The primer concentration was 100 nM for all the genes except for the *GLuA*, *PR1* and *actin* genes which optimized concentration was 300 nM. To prepare a calibration curve cDNA was cloned in in pSpark cloning vector (Canvax, Spain) an used to transform *E. coli* DH5 α , the number of extracted plasmid copies (QIAGEN Iberia, Spain) were quantified and appropriate dilutions were used to obtain the standard curve. The efficiency for each standard curve was calculated in to be sure that the efficiencies of the amplifications were similar. Then, a relative quantification of gene expression was done using the $\Delta\Delta$ Ct method (Livak and Schmittgen, 2001). Quantitative analysis was performed with the geneAmp7300 sequence detection system (PE Applied Biosystems, USA). The Ct values obtained for each treatment repetition were used to estimate the fold change value of the endogenous reference gene (*actin*) and the target plant defense gene. These results were used to calculate the ratios of the plant defense genes (relative to the *actin* gene for all treatments analyzed, and to control plants). The statistical significance of the results for the selected peptides was determined using the REST2009 Software (Pfaffl *et al.*, 2002)

In planta *E. amylovora* infection assays. The efficacy of peptides in the control of fire blight infections was determined using potted pear plants (cv. Conference) inocu-

lated with *E. amylovora*. Two-to-three-year-old pear plants grown in 20 cm diameter plastic pots in the greenhouse were used. Plants were pruned to leave 3-to-4 shoots per plant and were forced to bud in the greenhouse. Plants were fertilized once a week with a 200 ppm N-P-K solution (20-10-20) and were used when the shoots were about 3-to-4 cm in length, and had 5-to-6 young leaves per shoot. Fungicides or bactericides were not applied to prevent interference with the assays. Plants were treated with peptides BP13, BP100, BP143 or BPC200W. Different peptide treatment strategies were used, one based on two treatments, 7 and 2 days before pathogen inoculation (bpi), and a second one based on three treatments, 7, 2 and 0 days bpi. For inoculation, the three youngest expanded leaves of each shoot were wounded approximately in the middle of the leaf by a double transverse incision (*ca.* 1 mm) of the midrib that was inoculated with 10 μ l of a suspension of a single strain (EPS101) of *E. amylovora* at 10⁸ CFU ml⁻¹. Plants were incubated in a controlled environment greenhouse for nine days at 23 \pm 2°C during the day and 15 \pm 2°C at night and a photoperiod of 16 h light and 8 h dark. The experimental design consisted of three replicates of three plants per each treatment. Appropriate non-treated, non-inoculated and reference controls (BP100 at 125 μ M and Kasugamycin 8% at 2g l⁻¹ (Lainco, Spain) were used. A disease index, ranging from 0 (no symptoms) to 3 (severe lesions) was used to determine infection severity. The mean incidence was calculated for each replicate. Two independent experiments were performed.

The effect of treatments on plant material infection was determined using analysis of variance (ANOVA) with the general linear model (GLM) procedure of the Statistical Analysis System (SAS) (Version 8.2, SAS Institute, USA). Means were separated using the Tukey's test ($P < 0.05$).

RESULTS AND DISCUSSION

Rapid response to pathogens and stress in plants usually takes place during the first hour after perception, and delayed response may take hours or days (Albert, 2013). Relevant immediate responses consist of cell efflux of K⁺ and influx of Ca²⁺, production of reactive oxygen species (ROS) and ethylene, activation of mitogen-activated protein kinase (MAPK) and expression of defense-related genes (Albert, 2013; Benouaret *et al.*, 2014). Accordingly, alkalinization of the extracellular medium in plant cell cultures and production of ROS are considered indicators of the eliciting activity of compounds (Felix *et al.*, 1993, 1999; Guan *et al.*, 2013; Henry *et al.*, 2011; Miyashita *et al.*, 2011; Miyashita and Miyagawa, 2008; Yu *et al.*, 2008). In the case of peptides, several reports demonstrate that synthetic ultrashort lipopeptides and certain small peptides can activate the plant immune system (Brotman *et al.*, 2009; Miyashita and Miyagawa, 2008; Miyashita *et al.*, 2011). This is particularly relevant in the case of flagellin,

(Felix *et al.*, 1993, 1999), flagellin-derived peptides (Miyata *et al.*, 2006), linear lipopeptides (Brotman *et al.*, 2009; Henry *et al.*, 2011; Ongena *et al.*, 2007), cyclic lipopeptides (Tran *et al.*, 2006), and linear peptides (Miyashita *et al.*, 2011).

