Antimicrobial peptide KSL-W and analogues: Promising agents to control plant diseases

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Abstract

Recent strong restrictions on the use of pesticides has prompted the search for safer alternatives, being antimicrobial peptides promising candidates. Herein, with the aim of identifying new agents, 15 peptides reported as plant defense elicitors, promiscuous, multifunctional or antimicrobial were selected and tested against six plant pathogenic bacteria of economic importance. Within this set, KSL-W (KKVVFWVKFK-NH_2) displayed high antibacterial activity against all the tested pathogens, low hemolysis and low phytotoxicity in tobacco leaves. This peptide was taken as a lead and 49 analogues were designed and synthesized, including N-terminal deletion sequences, peptides incorporating a D-amino acid and lipopeptides. The screening of these sequences revealed that a nine amino acid length was the minimum for activity. The presence of a D-amino acid significantly decreased the hemolysis and endowed KSL-W with the capacity to induce the expression of defense-related genes in tomato plants. The incorporation of an acyl chain led to sequences with high activity against *Xanthomonas* strains, low hemolysis and phytotoxicity. Therefore, this study demonstrates that KSL-W constitutes an excellent candidate as new agent to control plant diseases and can be considered as a lead to develop derivatives with multifunctional properties, including antimicrobial and plant defense elicitation.

Keywords

D-Amino acids; Lipopeptides; Plant pathogens; Plant defense elicitors

Introduction

The continuous emergence of new pests caused by phytopathogenic bacteria and fungi gives rise to huge economic losses in agriculture [1-3]. The management of these diseases relies on extensive use of chemical pesticides, namely copper-based compounds and antibiotics [4]. However, the use of these products generates ethical, environmental and welfare concerns. In addition, the development of resistance by bacteria to antibiotics has further exacerbated the availability of efficient compounds for crop protection [5,6]. This situation is driving researchers to explore new and non-conventional approaches.

In this regard, antimicrobial peptides have captured attention as novel candidates to control plant diseases [7-10]. They have been found virtually in all higher organisms as an important component of innate immunity to microbial infections. Most antimicrobial peptides are positively charged and amphipathic, features that favor their interaction with and insertion into the anionic phospholipids of the bacterial membrane, leading to its final disruption [11-16]. In addition, it has been demonstrated that these peptides can also interact with intracellular targets [17,18].

In the pursuit of efficient agents to control the plant diseases caused by the bacteria *Erwinia amylovora*, *Xanthonomas axonopodis* pv. vesicatoria, and *Pseudomonas syringae* pv. syringae, we have identified linear undecapeptides, cyclic decapeptides and triazolyl or acylated derivatives with high antibacterial activity, low hemolysis and phytotoxicity, and reasonable susceptibility to protease degradation [19-22]. Moreover, we have recently investigated a new strategy for plant disease management based on the use of peptides as plant defense elicitors. Interestingly, we have found sequences from the aforementioned families that are able to induce defense responses on tobacco cells and tomato plants, and that efficiently control fire blight infections on pear [23].

The finding of new antimicrobial peptides active *in vivo* against plant pathogens demands the accessibility to a wide range of sequences with high activity *in vitro*. Realizing this need, we selected sequences described in the literature as plant defense elicitors, promiscuous, multifunctional or antimicrobial to be further tested against our target plant pathogens.

Among peptide elicitors, we chose PIP-1 (YGIHTH-NH_2), identified through combinatorial chemistry, and Pep-13 (VWNQPVRGFKVYE-OH), a pathogen-associated molecular pattern (PAMP) from *Phytophthora sojae*, which trigger multiple defense responses in plant cells [24-28]. The promiscuous peptides studied were the AMPs *Cn*-AMP1 (SVAGRAQGM-NH_2), *Cn*-AMP2 (TESYFVFSVGM-NH_2) and *Cn*-AMP3 (YCSYTMEA-NH_2) identified from green coconut (*Cocos nucifera*) water [29,30].

Regarding multifunctional peptides, we considered QKALNEINQF-NH_2 (p10) and TKKTKLTEEEKNRL-NH_2 (p14) which were isolated from bovine milk [31,32]; temporin-1CEa (FVDLKKIANIINSIFGK-NH_2), identified from the skin secretions of the Chinese brown frog (*Rana chensinensis*) [33-34]; the lactoferrin-derived peptide WFRKQLKW-OH (L10) [35]; frenatin 2.1S (GLVGTLLGHIGKAILG-NH_2) and frenatin 2.2S (GLVGTLLGHIGKAILS-NH_2) which were isolated from skin secretions of the frog *Sphaenorhynchus lacteus* [36,37]. Apart from being able to develop different biological activities including antimicrobial activity, these peptides show, in general, low hemolysis.

