Derivatives of the Antimicrobial Peptide BP100 for Expression in Plant Systems

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Abstract

Production of antimicrobial peptides in plants constitutes an approach for obtaining them in high amounts. However, their heterologous expression in a practical and efficient manner demands some structural requirements such as a minimum size, the incorporation of retention signals to assure their accumulation in specific tissues, and the presence of protease cleavage amino acids and of target sequences to facilitate peptide detection. Since any sequence modification may influence the biological activity, peptides that will be obtained from the expression must be screened prior to the synthesis of the genes for plant transformation. We report herein a strategy for the modification of the antimicrobial undecapeptide BP100 that allowed the identification of analogues that can be expressed in plants and exhibit optimum biological properties. We prepared 40 analogues obtained by incorporating repeated units of the antimicrobial undecapeptide, fragments of natural peptides, one or two AGPA hinges, a Gly or Ser residue at the N-terminus, and a KDEL fragment and/or the epitope tag54 at the C-terminus. Their antimicrobial, hemolytic and phytotoxic activities, and protease susceptibility were evaluated. Best sequences contained a magainin fragment linked to the antimicrobial undecapeptide through an AGPA hinge. Moreover, since the presence of a KDEL unit or of tag54 did not influence significantly the biological activity, these moieties can be introduced when designing compounds to be retained in the endoplasmic reticulum and detected using a complementary epitope. These findings may contribute to the design of peptides to be expressed in plants.

Introduction

Antimicrobial peptides (AMPs) are short sequences containing less than 50 amino acids. They are considered a first line of defense in plants and animals or are produced by microorganisms participating in antibiotic processes [1]. There is broad literature review on AMPs produced in bacteria [2-4], fungi [5,6], insects [7,8], marine invertebrates [9], amphibian, mammals [10,11], and plants [12].

Due to their potential high biocompatibility, moderate biodegradability, and low resistance developed on target microorganisms, AMPs offer great perspectives as a novel class of antibiotics with application in several fields. They can be used to combat fungal and bacterial infections in humans [7,10] and plant diseases in crop protection [1,13,14]. Moreover, they can substitute or complement antibiotics in animal feed, biopreservatives in food, cosmetics and biomaterials, and antifoulings [15,16]. However, the exploitation of AMPs encounters several difficulties because they are produced at low concentrations in living organisms and often their antimicrobial activity is low to moderate. In addition, some of the AMPs showing high antimicrobial activity may be relatively toxic to non-target organisms (animals, humans, plants).

To overcome the above limitations, novel peptides have been designed based on structure-activity relationship studies in natural AMPs. Small truncated sequences containing the minimal domain for activity have been developed as well as chimeric constructions. De novo designed sequences, bearing structural features that are crucial for the activity of natural peptides, have also been reported. Combinatorial chemistry approaches are also powerful tools that have been used to optimize the biological activity profile of AMPs, and sequences with improved activity, decreased toxicity to non-target...
Peptides for Expression in Plants

organisms and low susceptibility to proteolytic hydrolysis have been identified. Following this rationale, we have designed chimeric peptides that are cecropin A-melittin hybrids and their biological activity has been optimized through the synthesis of a 125-member library (CECMEL111) [17]. From this library we have identified BP100 and several analogues active against bacterial and fungal pathophytophages with minimal inhibitory concentrations (MIC) lower than 10 μM [17-19]. This activity is relevant because it is of the same order than that of standard antibiotics and antifungals (e.g. penicillins, aminoglycosides, ketoconazole). Moreover, they showed an extremely high biocompatibility with an acute oral toxicity, determined as the LOD<sub>50</sub> higher than 2000 mg/Kg of body weight in mice [20].

BP100 and its derivatives have strong cationic charge and amphipathic arrangement that enable their interaction with biological membranes resulting in cell membrane disruption. Biophysical studies with BP100 using phospholipid bilayers similar to that of the bacterial cytoplasmic membrane showed vesicle permeabilization, membrane electroneutrality, and vesicle aggregation, but also translocation [21]. It has been also reported that BP100 is a fast and efficient cell-penetrating agent to deliver functional cargoes peptides into tobacco cells [22].

Exploitation of AMPs may be performed by expression in plants for self-defence against bacterial or fungal pathogens or through mass production to be used as active ingredients in antimicrobial formulations. Mass production can be accomplished through chemical or quimioenzymatic synthesis, or by means of microbial or plant biofactories. Chemical synthesis using solution or solid-phase protocols or employing enzymatic procedures is only economically feasible for the preparation of short peptides. In contrast, heterologous production of AMPs using living systems as biofactories offers a reliable and sustainable mean of exploitation of these peptides which can lead to high amounts of product. Microbial systems, such as Escherichia coli or Pichia pastoris, have been reported as efficient production platforms for several proteins [23,24]. Plants have also been used as biofactories for the production of medium to large size proteins due to their high added value for pharmaceutical applications [25-28].

Linear AMPs containing proteinogenic aminoacids can also be produced in plants [13,29]. However, to be heterologous expressed in plants, peptides must fulfill some structural requirements. Particularly, the size of the peptide has to be above a minimum expressability threshold [30]. Moreover, the peptide has to be targeted to subcellular organelles to guarantee its stability and avoid cell toxicity [31]. In addition, the expression and accumulation of the peptide in specific plant tissues is necessary to meet low cost production needs [32] and to decrease downstream processing operations [33,34]. To accomplish these requirements the following strategies may be used: (i) increase of the peptide length by n-merizations, chimeric enlargements or fusions [35], (ii) stabilization/distortion by incorporating an AGPA hinge between the peptide fragments [36], (iii) introduction of retention signals for the accumulation of peptides in subcellular organelles, such as the endoplasmic reticulum by the addition of the KDEL sequence at the C-terminus [13,37], (iv) incorporation of protease cleavage amino acids for processing fusions [38], and (v) of target sequences to allow peptide detection and/or purification [39,40].

Taking into account these considerations, to achieve an efficient production of the CECMEL111 peptides in living systems and, particularly, in plants, the sequences to be expressed should incorporate the above structural features. Unfortunately, any sequence modification may have dramatic consequences in the peptide properties, including antimicrobial, hemolytic and phytotoxic activities as well as protease susceptibility, as it has been previously described [17,19,20]. Therefore, the biological activity of the sequences that will be obtained from the expression process must be screened prior to the synthesis of the corresponding genes and the cloning systems for plant transformation.