In the present work, we have selected a series of peptides from different families previously described (Table 1) (Badosa *et al.*, 2007; Güell *et al.*, 2011, 2012, 2013, 2017; Monroc *et al.*, 2006a, 2006b; Vilà *et al.*, 2013, 2016). These selected sequences included linear peptides derived from the CECMEL11 library, cyclic peptides derived from the CYCLO10 library and from iturins, linear and cyclic peptidotriazoles, and cyclic lipopeptides. These peptides exhibited a distinct activity profile against the plant pathogenic bacteria *E. amylovora*, *Pseudomonas syringae* pv. *syringae* and *Xanthomonas axonopodis* pv. *vesicatoria*, as well as different hemolytic activity.

The first screening to identify peptides with putative elicitor activity was performed by analyzing *in vitro* in tobacco cell cultures (BY2 line) for alkalization of the extracellular medium and production of hydrogen peroxide (Fig. 1 and 2). In the alkalization assay, the control peptide flg15 produced an increase of pH > 0.4 units, whereas peptides BP14, BP285, BP143, BP13 and BPC200W caused an increase of the pH between 0.1 and 0.15 units. In Fig. 1 the kinetics obtained for peptides flg15, BP13, BP143 and BPC200W in one of the alkalization assays is depicted. To evaluate the peptide-induced oxidative burst we used flg15 and flg21 as positive controls. Fig. 2 shows the results of the six linear and four cyclic peptides that induced hydrogen peroxide production in BY2 cells. Using a threshold concentration of 1 μM and compared to flg15 and flg21, only the linear peptides BP13 and BP272, and the cyclic peptides BPC148W and BPC200W were effective.

Analysis of the above data in BY2 cells revealed that BP13 and BPC200W gave good results in both assays. Moreover, also BP143 led to a pH increase of the extracellular medium in these cells. Thus, these peptides were chosen for further studies. However, other peptides from our libraries cannot be excluded, because they may be non-active in tobacco or in the above tests, but active in other plant species. For example, the endogenous octapeptide GmPep914 from soybean (a cryptic peptide derived from proteins with separate primary functions), which is induced as a wound response, produces alkalization in soybean cell cultures, but not in tobacco or *Arabidopsis* cell cultures (Yamaguchi and Huffaker, 2011; Yamaguchi *et al.*, 2011). Also, certain peptides can act as elicitors by inducing ISR at concentrations that produce no or a slight increase in pH, but also having inhibitory effect in pathogens (Brotman *et al.*, 2009).

Next, we analyzed if peptides BP13, BP143 and BPC200W were able to induce the expression of genes related to plant defense responses (Table 2). In addition to these three peptides, BP100 was included in the study because it is a representative peptide from the CECMEL11 library

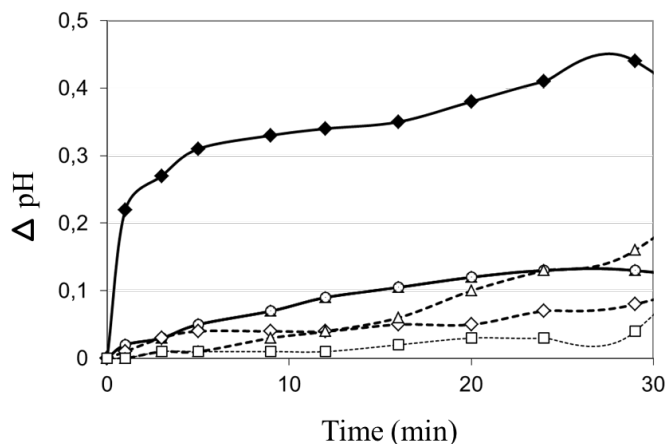


Fig. 1. Kinetics of the alkalization in tobacco cells cultures (BY2) treated with the peptides BP13 (○), BP143 (◇), BP200W (△), and flg15 at 20 μM (◆). Water (□) was used as control.