As antimicrobial peptides we selected Api88 (Gu-ONNRPVYIPRPRPPHPRL-NH_2), a Pro-rich antimicrobial peptide derived from apidaecin 1b [38] and JCpep7 (KVFLGLK-OH), a short cationic antimicrobial peptide isolated from *Jatropha curcas* [39]. We also included in the study the synthetic decapeptide KSL (KKVVFKVKFK-NH_2), identified from a combinatorial library, and its improved analogue KSL-W (KKVVFWVKFK-NH_2) [40-42]. They show a wide range of antimicrobial activity and display low hemolysis. Moreover, they effectively block oral biofilm formation and inhibit the

activity of several oral bacteria involved in caries development [41-45]. In addition, it has been shown that KSL-W is also effective towards other bacteria associated with wound healing [46].

Herein, to achieve our goal of developing novel antimicrobial agents, the selected peptides were screened against the plant pathogenic bacteria *Erwinia amylovora*, *Xanthomonas arboricola* pv. pruni, *Xanthonomas axonopodis* pv. vesicatoria, *Xanthomonas fragariae*, *Pseudomonas syringae* pv. actinidiae and *Pseudomonas syringae* pv. syringae. We also examined their hemolytic activity and phytotoxicity. The best candidates were taken as leads to prepare new analogues which were also screened for their antibacterial activity and toxicity. Moreover, we sought to determine the potential of these analogues to express defense-related genes on tomato plants.

Materials and Methods

Manual solid-phase synthesis was performed in polypropylene syringes (2 or 5 mL) fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by suction. Most chemicals were from commercial suppliers Sigma-Aldrich (Madrid, Spain), Iris Biotech GmbH (Marktredwitz, Germany), NovaBiochem (Schwalbach, Germany), Merck (Mollet del Vallès, Spain), Fischer Scientific (Madrid, Spain), Carlo Erba (Sabadell, Spain), and used without further purification.

Peptides were analyzed using standard analytical HPLC conditions with a Dionex liquid chromatography instrument composed of an UV/Vis Dionex UVD170U detector, a P680 Dionex bomb, an ASI-100 Dionex automatic injector, and CHROMELEON 6.60 software. Detection was performed a wavelength of 220 nm. Solvent A was 0.1% aqueous TFA and solvent B was 0.1% TFA in CH_3CN. Analysis was carried out with a Kromasil 100 C18 (4.6 mm x 40 mm x 3.5 μm) column with a 2-100% B over 7 min at a flow rate of 1 mL/min.

All peptides were purified on a Combi*Flash* Rf200 automated flash chromatography system using Redi*Sep* Rf Gold reversed-phase C18 column packed with high performance C18 derivatized silica.

ESI-MS analyses were performed at the Serveis Tècnics de Recerca of the University of Girona with an Esquire 6000 ESI ion Trap LC/MC (Bruker Daltonics) instrument equipped with an electrospray ion source. The instrument was operated in the positive ESI(+) ion mode. Samples (5 μL) were introduced into the mass spectrometer ion source directly through an HPLC autosampler. The mobile phase (80:20 CH_3CN/H_2O at a flow rate of 100 μL/min) was delivered by a 1200 Series HPLC pump (Agilent). Nitrogen was employed as both the drying and nebulising gas.

HRMS were recorded on a Bruker MicroTof-QIITM instrument using ESI ionization source at the Serveis Tècnics de Recerca of the University of Girona. Samples were introduced into the mass

spectrometer ion source by direct infusion using a syringe pump and were externally calibrated using sodium formate. The instrument was operated in the positive ion mode.