In the present work, we designed, synthesized and evaluated 40 sequences derived from BP100 for their antimicrobial, hemolytic and phytotoxic activities, and protease susceptibility. These analogues were designed based on the structural requirements that they must possess to be expressed in plants. Finally, the best candidates are proposed for further development of transgenic plants for their production.

Materials and Methods

Peptide synthesis

All peptides were synthesized manually by the solid-phase method using 9-fluorenylmethoxycarbonyl (Fmoc)-type chemistry, tert-butyloxy carbonyl side-chain protection for Lys and Trp, tert-butyl (tBu) for Tyr, Glu, Asp, Thr, Gin and Ser, and trityl for His. An aminomethyl ChemMatrix resin (0.59 mmol/g) was used as solid support. The linker 3-(4-hydroxymethylphenoxo)propionic acid (PAC) was employed to obtain C-terminal peptide acids. The PAC linker (3 equiv) was coupled to the resin with N-[1H-benzo triazol-1-yl] (dimethylamino)methylene-N-methyl methanaminium hexafluorophosphate N-oxide (HBTU) (3 equiv) and N,N-diisopropylcarbodiimide (DIPCDI) (5 equiv), 1-hydroxy benzotriazole (HOBt) (5 equiv) and N,N-dimethylaminopyridine (DMAP) (1.25 equiv) in DMF for 15 h. After the coupling, an Fmoc test was performed to check the resin loading. The resin was then acylated by treatment with a mixture of acetic anhydride–pyridine–CH<sub>2</sub>Cl<sub>2</sub> (1:1:1; 2 × 30 min). Couplings of the other Fmoc-amino acids (4 equiv) were mediated by HBTU (3.8 equiv), HOBt (4 equiv) and DIEA (7.8 equiv) in DMF for 1 h, and monitored by the ninhydrin test. The coupling of the first amino acid (5 equiv) was performed using N,N-diisopropylcarbodiimide (DIPCDI) (5 equiv), 1-hydroxy benzotriazole (HOBt) (5 equiv) and N,N-dimethylaminopyridine (DMAP) (1.25 equiv) in DMF for 15 h. After the coupling, an Fmoc test was performed to check the resin loading. The resin was then acylated by treatment with a mixture of acetic anhydride–pyridine–CH<sub>2</sub>Cl<sub>2</sub> (1:1:1; 2 × 30 min). Couplings of the other Fmoc-amino acids (4 equiv) were mediated by HBTU (3.8 equiv), HOBt (4 equiv) and DIEA (7.8 equiv) in DMF for 1 h, and monitored by the ninhydrin test. Fmoc group was removed by treating the resin with a mixture of piperidine–DMF (3:7; 2 + 10 min). Peptides were individually cleaved from the resin with trifluoroacetic acid (TFA)-H<sub>2</sub>O−trisopropylsilane (95:2.5:2.5; 2 h). Following TFA evaporation and diethyl ether extraction, the crude peptides were dissolved in H<sub>2</sub>O and lyophilized.

Peptides were analyzed under standard analytical high-performance liquid chromatography (HPLC) conditions with a
Dionex liquid chromatography instrument (Conditions A-D). Detection was performed at 220 nm. Solvent A was 0.1% aqueous TFA and solvent B was 0.1% TFA in CH₃CN. Conditions A: Analysis was carried out with a Kromasil 100 C₁₈ (4.6 mm × 40 mm, 3.5 μm) column with a 2–100% B over 7 min at a flow rate of 1 ml/min. Conditions B: Analysis was carried out with a Kromasil 100 C₁₈ (4.6 mm × 40 mm, 3.5 μm) column with a 30–50% B over 6 min at a flow rate of 1 ml/min. Conditions C: Analysis was carried out with a Kromasil 100 C₁₈ (4.6 mm × 40 mm, 3.5 μm) column with a 60–70% B over 6 min at a flow rate of 1 ml/min. Conditions D: Analysis was carried out with a Kromasil 100 C₁₈ (4.6 mm × 250 mm, 3.5 μm) column with a 2–100% B over 30 min at a flow rate of 1 ml/min. Other peptides were analyzed under standard analytical HPLC conditions with an Agilent Technologies 1200 Series liquid chromatography instrument (Conditions E). Detection was performed at 220 nm. Solvent A was 0.1% aqueous TFA and solvent B was 0.1% TFA in CH₃CN. Conditions E: Analysis was carried out with a Kromasil 100 C₁₈ (4.6 mm × 40 mm, 3.5 μm) column with a 2–100% B over 5 min at a flow rate of 1 ml/min.

Electrospray ionization mass spectrometry (ESI-MS, Bruker Daltonics, USA) and matrix-assisted laser desorption ionization with time-of-flight analysis (MALDI-TOF, Bruker, USA) were used to confirm peptide identity.

Peptides BP216, BP217, BP235 and BP236 were purchased from CASLO Laboratory ApS (Lyngby, Denmark) at >90% purity.

Bacterial strains and growth conditions

For the analysis of the in vitro activity of peptides, the following plant pathogenic bacterial strains were used: E. amylovora PMV6076 (Institut National de la Recherche Agronomique, Angers, France), P. syringae pv. syringae EPS94 (Institut de Tecnologia Agroalimentària, Universitat de Girona, Spain) and X. axonopodis pv. vesicatoria 2133-2 (Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain). All bacteria were stored in Luria Bertani (LB) broth supplemented with glycerol (20%) and maintained at -80 °C. All strains were scraped from LB agar plates incubated at 25 °C after growing for 24 h in the case of E. amylovora and P. syringae pv. syringae, and for 48 h for X. axonopodis pv. vesicatoria. The cell material was suspended in sterile water to obtain a suspension of 10⁸ CFU ml⁻¹.