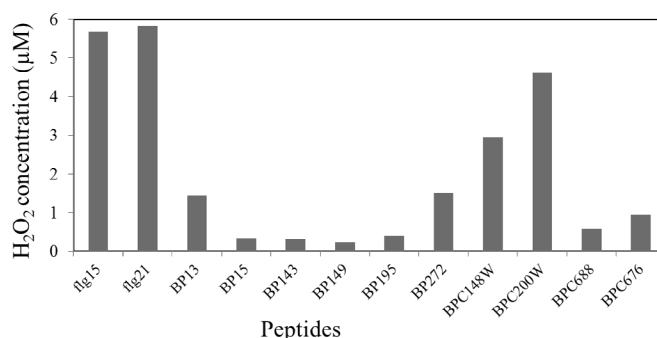


Fig. 2. Hydrogen peroxide accumulation in tobacco cell suspensions after treatment with selected peptides. Flagellins flg15 and flg21 were used as reference, and water as control.

that is effective against *E. amylovora* infections in plant assays (Güell *et al.*, 2011). Moreover, flg15 was used as positive control. The selected genes are involved in the pathways of the salicylic, jasmonic acid and ethylene, as well as in saline stress and wound damage. Seven primers were taken from the literature and four were developed in this work (Table 2). The *actin* gene was used as an endogenous constitutive gene to normalize the results of expression. The corresponding cDNA was obtained from all primers and was cloned to obtain calibration curves. Then, it was demonstrated that the efficiency of the primers was similar at the optimized concentrations, ranging from 100 to 300 nM, a criteria to properly apply the $\Delta\Delta\text{Ct}$ method (Livak and Schmittgen, 2001).

The relative quantification for the expression of the selected genes by the $\Delta\Delta\text{Ct}$ method, using tomato plants as the experimental system, is shown in Table 3. It is clearly observed that treatment with the reference products (SA, JA, E) caused the overexpression of several genes. Salicylic acid (SA) overexpressed *PR1*, *PR3* and *TAS*. Jasmonic acid (JA) induced expression of *PR3*, *LOX*, *GluB* and *PinII*. Ethylene (E) treatment induced *PR3*, *LOX* and *GluB*. These results confirm that both pathways, SA and JA,

Table 2. Genes and primer sequences used in this study.

Pathway/Stress	Gene	Code	Sequence (5'3')	Reference
Salicylic acid	<i>Chitinase A</i>	Chi3-f	TGCAGGAACATTCACTGGAG	(Aimé <i>et al.</i> , 2008)
		Chi3-r	TAACGTTGTGGCATGATGGT	
	<i>Acidic β-1,3-endoglucanase</i>	GluA-f	GGTCTCAACCGCGACATATT	(Aimé <i>et al.</i> , 2008)
		GluA-r	CACAAGGGCATCGAAAAGAT	
	<i>Pathogenesis-related protein-1</i>	PR1-f	TCTTGTGAGGCCAAAATTC	(Aimé <i>et al.</i> , 2008)
		PR1-r	ATAGTCTGGCCTCTCGGACA	
Ethylene	<i>Basic β-1,3-endoglucanase</i>	GluB-f	TTGTGCCACCAACATTCAACA	This study
		GluB-r	ACCATCTCGCGTGTCCATC	
Jasmonic acid	<i>Lipoxygenase</i>	LOX-f	CAAGCATGGGGTGGGCTTAT	This study
		LOX-r	CATGGTCTCGACCCGTAAA	
	<i>Phenylalanine ammonia lyase</i>	PAL-f	CACAGTGGCTTGGACCTCAA	This study
		PAL-r	ACCGTGTAAGGCCTTGTCTCT	
Salt	<i>Na⁺/H⁺ antiporter</i>	NHX1-f	ATGTTGTTGGTGCCGTCG	(He and Huang, 2013)
		NHX1-r	AGGCTGCTCGTCCTGATT	
	<i>Heat shock protein</i>	S13-f	CAAACATGTGATTGGATAAAGAAACG	(Zhou <i>et al.</i> , 2007)
	S13-r	CTGACCAACCAAACCTTCTGAT		
	<i>Dehydrin</i>	TAS14-f	CTGGTGGAGAATATGGAAGTCAAGGC	(Muñoz-Mayor <i>et al.</i> , 2012)
		TAS14-r	CTTCATGTTGTCCAGGCATCTTCTCC	
Wound	<i>Protein inhibitor II</i>	PinII-f	TGATGCCAAGGCTTGTACTAGAGA	(Herman <i>et al.</i> , 2007)
		PinII-r	AGCGGACTTCCTTCTGAACGT	
Endogenous reference gene	<i>Actin</i>	Act-f	CACTGTATGCCAGTGGTTCGT	This study
		Act-r	GACGGAGAATGGCATGTGGA	