General procedure for the solid-phase synthesis of peptides

Peptides were synthesized manually by the solid-phase method using standard Fmoc/^{t}Bu strategy. A Fmoc-Rink-MBHA resin (0.55 mmol/g), a PAC-MBHA resin (0.4 mmol/g), a Fmoc-Rink-ChemMatrix resin (0.66 mmol/g) or a PAC-ChemMatrix resin (0.66 mmol/g) was used as solid support. The ChemMatrix resins were selected for the synthesis of peptides containing more than 11 residues. The PAC-derivatized resins were employed to prepare C-terminal carboxylic acid peptides whereas the Rink-derivatized ones served for C-terminal peptide amides. Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Phe-OH, Fmoc-Ile-OH, Fmoc-Tyr(^{t}Bu)-OH, Fmoc-Val-OH, Fmoc-Trp(Boc)-OH, Fmoc-His(Trt)-OH, Fmoc-Ala-OH, Fmoc-Pro-OH, Fmoc-Glu(O^{t}Bu)-OH, Fmoc-Thr(^{t}Bu)-OH, Fmoc-Met-OH, Fmoc-Gly-OH, Fmoc-Gln(Trt)-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Ser(^{t}Bu)-OH, Fmoc-Asp(O^{t}Bu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Hyp(^{t}Bu)-OH, Fmoc-D-Lys(Boc)-OH, Fmoc-D-Phe-OH, Fmoc-D-Val-OH, Fmoc-D-Trp(Boc)-OH and Fmoc-Cys(^{t}Bu)-OH were used as amino acid derivatives. The coupling of the first amino acid (5 equiv.) onto the PAC-derivatized resins was performed in presence of *N,N'*-diisopropylcarbodiimide (DIC) (5 equiv.), 4-dimethylaminopyridine (DMAP) (0.5 equiv.) and *N,N'*-diisopropylethylamine (DIEA) (1 equiv.) in DMF at room temperature for 2 h under stirring. This treatment was repeated twice and, then, the resin was washed with DMF (6 × 1 min) and CH_2Cl_2 (3 × 1 min) and dried with diethyl ether (3 × 2 min). The completion of the coupling was checked using a Fmoc test. Then, the resin was acetylated with acetic anhydride/pyridine/CH_2Cl_2 (1.35:1.35:18, 2 × 30 min) followed by washes with CH_2Cl_2 (3 × 2 min), DMF (3 × 2 min), MeOH (2 × 2 min), CH_2Cl_2 (2 × 2 min) and DMF (6 × 1 min). Peptide

elongation was carried out through sequential Fmoc removal and coupling steps of the corresponding Fmoc-protected amino acid. Fmoc group removal was performed with piperidine/DMF (3:7, 2 + 10 min). Couplings of the Fmoc-amino acids (4 equiv.) were mediated by ethyl 2-ciano-2-(hydroxyimino) acetate (Oxyma) (4 equiv.) and DIC (4 equiv.) in DMF at room temperature for 1 h under stirring. The completion of the couplings was checked using the Kaiser or the chloranil test [47,48]. After each coupling and deprotection step, the resin was washed with DMF (6 × 1 min) and CH_2Cl_2 (2 × 1 min).

Once the peptidyl sequence was completed, the resin was treated with piperidine/N-methylpyrrolidinone (NMP) (3;7, 2 + 10 + 10 min), washed with NMP (6 × 1 min), CH_2Cl_2 (3 × 1 min) and diethyl ether (3 × 2 min), and air dried. Finally, the resulting resin was treated with trifluoroacetic acid (TFA)/H_2O/triisopropylsilane (TIS) (95:2.5:2.5) for 2 h at room temperature. In the case of peptides containing a tryptophan residue a mixture of TFA/phenol/H_2O/TIS (92.5:2.5:2.5:2.5) was used. For peptides containing a methionine residue a solution of TFA/dithiothreitol (DTT)/H_2O/TIS (92.5:2.5:2.5:2.5) was employed. Following TFA evaporation and diethyl ether extraction, the crude peptide was dissolved in H_2O, lyophilized, purified with a CombiFlash, analysed by HPLC, and characterized by ESI-MS and HRMS.

Solid-phase synthesis of lipopeptides derived from KSL-W containing an acyl group at the N-terminus

The synthesis was performed using a Fmoc-Rink-MBHA resin (0.55 mmol/g) following the general procedure described above. Fmoc-Lys(Boc)-OH, Fmoc-Val-OH, Fmoc-Phe-OH, and Fmoc-Trp(Boc)-OH were used as amino acid derivatives. Once the peptidyl sequence was

completed, the N-terminal Fmoc group was removed and the resulting peptidyl resin was acylated with the corresponding fatty acid (10 equiv.), DIC (10 equiv.) and Oxyma (10 equiv.) in NMP for 1 h under stirring. The resin was washed with NMP (6 × 1 min) and CH_2Cl_2 (2 × 1 min). The completion of the reaction was checked using the Kaiser test [47]. The resulting peptide was cleaved from the resin, purified, analysed by HPLC and characterized by mass spectrometry.

Solid-phase synthesis of lipopeptides derived from KSL-W containing an acyl group at the side chain of a lysine residue

The synthesis was performed using a Fmoc-Rink-MBHA resin (0.55 mmol/g) following the general procedure described above. Fmoc-Lys(Boc)-OH, Fmoc-Lys(ivDde)-OH, Fmoc-Val-OH, Fmoc-Phe-OH, and Fmoc-Trp(Boc)-OH were used as amino acid derivatives. Once the peptidyl sequence was completed, the N-terminal Fmoc group was removed and the resulting peptidyl resin was acetylated with acetic anhydride/pyridine/CH_2Cl_2 (1:1:1, 2 × 30 min), washed with NMP (6 × 1 min) and CH_2Cl_2 (3 × 1 min), and air dried. The completion of the reaction was checked with the Kaiser test [47]. Then, the peptidyl resin was treated with $NH_2NH_2{\cdot}H_2O$/NMP (5:95, 10 × 20 min) under stirring and washed with NMP (2 × 1 min), CH_2Cl_2 (2 × 1 min), MeOH (2 × 1 min) and NMP (2 × 1 min). The resulting resin was acylated by treatment with the corresponding fatty acid (10 equiv.), DIC (10 equiv.) and Oxyma (10 equiv.) in NMP for 1 h under stirring. The resin was then washed with NMP (6 × 1 min) and CH_2Cl_2 (2 × 1 min). The completion of the reaction was checked by the Kaiser test [47]. The resulting peptide was cleaved from the resin, purified, analysed by HPLC and characterized by mass spectrometry.