Antibacterial activity

Lyophilized peptides were solubilized in sterile distilled water to a concentration of 1000 μM and filter sterilized through a 0.22-μm-pore-size filter. For MIC assessment, dilutions of the synthetic peptides were made to obtain a final concentration of 200, 100, 75, 50, 25 and 12.5 μM. Twenty microlitres of each dilution were mixed in a microtiter plate well with 20 μl of the corresponding suspension of the bacterial indicator at 10⁷ CFU ml⁻¹ and with 160 μl of Trypticase Soy Broth (TSB) (BioMerieux, France) to a total volume of 200 μl. Final peptide concentrations assayed were 20, 10, 7.5, 5, 2.5 and 1.25 μM. Tag54 and tag54-2 were tested at 100 μM. Three replicates for each strain, peptide and concentration were used. Positive controls contained water instead of peptide and negative controls contained peptide without bacterial suspension. Microbial growth was automatically determined by optical density measurement at 600 nm (Bioscreen C, Labsystem, Finland). Microplates were incubated at 25 °C with 20 s shaking before hourly absorbance measurement for 48 h. The experiment was repeated twice. The MIC was taken as the lowest peptide concentration with no growth at the end of the experiment.

Hemolytic activity

The hemolytic activity of peptides was evaluated by determining hemoglobin release from erythrocyte suspensions of fresh human blood (5%, vol/vol). Blood was aseptically collected using a BD vacutainer K2E System with EDTA (Belliver Industrial State, Plymouth, U.K.) and stored for less than 2 h at 4 °C. Blood was centrifuged at 6,000 × g for 5 min, washed three times with TRIS buffer (10 mM TRIS, 150 mM NaCl, pH 7.2) and ten-fold diluted in the same buffer. Peptides were solubilized in TRIS buffer to a concentration of 500, 300 and 100 μM. Sixty five microlitres of human red blood cells were mixed with 65 μl of the peptide solution (final concentration of 250, 150 and 50 μM) in a 96-well reaction plate and incubated under continuous shaking for 1 h at 37 °C. Then, the plates were centrifuged at 3,500 × g for 10 min. Eighty microlitre aliquots of the supernatant were transferred to 100-well microplates and diluted with 80 μl of sterile distilled water. Three replicates for each peptide were used. Hemolysis was measured as the absorbance at 540 nm with a microplate reader. Complete hemolysis was determined in TRIS buffer plus melittin at 100 μM as a positive control. The percentage of hemolysis (H) was calculated using the equation: 

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H = 100 \times \frac{(Op - Ob)/(Om - Ob)},
\]

where Op is the density for a given peptide concentration, Ob for the buffer, and Om for the melittin positive control.

Phytotoxicity

A set of 16 selected peptides BP100, BP134, BP173, BP178, BP183, BP188, BP192, BP209, BP210, BP211, BP213, BP214, BP215, BP216, BP217 and BP235 were evaluated for their phytotoxicity. Tobacco plants (Nicotiana benthamiana) were grown from seed in the glasshouse and used between 20 and 30 days old. One hundred μl of the peptides at concentrations from 50 to 250 μM, depending on the peptide, were inoculated into the mesophylls of tobacco leaves as described previously [41], and plants were incubated again for three days. Up to six independent inoculations were carried out in a single leaf, and at least three independent inoculations were performed per peptide and concentration, randomly distributed in different leaves and plants. Toxicity was measured as the lesion diameter.

Ethics statement

The use of human blood samples was solely to assess the hemolytic activity of the peptides, and was not used for other type of research with ethics concerns. The president of the Research Committee of the University of Girona confirmed that ethics approval was not required for the collection of blood samples.
**Table 1.** Sequences, number of amino acids, retention times and purities on HPLC, and mass spectrometry data of peptides from the first set.

| Peptide | Sequence | #Aa | tR (min)* | Purity (%) | Theoretical monoisotopic m/z | Observed m/z* |
|---------|----------|-----|----------|------------|-------------------------------|---------------|
| BP134 monomers | KKLFKKILKYL-OH | 11 | 6.24** | 90 | 1421.9 | 1422.1 [M+H]** |
| BP199 | KKLFKKILKYL-AGPA-OH | 15 | 6.60** | 89 | 1718.1 | 1718.3 [M+H]** |
| BP214 | KKLFKKILKYL-KDEL-OH | 15 | 3.14** | 88 | 1907.2 | 1907.2 [M+H]** |
| BP134 dimers | KKLFKKILKYL-KKLFKKILKYL-OH | 22 | 7.48* | 85 | 2824.9 | 2824.9 [M+H]** |
| BP202 | KKLFKKILKYL-AGPA-KKLFKKILKYL-OH | 26 | 3.58* | 99 | 3121.1 | 3121.3 [M+H]** |
| BP193 | G-KKLFKKILKYL-AGPA-KKLFKKILKYL-OH | 27 | 7.18* | 89 | 3179.1 | 3178.8 [M+H]** |
| BP195 | S-KKLFKKILKYL-AGPA-KKLFKKILKYL-OH | 27 | 7.19* | 93 | 3209.1 | 3208.7 [M+H]** |
| BP200 | KKLFKKILKYL-AGPA-KKLFKKILKYL-AGPA-OH | 30 | 7.53* | 88 | 3417.2 | 3417.4 [M+H]** |
| BP198 | KKLFKKILKYL-KKLFKKILKYL-KDEL-OH | 26 | 4.08* | 99 | 3311.3 | 3311.5 [M+H]** |
| BP192 | KKLFKKILKYL-AGPA-KKLFKKILKYL-KDEL-OH | 30 | 7.27* | 95 | 3607.5 | 3608.0 [M+H]** |
| BP213 | KKLFKKILKYL-AGPA-LYKLIKKFLKK-KDEL-OH | 30 | 3.48* | 90 | 3666.3 | 3666.3 [M+H]** |
| BP194 | G-KKLFKKILKYL-AGPA-KKLFKKILKYL-KDEL-OH | 31 | 7.33* | 91 | 3664.6 | 3665.0 [M+H]** |
| BP196 | S-KKLFKKILKYL-AGPA-KKLFKKILKYL-KDEL-OH | 31 | 7.35* | 71 | 3694.6 | 3695.0 [M+H]** |
| BP236 | KKLFKKILKYL-AGPA-KKLFKKILKYL-AGPA-KDWEHLKDWEHLKDWEHL-KDEL-OH | 52 | 20.30** | 99 | 6330.7 [M+H]** | 1266.9 [M+5H]** |
| BP134 trimers | KKLFKKILKYL-KKLFKKILKYL-KKLFKKILKYL-OH | 33 | 9.27** | 52 | 4228.8 | 4229.3 [M+H]** |
| BP201 | KKLFKKILKYL-AGPA-KKLFKKILKYL-AGPA-KKLFKKILKYL-OH | 41 | 8.13** | 67 | 4821.1 | 4821.4 [M+H]** |
| BP216 | KKLFKKILKYL-AGPA-KKLFKKILKYL-AGPA-KKLFKKILKYL-KDEL-OH | 45 | 4.18** | 99 | 5307.8 [M+H]** | 885.3 [M+6H]** |