Table 3. Expression of genes related to defense/stress response in tomato after the treatment with the reference products salicylic acid (SA), jasmonic acid (JA) and ethylene (E), and with the selected peptides. Fold induction above 2 is considered as overexpression in the relative quantification by the $\Delta\Delta$ Ct method. Significant values are indicated with an asterisk.

Gene	REFERENCE PRODUCTS			PEPTIDES				
	SA	JA	E	flg15	BPC200W	BP13	BP143	BP100
<i>PAL</i>	0.4	1.4	0.7	1.5	0.9	1.2	1.0	1.2
<i>PR1</i>	25.9*	0.4	1.3	55.9*	1.0	1.6	0.4	2.0
<i>Chi3, PR3</i>	205.0*	138.2*	111.5*	14.8*	2.6*	6.6*	0.6	1.3
<i>GluA, PR2</i>	1.0	0.1	0.5	7.1*	4.5*	7.8*	0.9	1.5
<i>LOX</i>	0.1	4.1*	6*	3.3*	2.6*	1.9	0.5	1.2
<i>GluB</i>	1.5	9.1*	6.5*	17.8*	5.1*	1.3	0.4	1.0
<i>PinII</i>	0.3	193.8*	0.2	6.1*	0.3	1.1	6.0*	4.9*
<i>S13</i>	n.d.	n.d.	n.d.	1.0	1.4	1.3	0.9	1.0
<i>TAS</i>	2.0*	0.1	0.5	1.5	4.7	4.2*	2.2*	1.4
<i>NHX1</i>	n.d.	n.d.	n.d.	1.9	1.0	1.7	0.8	1.2

n.d., not determined.

share genes, as it has been reported (Pieterse *et al.*, 2012). The reference peptide flg15 overexpressed more genes (also *PR2*) and more intensely than the genes overexpressed in the SA, JA and E pathways by the corresponding inducers. BPC200W was the peptide that shared more overexpressed genes with flg15 (4 out of 6 genes: *PR3*, *LOX*, *GluB* and *PR2*), although with lower values. BP13 treatment only shared overexpression of genes *PR2* and *PR3* (2 out of 6) with flg15, but included *TAS*. Peptides BP100 and BP143 shared the overexpression of *PinII* with flg15. In addition, BP143 overexpressed *TAS*. Therefore, all peptides showed elicitation activity in tomato plants, but BP200W and BP13 were the most active, in comparison to the reference treatments.

In the present work, we used a preliminary approach based on genes reported to be involved in the salicylic,

jasmonic and saline stress in tomato. However, more robust strategies like microarrays or RNAseq are now being used, but are not the object of the present report.

Finally, peptides BP13, BP143, BPC200W and the reference peptide BP100, were used to perform *in planta* assays to study their effect to control *E. amylovora* infections (Fig. 3). Pear potted plants were used as host, under controlled greenhouse conditions. Two different schedules of treatments were used. In the first case, peptides were applied 7 and 2 days before pathogen inoculation (bpi). In the second case, an additional treatment 1h bpi was done. Peptides BP100 and BP143 had a significant effect in the second strategy, but not in the first one. In contrast, peptides BP13 and BPC200W showed little differences depending on the strategy, as they were both effective. Peptides BP13 and BPC200W showed a low activity against