Bacterial strains and growth conditions

The following plant pathogenic bacterial strains were used: *Erwinia amylovora* PMV6076 (Institut National de la Recherche Agronomique, Angers, France), *Pseudomonas syringae* pv. syringae EPS94 (Institut de Tecnologia Agroalimentària, Universitat de Girona, Spain), *Xanthomonas axonopodis* pv. vesicatoria 2133-2, *Pseudomonas syringae* pv. actinidiae Psa3700.1.1, *Xanthomonas fragariae* Xf349-9A (Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain), and *Xanthomonas arboricola* pv. pruni CFBP5563 (Collection Française de Bactéries associées aux Plantes, Angers, France). All bacteria except for *X. fragariae* were stored in Luria Bertani (LB) broth supplemented with glycerol (20%) and maintained at -80 ºC. For *X. fragariae*, Medium B [49] was used instead of LB. *E. amylovora*, *X. arboricola* pv. pruni*, P. syringae* pv. syringae and *P. syringae* pv. actinidiae were scraped from the agar media after growing for 24 at 25ºC, and *X. axonopodis* pv. *vesicatoria* and *X. fragariae* after growing for 48 h at 25 ºC. The cell material was suspended in sterile water to obtain a suspension of 10^8 CFU mL^{-1}.

Antimicrobial activity

Lyophilized peptides were solubilized in sterile Milli-Q water to a final concentration of 1 mM and filter sterilized through a 0.22-μm pore filter. For minimum inhibitory concentration (MIC) assessment, dilutions of the peptides were made to obtain a stock concentration of 500, 250, 125, 62, 31, 16 and 8 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 10^8 CFU

mL^{-1}, 160 µL of Trypticase Soy Broth (TSB) (BioMèrieux, France) to a total volume of 200 µL. Three replicates for each strain, peptide and concentration were used. Bacterial growth was determined by optical density measurement at 600 nm (Bioscreen C, Labsystem, Helsinki, Finland). Microplates were incubated at 25 ºC with 20 sec 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 the peptides was evaluated by determining hemoglobin release from erythrocyte suspensions of horse blood (5% vol/vol)(Oxoid) as previously described [20]. Blood was centrifuged at 6000g for 5 min, washed three times with TRIS buffer (10 mM TRIS, 150 mM NaCl, pH 7.2) and ten diluted.

Peptides were solubilized in TRIS buffer and mixed with horse erythrocytes and the final concentrations tested were 375, 250, 150 and 50 µM. The mixture was incubated under continuous shaking for 1 h at 37 ºC. Then, the tubes were centrifuged at 3500g for 10 min, 80 µL aliquots of the supernatant transferred to 100-well microplates (Bioscreen), diluted with 80 µL water, and the absorbance measured at 540 nm (Bioscreen). Complete hemolysis was obtained by the addition of melittin at 100 µM (Sigma-Aldrich Corporation, Madrid, Spain). The percentage of hemolysis (*H*) was calculated using the equation: $H = 100\times[(Op-Ob)/(Om-Ob)]$, where *Op* was the density for a given peptide concentration, *Ob* for the buffer, and *Om* for the melittin positive control.

Effect of peptide infiltration on tobacco leaves

Peptides were evaluated for their effect upon infiltration on tobacco leaves as described previously [21]. Peptide solutions of 50, 150 and 250 μM were infiltrated (100 μL) into the mesophylls of fully expanded tobacco leaves. 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. Control infiltrations with water (negative control) or mellitin (positive control) at the same molar concentration were performed. The appearance of symptoms on the leaves was followed for 48 h after infiltration and measured as a lesion diameter.