**Tags**

- tag54 | KDWEHLKDWEHLKDWEHL-OH | 18 | 2.95** | 99 | 2443.7 | 2443.2 [M+H]** |
- tag54-2 | KDWEHLKDWEHLKDWEHL-KDEL-OH | 22 | 2.96** | 99 | 2629.2 | 2629.5 [M+H]** |

* tR = HPLC elution time
* Percentage determined by HPLC at 220 nm from the crude reaction mixture.
* All peptides were analyzed by MALDI-TOF except for BP134, BP216, BP236, tag54, and tag54-2 that were analyzed by ESI-MS.
* RP-HPLC analysis using conditions A (see Materials and Methods).
* RP-HPLC analysis using conditions B (see Materials and Methods).
* RP-HPLC analysis using conditions C (see Materials and Methods).
* RP-HPLC analysis using conditions D (see Materials and Methods).

Results

**Design and synthesis of peptides**

Peptides were designed and synthesized in order to obtain sequences with optimized properties for plant expression at high yield and for accumulation in different subcellular plant cell compartments. Peptide sequences were based on the antimicrobial peptide BP134 (KKLFKKILKYL-OH), a C-terminal carboxylic acid derivative of BP100 [17]. A total of 40 peptides of 15 to 52 amino acids in length were prepared. One set of peptides contained one, two or three units of BP134 with: (i) melittin(10-19) or melittin(1-13); (ii) magainin(4-10); (iii) magainin(1-10); and (iv) cecropin A(25-37) (Table 2). Analogues of these peptides were also obtained by introducing: (i) one or two AGPA hinges as stabilization/distortion moiety; (ii) a KDEL fragment at the C-terminus as a signal for permanent retention of peptides in the endoplasmic reticulum; (iii) a Gly or a Ser residue at the N-terminus as a TEV protease recognition site; and (iv) the epitope tag KDWEHLKDWEHLKDWEHL (tag54) at the C-terminus for peptide detection or purification.

The synthesis was performed following a standard Fmoc/Ibu solid-phase peptide synthesis methodology to yield C-terminal carboxylic acid sequences; overall purity was among 71-99%, except for three sequences that were obtained in 52-68% purity (Tables 1 and 2). Their molecular weights were confirmed by mass spectrometry.

**Antibacterial activity**

The peptides synthesized were tested for in vitro growth inhibition of *X. axonopodis* pv. vasculatoria, *P. syringae* pv. syringae and *E. amylovora* at 1.25, 2.5, 5.0, 7.5, 10, and 20 μM and compared to that of BP134 (Tables 3 and 4).

From the analysis of the first set of peptides incorporating repeating units of BP134, we observed that the dimer BP203 displayed higher activity than the trimer BP204 against the three pathogens (Table 3). Moreover, BP203 was more active than the monomer BP134 against *X. axonopodis* pv. vasculatoria (1.25-2.5 μM vs 10-20 μM) and *P. syringae* pv.
Table 2. Sequences, number of amino acids, retention times and purities on HPLC, and mass spectrometry data of peptides from the second set.

| Peptide Sequence                                                                 | #Aa | t_R (min) | Purity (%) | Theoretical monoisotopic m/z | Observed m/z 5+ |
|---------------------------------------------------------------------------------|-----|-----------|------------|------------------------------|-----------------|
| BP134-melittin(10-19) or BP134-melittin(1-13)                                   |     |           |            |                              |                 |
| BP170 KKLFKKLKYL-TTGLPALISW-OH                                                   | 21  | 6.95      | 90         | 2461.1                       | 2461.4 [M+H]+   |
| BP171 KKLFKKLKYL-AGPA-TTGLPALISW-OH                                             | 25  | 7.13      | 87         | 2757.7                       | 2757.8 [M+H]+   |
| BP172 G-KKLFKKLKYL-AGPA-TTGLPALISW-OH                                           | 26  | 3.41      | 85         | 2814.7                       | 2814.6 [M+H]+   |
| BP173 S-KKLFKKLKYL-AGPA-TTGLPALISW-OH                                           | 26  | 3.41      | 85         | 2844.7                       | 2844.5 [M+H]+   |
| BP174 KKLFKKLKYL-TTGLPALISW-OH                                                  | 25  | 7.14      | 87         | 2946.8                       | 2946.8 [M+H]+   |
| BP175 KKLFKKLKYL-AGPA-TTGLPALISW-OH                                             | 29  | 7.04      | 80         | 3242.9                       | 3243.0 [M+H]+   |
| BP176 KKLFKKLKYL-GIGAVLKLTTGL-KDEL-OH                                           | 28  | 5.23      | 98         | 3129.9                       | 3130.0 [M+H]+   |
| BP177 KKLFKKLKYL-TTGLPALISW-AGPA-SILAPLGTTL-YKLLKFLKK-KDEL-OH                   | 48  | 25.22     | 99         | 5315.7 [M+H]+                | 1063.9 [M+5H]+  |
| BP134-magainin(4-10)                                                           |     |           |            |                              |                 |
| BP180 KKLFKKLKYL-KFLHSAK-OH                                                     | 18  | 6.55      | 99         | 2233.4                       | 2233.4 [M+H]+   |
| BP181 KKLFKKLKYL-AGPA-KFLHSAK-OH                                                | 22  | 6.51      | 99         | 2529.6                       | 2529.6 [M+H]+   |
| BP182 G-KKLFKKLKYL-AGPA-KFLHSAK-OH                                              | 23  | 3.15      | 83         | 2586.6                       | 2586.6 [M+H]+   |
| BP183 S-KKLFKKLKYL-AGPA-KFLHSAK-OH                                              | 23  | 3.16      | 82         | 2616.8                       | 2616.8 [M+H]+   |
| BP184 KKLFKKLKYL-KFLHSAK-KDEL-OH                                               | 22  | 6.13      | 99         | 2718.4                       | 2718.7 [M+H]+   |
| BP185 KKLFKKLKYL-KFLHSAK-KDEL-OH                                               | 26  | 6.11      | 73         | 3014.7                       | 3014.8 [M+H]+   |
| BP186 KKLFKKLKYL-AGPA-KDEL-OH                                                   | 48  | 17.32     | 99         | 5738.8 [M+H]+                | 1148.5 [M+5H]+  |
| BP134-magainin(1-10)                                                           |     |           |            |                              |                 |
| BP178 KKLFKKLKYL-GIGKFLHSAK-OH                                                  | 21  | 6.48      | 72         | 2460.1                       | 2460.3 [M+H]+   |
| BP179 KKLFKKLKYL-AGPA-GIGKFLHSAK-OH                                             | 25  | 6.52      | 80         | 2756.7                       | 2756.7 [M+H]+   |
| BP180 G-KKLFKKLKYL-AGPA-GIGKFLHSAK-OH                                           | 26  | 3.29      | 93         | 2813.7                       | 2813.5 [M+H]+   |
| BP181 S-KKLFKKLKYL-AGPA-GIGKFLHSAK-OH                                           | 26  | 3.30      | 92         | 2843.7                       | 2843.6 [M+H]+   |
| BP182 KKLFKKLKYL-GIGKFLHSAK-KDEL-OH                                            | 25  | 6.44      | 99         | 2945.7                       | 2945.7 [M+H]+   |
| BP183 KKLFKKLKYL-AGPA-GIGKFLHSAK-KDEL-OH                                        | 29  | 6.50      | 90         | 3242.0                       | 3242.7 [M+H]+   |
| BP134-cecropin A(25-37)                                                        |     |           |            |                              |                 |
| BP188 KKLFKKLKYL-AAVAVGQATQIAK-KDEL-OH                                          | 28  | 5.14      | 99         | 3143.9                       | 3144.0 [M+H]+   |
| BP189 KKLFKKLKYL-AGPA-AAVAVGQATQIAK-KDEL-OH                                     | 31  | 3.30      | 92         | 3369.0                       | 3369.0 [M+H]+   |
| BP190 AAVAVGQATQIAK-KKLFKKLKYL-KDEL-OH                                         | 28  | 5.05      | 98         | 3143.9                       | 3144.0 [M+H]+   |