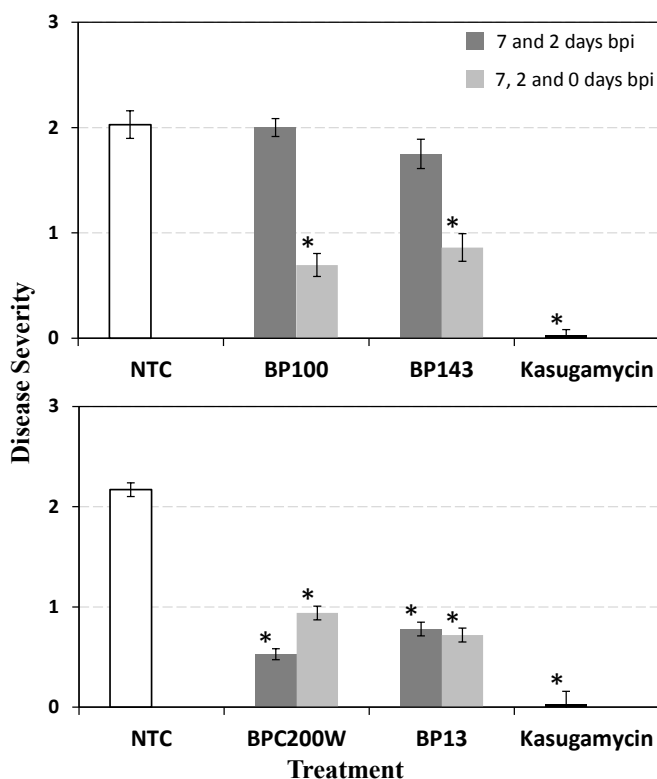


Fig. 3. Protection against *E. amylovora* infection by peptides BP13, BP100, BP143 and BP200W. Two treatment schedules were used in relation to the pathogen inoculation: 7, and 2 days bpi; and 7, 2 and 0 days bpi. Standard deviations are indicated on bars. Significantly different means from non-treated controls (NTC) are indicated with an asterisk.

E. amylovora (MIC 25-50 to 75-100 μ M, respectively). All these peptides are susceptible to protease activity and easily interact with leaf tissue structures and components due to their cationic and amphipathic properties (Badosa *et al.*, 2007; Güell *et al.*, 2011; Monroc *et al.*, 2006b). Therefore, they are probably inactivated in treatments performed 7 or 2 days before pathogen inoculation, but can be active directly against the pathogen when applied simultaneously. Thus, the finding of a significant disease control by peptides BP13 and BPC200W when applied 7 and 2 days bpi can be interpreted as a consequence of their induction of plant host defenses.

In conclusion, results shown in the present work demonstrate that linear synthetic peptides from the CEC-MEL11 library and cyclic decapeptides derived from the CYCLO10 library, with low antibacterial activity, can induce defense responses on tobacco cells and tomato plants, and efficiently control fire blight infections on pear. These results open the possibility of application of this strategy for future implementation in fire blight management. However, there are still limitations related to regulatory issues and production that have to be addressed before their use.

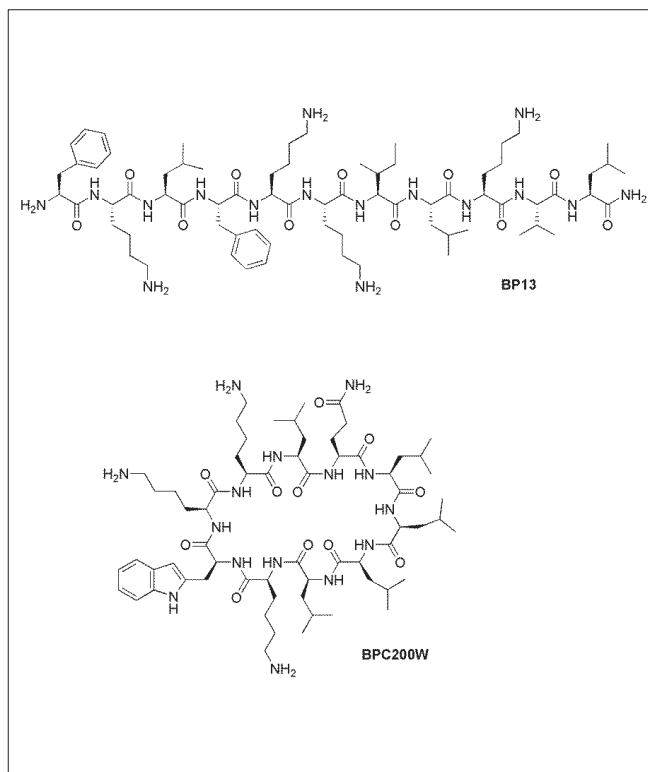


Fig. 4. Structure of the linear peptide BP13 and the cyclic peptide BPC200W.

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