Effect of peptide treatment on induction of defense gene expression of tomato plants

Seeds of tomato cv. Rio Grande plants were sown in a hydroponic seed plugs (rockwool), germinated and grown under controlled greenhouse conditions (25 ± 2 ºC, 16 h light/ 15 ± 2 ºC, 8 h dark, and 60% RH). Two-week old seedlings (two cotyledons) were transplanted into rockwool plugs (7.5×7.5×6.5 cm, Gordan Iberica). The experimental design consisted of three replicates of three plants per treatment. After two weeks, tomato leaves were sprayed with aqueous solutions of the peptide KSL-W, its derivatives **BP442-BP451**, PIP-1, Pep-13 or **flg15** at 125 μM, jasmonic acid at 2.5 mM (Sigma-Aldrich, Sant Louis, Missouri, UE) or acybenzolar-*S*-methyl at 300 mg/L (Syngenta, Basel, Switzerland) until the run-off point. Water-sprayed plants were used as untreated control. Twenty-four hours after product application, leaf samples were collected and processed to extract RNA for RT-qPCR assays. Plant material was ground to a fine powder in liquid nitrogen with the Tissuelyzer II system (Qiagen). Total RNA was extracted from leaves using PureLink Plant RNA Reagent

(Invitrogen, Life Technologies) according to the manufacturer's manual. The RNA was solubilized in RNAse free water and was routinely subjected to DNAse treatment (Ambion® Turbo DNA-free™, Life Technologies) to remove any contaminant DNA. In each step, the RNA was quantified using Nanodrop N-2000 spectrophotometer, and its integrity was verified by denaturing agarose gel electrophoresis. First-strand of complementary DNA (cDNA) was generated from leaf RNA using reverse transcriptase (High Capacity cDNA Reverse Transcription Kit, Invitrogen) according to the manufacturer's manual.

To test gene defense induction in the treated tomato plants, a qPCR assay was performed. Quantitative PCR was carried out in a fluorometric thermal cycler (qPCR Quant Studio 5, Applied Biosystems) by using a Mix SYBR®Green PCR Master Mix (Applied Biosystems). The total reaction volume of this PCR reaction was 20 µL and the reaction mixture was 1 µL of each primer set at the adequate concentration, 10 µL of MixSyber Green, 6 µL of distilled water and 2 µL of cDNA. Melting curve analysis was performed after each amplification to verify amplification specificity. A constitutive gene (*actin* gene) was used as reference control, and the following genes implicated in plant defense response were analyzed: pathogenesis-related protein-1 (*PR1*), harpin (*Harp*), polyphenol oxidase (*PPO*), subtilisin-like protease (*Sub1*), blue copper binding-protein (*BCB*), osmotin (*Osm2*), acidic β-1,3 endoglucanase (*GluA*), lypoxigenase (*LOX*), protein inhibitor II (*PinII*), dehydrin (*Tas14*) and early-ripening tomato (*ERT3*). Specific oligonucleotides were used for the quantification of the target genes: *Harp* (f-ATTATGGCCCGTCCATTCCG; r-ATGCAATGACTCCGAGGACG), *GluA* (f-GGTCTCAACCGCGACATATT; r-CACAAGGGCATCGAAAAGAT), *PPO* (f-AGACGTAATTCCCACGTCCG; r-GGCACGGTACACCGAAGTTA), *Sub1* (f-ACCTAAAGGCGTTGTCGTGA; r-ACCCCAGACATTGAGCTGTT), *ERT3* (f-TCCGAAACAGTCACATCGCA; r-AGCATCTTCCGCGCTATCAA´), *BCB* (f-

TTGGCACACACTGTCAAGGT; r-ACTGGCCAATAGGGTCGTTG), *Osm2* (f-TCCAATTCAATGCACAGCCA; r-TAGGACCACATGGACCGTGA), and the oligonucleotides used for the genes *PR1*, *LOX*, *PinII* and *Tas14* [23]. For each gene system, the concentration of the primer pair was optimized. The primer concentration was 100 nM for all the genes except for the *GluA*, *Harp*, *PR1* and actin genes which optimized concentration was 300 nM. A calibration curve was prepared by cloning the corresponding DNA in the pSpark cloning vector (Canvax, Córdoba, Spain), which was then used to transform *E. coli* DH5α. The number of plasmid copies were quantified after purification from *E. coli* (QIAGEN Iberia, S. L., Madrid, Spain), and appropriate dilutions were prepared to obtain the standard curve. The efficiency for each standard curve was calculated to check that the efficiency within amplifications were similar. Relative quantification of gene expression was done using the *ΔΔCt* method [50]. The Ct values obtained for each repetition treatment were used to estimate the fold change value of the endogenous reference gene (actin) and the target plant defense genes. These results were used to calculate the ratios of the plant defense genes (relative to the actin gene, and for all treatments analyzed, including the control plants). The statistical significance of the results for the selected peptides was determined using the REST2009 Software [51].

Results

Design and solid-phase synthesis of the peptides

With the aim of identifying new antimicrobial peptides to fight against plant diseases, we selected 15 peptides reported as plant defense elicitors (PIP-1, Pep-13), promiscuous (C*n*-AMP1, C*n*-AMP2, C*n*-AMP3), multifunctional (p10, p14, temporin-1CEa, L10, frenatin 2.1S, frenatin 2.2S) or antimicrobial (Api88, JCpep7, KSL, KSL-W) (Table 1).