| a RP-HPLC retention time 
| b Percentage determined by HPLC at 220 nm from the crude reaction mixture 
| c All peptides were analyzed by MALDI-TOF except for peptides BP217 and BP235 that were analyzed by ESI-MS 
| d RP-HPLC analysis using conditions A (see Materials and Methods) 
| e RP-HPLC analysis using conditions B (see Materials and Methods) 
| f RP-HPLC analysis using conditions C (see Materials and Methods) 
| g RP-HPLC analysis using conditions D (see Materials and Methods) 
| h Percentage determined by MALDI-TOF except for peptides BP217 and BP235 that were analyzed by ESI-MS 
| i RP-HPLC analysis using conditions A (see Materials and Methods) 
| j RP-HPLC analysis using conditions B (see Materials and Methods) 
| k RP-HPLC analysis using conditions C (see Materials and Methods) 
| l RP-HPLC analysis using conditions D (see Materials and Methods) 

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The introduction of an AGPA moiety as a hinge between two BP134 units afforded peptides displaying higher activity (compare BP203 and BP202; BP198 and BP192; BP204 and BP201). In contrast, the incorporation of an AGPA moiety at the C-terminus of BP134 or of BP202 led to peptides BP199 and BP200, respectively, with enhanced or similar activity against X. axonopodis pv. vesicatoria (2.5-5.0 μM and <1.25 μM), and with decreased activity against the other two pathogens. When a KDEL moiety was introduced at the C-terminus of BP134, an increase of the activity was observed against the three bacteria (BP214, 1.25-5.0 μM). However, the presence of a KDEL unit at the C-terminus of a dimer sequence maintained or decreased the activity, as shown for BP203 vs BP198, BP202 vs BP192, BP193 vs BP194, and BP195 vs BP196. The same behaviour was observed for the trimer peptides BP201 and BP216. The incorporation of a Gly or a Ser residue at the N-terminus of BP192 (5.0-10 μM) and BP202 (<1.25-5.0 μM) rendered peptides BP193-BP196 with higher MIC values (7.5-20 μM). The epitope tag peptides tag54 and tag54-2, the latter incorporating a KDEL unit at the C-terminus, were not active against the three bacteria. However, the introduction of tag54-2 into BP200 resulted in peptide BP236 with increased activity against P. syringae pv. syringae (2.5-5.0 μM) and E. amylovora (10-20 μM), and with reduced activity against X. axonopodis pv. vesicatoria (2.5-5.0 μM). Peptide dimer BP213, bearing a reversed peptide sequence from BP134 at the C-terminus, was slightly more active than BP192 against the three pathogens (1.25-5.0 μM vs 5.0-10 μM). From this set of
Table 3. Antibacterial activity (MIC) against three plant pathogenic bacteria and hemolytic activity of peptides from the first set.

| Peptide  | MIC (μM) | Xav<sup>a</sup> | Pss<sup>a</sup> | Es<sup>a</sup> | 50 μM | 150 μM | 250 μM | Hemolysis<sup>b</sup> (%) |
|----------|----------|-----------------|-----------------|--------------|-------|--------|--------|--------------------------|
| BP134    |          |                 |                 |              |       |        |        |                          |
| BP199    |          |                 |                 |              |       |        |        |                          |
| BP214    |          |                 |                 |              |       |        |        |                          |
| BP134 dimers |       |                 |                 |              |       |        |        |                          |
| BP203    |          |                 |                 |              |       |        |        |                          |
| BP202    |          |                 |                 |              |       |        |        |                          |
| BP193    |          |                 |                 |              |       |        |        |                          |
| BP195    |          |                 |                 |              |       |        |        |                          |
| BP200    |          |                 |                 |              |       |        |        |                          |
| BP198    |          |                 |                 |              |       |        |        |                          |
| BP192    |          |                 |                 |              |       |        |        |                          |
| BP213    |          |                 |                 |              |       |        |        |                          |
| BP194    |          |                 |                 |              |       |        |        |                          |
| BP196    |          |                 |                 |              |       |        |        |                          |
| BP236    |          |                 |                 |              |       |        |        |                          |
| BP134 trimers |      |                 |                 |              |       |        |        |                          |
| BP204    |          |                 |                 |              |       |        |        |                          |
| BP201    |          |                 |                 |              |       |        |        |                          |
| BP216    |          |                 |                 |              |       |        |        |                          |