The peptide with the best biological activity profile, KSL-W (see below), was taken as a lead and, based on its structure, 49 analogues were designed (Table 1). N-Terminal deletion derivatives containing 4 to 9 amino acids were synthesized to examine whether the entire sequence of KSL-W is necessary for its full antibacterial activity. Moreover, each amino acid of KSL-W was substituted with its D-enantiomer because it constitutes a strategy to protect peptides against enzymatic hydrolysis while reducing their hemolytic activity [52]. In addition, lipopeptides were designed by acylating the N-terminus or the side-chain of a lysine residue placed at each position of the sequence with butyric, hexanoic or lauric acid. Thus, the influence on the biological activity of the hydrophobic chain length and its position was evaluated. The incorporation of an acyl chain to a peptide has been described as an approach to increase the affinity of peptides towards microbial membranes enhancing their antimicrobial activity [21,53-55].

Table 1. Sequences of peptides synthesized in this study

Peptide	Sequence	Peptide	Sequence
Plant defense elicitors		*KSL-W derivatives with an acyl chain*	
PIP-1	YGIHTH-NH_2	**BP409**	C_5H_{11}CO-KKVVFWVKFK-NH_2
Pep-13	VWNQPVRGFKVYE-OH	**BP410**	C_3H_7CO-KKVVFWVKFK-NH_2
		BP411	$C_{11}H_{23}$CO-KKVVFWVKFK-NH_2
Promiscuous peptides		**BP412**	KKVVFWVKFK(COC_5H_{11})-NH_2
Cn-AMP1	SVAGRAQGM-NH_2	**BP413**	KKVVFWVKFK(COC_3H_7)-NH_2
Cn-AMP2	TESYFVFSVGM-NH_2	**BP414**	KKVVFWVKFK($COC_{11}H_{23}$)-NH_2
Cn-AMP3	YCSYTMEA-NH_2	**BP415**	KKVVFWVKK(COC_5H_{11})K-NH_2
		BP416	KKVVFWVKK(COC_3H_7)K-NH_2
Multifunctional peptides		**BP417**	KKVVFWVKK($COC_{11}H_{23}$)K-NH_2
p10	QKALNEINQF-NH_2	**BP418**	KKVVFWVK(COC_5H_{11})FK-NH_2
p14	TKKTKLTEEEKNRL-NH_2	**BP419**	KKVVFWVK(COC_3H_7)FK-NH_2
Temporin-1CEa	FVDLKKIANIINSIFGK-NH_2	**BP420**	KKVVFWVK($COC_{11}H_{23}$)FK-NH_2
L10	WFRKQLKW-OH	**BP421**	KKVVFWK(COC_5H_{11})KFK-NH_2
Frenatin 2.1S	GLVGTLLGHIGKAILG-NH_2	**BP422**	KKVVFWK(COC_3H_7)KFK-NH_2
Frenatin 2.2S	GLVGTLLGHIGKAILS-NH_2	**BP423**	KKVVFWK($COC_{11}H_{23}$)KFK-NH_2
		BP424	KKVVFK(COC_5H_{11})VKFK-NH_2
Antimicrobial peptides		**BP425**	KKVVFK(COC_3H_7)VKFK-NH_2
dGu-Api88	ONNRPVYIPRPRPPHPRL-NH_2[a]	**BP426**	KKVVFK($COC_{11}H_{23}$)VKFK-NH_2
JCpep7	KVFLGLK-OH	**BP427**	KKVVK(COC_5H_{11})WVKFK-NH_2
KSL	KKVVFKVKFK-NH_2	**BP428**	KKVVK(COC_3H_7)WVKFK-NH_2
KSL-W	KKVVFWVKFK-NH_2	**BP429**	KKVVK($COC_{11}H_{23}$)WVKFK-NH_2
		BP430	KKVK(COC_5H_{11})FWVKFK-NH_2
KSL-W derivatives with a D-amino acid		**BP431**	KKVK(COC_3H_7)FWVKFK-NH_2
BP442	KKVVFWVKFk-NH_2	**BP432**	KKVK($COC_{11}H_{23}$)FWVKFK-NH_2
BP443	KKVVFWVKfK-NH_2	**BP433**	KKK(COC_5H_{11})VFWVKFK-NH_2
BP444	KKVVFWVkFK-NH_2	**BP434**	KKK(COC_3H_7)VFWVKFK-NH_2
BP445	KKVVFWvKFK-NH_2	**BP435**	KKK($COC_{11}H_{23}$)VFWVKFK-NH_2
BP446	KKVVFwVKFK-NH_2	**BP436**	KK(COC_5H_{11})VVFWVKFK-NH_2
BP447	KKVVfWVKFK-NH_2	**BP437**	KK(COC_3H_7)VVFWVKFK-NH_2
BP448	KKVvFWVKFK-NH_2	**BP438**	KK($COC_{11}H_{23}$)VVFWVKFK-NH_2
BP449	KKvVFWVKFK-NH_2	**BP439**	K(COC_5H_{11})KVVFWVKFK-NH_2
BP450	KkVVFWVKFK-NH_2	**BP440**	K(COC_3H_7)KVVFWVKFK-NH_2
BP451	kKVVFWVKFK-NH_2	**BP441**	K($COC_{11}H_{23}$)KVVFWVKFK-NH_2

[a]O stands for 4-hydroxyproline.

Peptides were manually synthesized on a MBHA or a ChemMatrix resin following a standard Fmoc/tBu strategy. A Fmoc-Rink amide or a PAC linker was used to obtain C-terminal peptide amides or C-terminal carboxylic acid peptides, respectively. Couplings of the conveniently protected Fmoc-amino acids were mediated by Oxyma and DIC in DMF. Peptides were cleaved from the resin by acidolytic treatment, purified by reverse-phase column chromatography and were obtained in excellent purities (90->99%), as determined by analytical HPLC (Tables A and B in Supplementary Material). Their identity was confirmed by mass spectrometry.

For the synthesis of the N-terminal acylated sequences, once peptide chain elongation was completed, the N-terminal Fmoc group was removed and the free amino group was derivatized with butanoic, hexanoic or lauric acid. In the case of the side-chain acylated peptides, the lysine to be derivatized was incorporated as Fmoc-Lys(ivDde)-OH. After completion of the peptide sequence, the N-terminus was acetylated and the 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidine)-3-methylbutyl (ivDde) group was selectively removed. The resulting free amino group was acylated with the corresponding fatty acid. After acidolytic cleavage, lipopeptides were purified by reverse phase column chromatography being obtained in excellent HPLC purities (93->99%) and characterized by mass spectrometry (Table C in Supplementary Material).

Biological activity of selected peptides

Fifteen selected peptides from the literature were tested for *in vitro* growth inhibition of the plant pathogenic bacteria *E. amylovora*, *P. syringae* pv. syringae, *P. syringae* pv. actinidiae, *X. arboricola* pv. pruni, *X. fragariae* and *X. axonopodis* pv. vesicatoria at 0.8, 1.6, 3.1, 6.2, 12.5, 25 and 50 μM (Table 2).

Table 2. Antimicrobial activity (MIC), hemolysis and phytotoxicity of the set of peptides selected from the literature.

Peptide	MIC (μM)						Hemolysis[b] (%)	Tobacco lesion[c] (cm)
	Ea[a]	*Pss*[a]	*Psa*[a]	*Xap*[a]	*Xf*[a]	*Xav*[a]	375 μM	250 μM
Plant defense elicitors								
PIP-1	>50	>50	>50	>50	>50	>50	0 ± 0.1	0 ± 0
Pep-13	>50	>50	>50	>50	>50	>50	0 ± 0.1	0 ± 0
Promiscuous peptides								
Cn-AMP1	>50	>50	>50	>50	>50	>50	0 ± 0.4	0.11 ± 1.0
Cn-AMP2	>50	>50	12.5-25	12.5-25	>50	>50	8 ± 2.7	0 ± 0
Cn-AMP3	>50	>50	>50	>50	>50	>50	95 ± 2.5	0.11 ± 0
Multifunctional peptides								
p10	>50	>50	>50	>50	>50	>50	0 ± 0.1	0 ± 0
p14	>50	>50	>50	>50	>50	>50	0 ± 0.2	0 ± 0
Temporin-1CEa	25-50	>50	>50	3.1-6.2	>50	3.1-6.2	100 ± 2.6	0.71 ± 0.1
L10	>50	>50	>50	>50	>50	>50	1 ± 0.2	0 ± 0
Frenatin 2.1S	25-50	>50	>50	>50	>50	3.1-6.2	86 ± 2.4	1.10 ± 0.1
Frenatin 2.2S	25-50	>50	>50	6.2-12.5	25-50	6.2-12.5	84 ± 1.8	0.97 ± 0.2
Antimicrobial peptides								
dGu-Api88	25-50	>50	12.5-25	>50	>50	>50	1 ± 0.2	0 ± 0
JCpep7	>50	>50	>50	>50	>50	>50	5 ± 0.4	0 ± 0
KSL	>50	6.2-12.5	1.6-3.1	6.2-12.5	6.2-12.5	12.5-25	5 ± 1.3	0.34 ± 1.0
KSL-W	6.2-12.5	6.2-12.5	1.6-3.1	0.8-1.6	3.1-6.2	6.2-12.5	20 ± 1.8	0.62 ± 0.4

[a]*Ea*, *Erwinia amylovora; Pss*, *Pseudomonas syringae* pv. syringae; *Psa*, *Pseudomonas syringae* pv. actinidiae; *Xap*, *Xanthomonas arboricola* pv. pruni; *Xf*, *Xanthomonas fragariae*; *Xav*, *Xanthomonas axonopodis* pv. vesicatoria. [b]Percent hemolysis at 375 μM plus confidence interval ($\alpha = 0.05$). [c]Phytotoxicity at 250 μM determined as the lesion diameter (cm) in infiltrated tobacco leaves plus confidence interval.