<sup>a</sup>Xav, Xanthomonas axonopodis pv. vesicatoria; Pss, Pseudomonas syringae pv. syringae; Es, Erwinia amylovora
<sup>b</sup>Hemolysis plus confidence interval (α=0.05)

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Table 4. Antibacterial activity (MIC) against three plant pathogenic bacteria and hemolytic activity of peptides from the second set.

| Peptide  | MIC (μM) | Xav<sup>a</sup> | Pss<sup>a</sup> | Es<sup>a</sup> | 50 μM | 150 μM | 250 μM | Hemolysis<sup>b</sup> (%) |
|----------|----------|-----------------|-----------------|--------------|-------|--------|--------|--------------------------|
| BP134-melittin(10-19) or BP134-melittin(1-13) |          |                 |                 |              |       |        |        |                          |
| BP170    |          |                 |                 |              |       |        |        |                          |
| BP171    |          |                 |                 |              |       |        |        |                          |
| BP207    |          |                 |                 |              |       |        |        |                          |
| BP208    |          |                 |                 |              |       |        |        |                          |
| BP172    |          |                 |                 |              |       |        |        |                          |
| BP173    |          |                 |                 |              |       |        |        |                          |
| BP189    |          |                 |                 |              |       |        |        |                          |
| BP217    |          |                 |                 |              |       |        |        |                          |
| BP134-magainin(4-10) |      |                 |                 |              |       |        |        |                          |
| BP180    |          |                 |                 |              |       |        |        |                          |
| BP181    |          |                 |                 |              |       |        |        |                          |
| BP211    |          |                 |                 |              |       |        |        |                          |
| BP212    |          |                 |                 |              |       |        |        |                          |
| BP182    |          |                 |                 |              |       |        |        |                          |
| BP183    |          |                 |                 |              |       |        |        |                          |
| BP235    |          |                 |                 |              |       |        |        |                          |
| BP134-magainin(1-10) |      |                 |                 |              |       |        |        |                          |
| BP176    |          |                 |                 |              |       |        |        |                          |
| BP175    |          |                 |                 |              |       |        |        |                          |
| BP209    |          |                 |                 |              |       |        |        |                          |
| BP210    |          |                 |                 |              |       |        |        |                          |
| BP179    |          |                 |                 |              |       |        |        |                          |
| BP178    |          |                 |                 |              |       |        |        |                          |
| BP134-magainin(25-37) |      |                 |                 |              |       |        |        |                          |
| BP188    |          |                 |                 |              |       |        |        |                          |
| BP215    |          |                 |                 |              |       |        |        |                          |
| BP190    |          |                 |                 |              |       |        |        |                          |

<sup>a</sup>Xav, Xanthomonas axonopodis pv. vesicatoria; Pss, Pseudomonas syringae pv. syringae; Es, Erwinia amylovora
<sup>b</sup>Hemolysis plus confidence interval (α=0.05)

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Peptides, the most active sequences were BP202, BP213 and BP214.

Concerning the second set of peptides, incorporating a combination of one unit of BP134 with fragments of the natural antimicrobial peptides melittin, magainin and cecropin A, we observed that peptide dimers BP170, BP176 and BP180 displayed lower MIC values (1.25-7.5 μM) than BP134 (Table 4). In general, the incorporation of an AGPA moiety into the sequence of peptides BP170, BP172, BP176, BP179, BP180, BP182, and BP188 did not significantly influence the activity rendering peptides BP171, BP173, BP175, BP178, BP181, BP183 and BP215, respectively, with MIC values of 1.25-10 μM. Sequences BP172, BP173, BP178, BP179, BP182 and BP183, bearing a KDEL moiety at the C-terminus, were as active or slightly less active (1.25-10 μM) than the corresponding peptides BP170, BP171, BP175, BP176, BP180 and BP181 (1.25-7.5 μM). In general, the derivatization of BP171, BP175 and BP181 at the N-terminus with a Gly or a Ser residue resulted in peptides BP207-BP212 with slightly lower MIC values (<1.25-5.0 μM). Peptide BP235, derived from BP134 and magainin(4-10) and bearing two AGPA moieties, tag54-2, and KDEL, showed similar activity than BP181 (2.5-5.0 μM). Peptide BP217, which resulted from the combination of BP170 and a reversed peptide sequence from BP170 linked with an AGPA moiety and bearing KDEL at the C-terminus, displayed similar activity than BP170 (<1.25-5.0 μM). The analogue BP172, resulting from the combination of BP134 and melittin(10-19), was more active (1.25-5.0 μM) than BP189 bearing a melittin(1-13) fragment. When the BP134 and cecropin A(25-37) fragments in BP188 (1.25-5.0 μM) were inverted, the resulting peptide BP190 was poorly active (5.0 - >20 μM). The best peptides of this second set were BP207, BP208, BP209, BP210, and BP211 which also displayed higher activity than the most active peptides from the first set.

Hemolytic activity

The toxicity of peptides to eukaryotic cells was determined as the ability to lyse erythrocytes in comparison to melittin.
Percent hemolysis at 50, 150, and 250 μM is shown in Tables 3 and 4.

Among the peptides of the first set, the two BP134 monomeric analogues BP199 and BP214 were not hemolytic even at 250 μM (0-9%), whereas the dimer BP203 and the trimer BP204 were highly hemolytic (95-100%) (Table 3). Peptides incorporating an AGPA moiety either as a hinge or at the C-terminus (BP192 and BP199-BP202) exhibited similar hemolytic activity than the corresponding peptides that do not incorporate this moiety (BP134, BP198, BP203, and BP204).

The incorporation of a KDEL moiety at the C-terminus of BP134, BP193, BP201, BP202, and BP203 resulted in peptides BP214, BP194, BP196, BP216, BP192, and BP198, respectively, that did not display a clear hemolytic activity pattern. The modification of the N-terminus of peptide BP202 with a Gly or a Ser residue led to peptides BP193 and BP195 with lower hemolysis (71-74% at 250 μM). In contrast, when the same derivatization was performed on BP192, the resulting peptides BP194 and BP196 were slightly more hemolytic (74-92% at 250 μM). The presence of the epitope tag tag54-2 (not hemolytic) at the C-terminus of BP236 did not significantly influence the hemolytic activity as compared to BP200 (92% vs 81% at 250 μM). Peptide dimer BP213, bearing a reversed peptide sequence from BP134 at the C-terminus, was more hemolytic (98% at 250 μM) than its analogue BP192 (69% at 250 μM). Peptide monomers BP199 and BP214 were the least hemolytic sequences from this set (0-9% at 250 μM) and BP192, BP193, BP195, BP196 and BP198 displayed <75% hemolysis at 250 μM.

Regarding the second set of peptides (Table 4), the combination of one unit of BP134 with melittin(10-19), magainin(4-10) and magainin(1-10) resulted in analogues BP170, BP180 and BP176, respectively, with a significantly higher hemolysis percentage (58-98% at 250 μM) than that of BP134 (18% at 250 μM). Among them, the most hemolytic was BP170. Peptides containing an AGPA moiety as a hinge, BP171, BP173, BP175, BP178, BP181, BP183 and BP215, were less hemolytic (0-40% at 250 μM) than the corresponding analogues BP170, BP172, BP176, BP179, BP180, BP182, and BP188 (35-98% at 250 μM). The incorporation of a KDEL moiety at the C-terminus of BP170, BP171, BP175, BP176, BP180 and BP181 rendered peptides BP172, BP173, BP178, BP179, BP182 and BP183 with similar or lower hemolysis.

When peptides BP171, BP175 and BP181 were modified at the N-terminus with a Gly or a Ser residue, the resulting sequences BP207-BP212 showed a comparable hemolytic percentage being those incorporating a Ser residue the most hemolytic. BP181 and its analogue BP235, incorporating a BP134 unit and a magainin(4-10) fragment together with two AGPA moieties and tag54-2, were not hemolytic even at 250 μM. Similarly to BP170, its analogues BP189 and BP217 were highly hemolytic. The derivatives containing a BP134 unit and a cecropin A(25-37) fragment, BP188 and BP190, displayed similar hemolysis (42% and 32% at 250 μM, respectively). The least hemolytic peptides from this second set are BP178, BP181, BP183, BP209, BP211, BP212, BP215, and BP235 which displayed hemolysis ≤25% at 250 μM.

**Phytotoxicity**

All BP134 derived peptides were more phytotoxic than the original monomer (Figures 1 and 2). However, phytotoxicity was only observed at concentrations 10-50 times higher than the MIC, as in the case of hemolytic activity. Tobacco leaves responded in a very quick and selective manner to peptides, and a clear dose-response effect was observed for some of the best antibacterial peptides (BP134, BP209, BP210, and BP211), with practically a linear increase from 50 to 250 μM (Figure 1). Melittin was the most phytotoxic peptide, inducing lesions of around 2 cm diameter. The most phytotoxic BP134 derivatives were BP214 (BP134-KDEL), BP192 and BP213 (BP134 dimer derivatives), BP217 (BP134-melittin(10-19) dimer derivative), BP178 (BP134-AGPA-magainin(1-10)-KDEL), and BP188 (BP134-cecropin A(25-37)-KDEL) (Figure 2). Peptides with moderate phytotoxicity were BP100, BP173 (BP134-AGPA-melittin(10-19)-KDEL), BP183, BP211, and BP235 (BP134-AGPA-magainin(4-10) derivatives), BP209 and BP210 (BP134-AGPA-magainin(1-10) derivatives), BP215 (BP134-AGPA-mecillin(25-37)-KDEL), and BP216 (BP134 trimer derivative).

**Relationship between phytotoxicity and hemolytic activity**

When phytotoxicity was plotted against hemolytic activity (Figure 2) two different patterns were observed. Peptides BP192, BP213, BP216 and BP217 were strongly phytotoxic and hemolytic, with similar values as melittin at the same concentrations. In contrast, peptides BP173, BP178, BP183, BP188, BP209, BP210, BP211, BP214, BP215, and BP235 were low or very low hemolytic but they unexpectedly caused moderate to significant lesions in tobacco leaves upon infiltration.

**Discussion**

The undecapeptide BP100 (KKLFKKILKYL-NH₂), identified from a library of cecropin A-melittin hybrid peptides, has significant activity against X. axonopodis pv. vesicatoria, P. syringae pv. syringae and E. amylovora (MIC of 2.5-7.5 μM) and low hemolysis (22% at 150 μM) [17]. Since it is known that peptide expression by plants requires a minimum chain length and that these peptides are produced as C-terminal carboxylic acids, here we describe BP100 analogues containing from 15 to 52 residues and a carboxylic acid group at the C-terminus. The C-terminal carboxylic acid undecapeptide BP134 (KKLFKKILKYL-OH) was also included for comparison purposes. In designing BP134 analogues, we incorporated repeated units of BP134 or a combination of one BP134 sequence with a fragment of a natural antimicrobial peptide, in particular, melittin(10-19), melittin(1-13), magainin(4-10), magainin(1-10) or cecropin A(25-37). Moreover, we modified these peptides by introducing one or two AGPA hinges as stabilization/distortion moiety, a KDEL fragment at the C-terminus as a signal for permanent retention of peptides in the endoplasmic reticulum, a Gly or a Ser residue at the N-terminus as a TEV protease recognition site and the epitope tag KDWEHLKDWEHLKDWEHL (tag54) at the C-terminus for
peptide detection and purification. We studied the influence of these modifications on the antimicrobial and hemolytic activities.

The combination of BP134 with another unit of this peptide or with a fragment of melittin or magainin led to sequences with higher antibacterial activity, being the hybrid BP134-melittin(10-19) (BP170) the most active. Moreover, the elongation of a peptide sequence with an AGPA hinge, a KDEL unit or the tag epitope tag54-2 do not significantly influence peptide activity. Notably, the modification of the N-terminus in peptides BP171 (BP134-AGPA-melittin(10-19)), BP175 (BP134-AGPA-magainin(1-10)) and BP181 (BP134-AGPA-magainin(4-10)) with a Gly or a Ser residue led to an increase of the antibacterial activity. Peptides with this Gly or Ser residue are generated upon hydrolysis with the TEV protease over peptide-protein fusions in certain strategies for heterologous expression in plants [38]. The resulting sequences BP207-BP212 displayed similar activities and were among the best peptides identified in this study (MIC of <1.25 to 5.0 μM). These results also show the effect of small sequence modifications on the biological activity of these peptides [17,42-44] and are in agreement with previous studies reporting that the length and sequence are among the most important factors for biological activity of antimicrobial peptides [45].

The order of the peptide fragments is crucial for the antibacterial activity. For example, the hybrid BP188, containing the cationic peptide BP134 at the N-terminus and
the hydrophobic cecropin A(25-37) fragment at the C-terminus is highly active against the three pathogens, whereas the analogue BP190 containing cecropin A(25-37)-BP134 is only active against X. axonopodis pv. vesicatoria and with higher MIC values than BP188. This result is in agreement with the structural features of cecropins necessary for antibacterial activity which include a basic N-terminus and a hydrophobic C-terminus [46].

Interestingly, the peptides incorporating a combination of a normal and a reversed peptide sequence, like BP213 and BP217, showed higher or similar activity than the normal peptides BP192 and BP170, respectively. A similar behavior has been previously reported for reversed peptides derived from the antimicrobial peptides cecropin, melittin or magainin [47-51].

Peptide toxicity has been evaluated against red blood human cells thanks to the highly standardized methods available and because data can be compared straightforwardly with other reports, mainly dealing with human pathogens. In general, the elongation of the BP134 sequence with another BP134 unit or with a fragment of a natural antimicrobial peptide, produced an increase of the hemolytic activity. The most hemolytic peptides were those incorporating two BP134 units and those derived from melittin(10-19) and melittin(1-13), while the derivatives obtained from magainin and cecropin A fragments displayed the lowest hemolysis. Among the latter, six peptides were less hemolytic than BP134 (0-14% at 250 μM). The hemolysis observed for the analogues designed from melittin, magainin and cecropin A correlated with that of the natural peptides. In fact, melittin has been described to be very cytotoxic for erythrocytes, whereas magainin and cecropin A have no toxicity [52,53]. Surprisingly, even though BP134 is low hemolytic, the dimer BP203 and its analogues showed high hemolysis.

Figure 2. Badosa et al. The assay was performed at 50 (low, L), 150 (medium, M) and 250 μM (high, H). For some peptides not all concentrations were assayed.

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The incorporation of an AGPA hinge to peptides containing two units of BP134 did not influence the hemolytic activity. Notably, when this hinge was introduced in peptides including a melittin, magainin or cecropin A fragment, the cytotoxicity decreased significantly. The other modifications such as the derivatization with a Gly or a Ser residue at the N-terminus or with tag54-2 at the C-terminus resulted in peptides with comparable hemolysis. Peptides bearing a Gly residue were slightly less hemolytic than those containing a Ser. On the other hand, the elongation of a peptide with a reversed sequence afforded compounds highly hemolytic (BP213 and BP217). Interestingly, the most active peptides BP207-BP211 showed low hemolysis (6-68% at 250 μM), being BP209, BP210 (BP134-magainin(1-10) derivatives) and BP211 (BP134-magainin(4-10) derivative) the sequences with the best balance between antibacterial and hemolytic activities.

In relation to phytotoxicity, all peptides were more phytotoxic than BP134 and a dose-response direct relationship was observed between the concentration of peptide and the development of lesion in tobacco. However, best peptides in terms of high antibacterial and low hemolytic activities (BP209, BP210 and BP211) showed a moderate phytotoxicity. In agreement with our observation is the report that the constitutive expression of transgenes encoding certain BP134 analogues (BP192, BP213, BP216, BP217) has a negative impact on rice plant regeneration upon callus transformation [41].

Interestingly, several peptides incorporating a BP134 unit and melittin, magainin or cecropin A fragment, showed low hemolytic activity, but moderate or high phytotoxicity. It could be possible that these peptides have no targets into the erythrocyte membrane, but affect tobacco plant cell membranes. However, a second possibility is that these peptides are elicitors of the hypersensitivity reaction in tobacco leaves, but this cannot be distinguished from phytotoxicity based on lesion symptoms alone as used here, and would require additional analysis. The possibility of having peptides in the CECMEL11 library with defense elicitation properties is in agreement with other studies reporting elicitation of defense responses by peptides in BY2 tobacco cells and protoplasts [54] and in cucumber and Arabidopsis leaves [55].

In the present work, phytotoxicity has been assessed by leaf infiltration into tobacco as a model and it may differ in other plant systems that can be used to express BP134 derivatives. However, a significant correlation has been reported between toxicity in tobacco leaves and rice seed germination for 10 relevant peptides (BP134, BP235, BP183, BP173, BP178, BP215, BP217, BP213, and BP192) which have been included in the present report [56].

In summary, we have described a convenient strategy for the development of peptides to be expressed by plants with high antibacterial activity, low hemolysis and moderate phytotoxicity. The structural features that confer these biological properties are the presence of an AGPA hinge together with a Gly residue at the N-terminus as a protease recognition site. Moreover, since the presence of a KDEL unit or tag54-2 at the C-terminus of a peptide sequence do not influence significantly in its biological activity, these moieties can be introduced enabling the design of compounds that can be retained in the endoplasmic reticulum and recognized by a complementary epitope. Interestingly, the best peptides in terms of high antibacterial and low hemolytic activities, with moderate phytotoxicity were BP209, BP210 and BP211. Current research by our laboratory involves the expression of several of these peptides in rice plants using diverse strategies to direct expression either to the whole plant or to seed endosperm or embryo, that will permit to verify the main conclusions of the present study.

Author Contributions
Conceived and designed the experiments: E. Badosa E. Bardají LF MP EM. Performed the experiments: E. Badosa LM GM MT. Analyzed the data: E. Badosa LM GM MT E. Bardají LF MP EM. Contributed reagents/materials/analysis tools: E. Badosa E. Bardají LF MP EM. Wrote the manuscript: E. Badosa E. Bardají LF MP EM.

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