The plant defense elicitor peptides PIP-1 and Pep-13 were not active against these bacteria. Among the other sequences, seven exhibited activity against at least one of the above pathogens (MIC <25 μM). In particular, the promiscuous peptide C*n*-AMP2 showed MIC of 12.5 to 25 μM against *P. syringae* pv. actinidiae and *X. arboricola* pv. pruni. Among the multifunctional peptides, temporin-1CEa and frenatin 2.2S displayed MIC values ranging from 3.1 to 12.5 μM against *X. arboricola* pv. pruni and *X. axonopodis* pv. vesicatoria, and frenatin 2.1S was active against the latter pathogen with MIC of 3.1 to 6.2 μM. Concerning the tested antimicrobial peptides, the deguanylated Api88 analogue was active against *P. syringae* pv. actinidiae with MIC of 12.5 to 25 μM. Interestingly, KSL and KSL-W were highly active against the pathogens tested. KSL showed MIC values ranging from 1.6 and 25 μM against all the bacteria except for *E. amylovora*. KSL-W was more active with MIC values between 0.8 and 12.5 μM against all the pathogens, highlighting *P. syringae* pv. actinidiae and *X. arboricola* pv. pruni (MIC of 1.6 to 3.1 μM and of 0.8 to 1.6 μM, respectively).

The toxicity of these peptides to eukaryotic cells was determined as the ability to lyse erythrocytes in comparison to melittin. Percent hemolysis at 375 μM is shown in Table 2. Notably, the best peptides, KSL and KSL-W displayed also low hemolytic activity with a percent hemolysis of 5 and 20%, respectively, at this concentration.

This set of selected peptides was also screened for their effect on tobacco leaves by infiltrating each compound into the mesophylls of the leaves (Table 2). The nonspecific and nonselective toxic melittin was used as positive control, which caused a brown necrotic area of around 2 cm diameter at 250 μM after 48 h of infiltration. In contrast, KSL and KSL-W had a significantly lower effect than melittin at this concentration being the size of the lesions of 0.34 and 0.62 cm, respectively.

Taken together, these results allowed the identification of KSL-W, a short cationic peptide with a suitable activity profile to be considered as an excellent antimicrobial agent against plant pathogenic bacteria. It displayed high antibacterial activity, low hemolysis and phytotoxicity and, therefore, was selected for further studies.

Biological activity of KSL-W derivatives

The antibacterial activity of 49 KSL-W analogues was examined against *E. amylovora*, *P. syringae* pv. syringae, *P. syringae* pv. actinidiae, *X. arboricola* pv. pruni, *X. fragariae* and *X. axonopodis* pv. vesicatoria (Table 3 and Table D in Supplementary Material, Fig 1). Their toxicity against red blood cells and tobacco leaves was also evaluated (Figs 2 and 3). These analogues included 6 N-terminal deletion sequences, 10 peptides incorporating a D-amino acid and 33 lipopeptides.

The N-terminal deletion analogues containing 4 to 7 amino acids were inactive against all the pathogens (MIC >25 μM, data not shown). The peptide incorporating 8 amino acids showed activity against 4 pathogens with MIC of 6.2 to 25 μM. In the case of the 9-residue peptide, activity was observed against all the pathogens (MIC of 3.1 to 25 μM) except for *X. arboricola* pv. pruni.

Compared to KSL-W, in general, a decrease of the activity was observed for its D-diastereoisomers (Table 3). The most active analogues were **BP442**, **BP448**, **BP450**, and **BP451** bearing the D-amino acid at positions 10, 4, 2 and 1, respectively (MIC of 0.8 to 25 μM). Among them highlighted **BP442** which was as active as KSL-W against *P. syringae* pv. syringae*, X. arboricola* pv. pruni and *X. fragariae*, and more active against *X. axonopodis* pv.

vesicatoria (MIC of 1.6 to 3.1 μM). Moreover, all D-diastereoisomers were less hemolytic than the parent peptide with a percent hemolysis ≤12% at 375 μM. In addition, they were less phytotoxic than melittin, causing a necrotic area between 0.12 to 0.90 cm at 250 μM. Interestingly, the best peptide **BP442** was neither hemolytic (2% at 375 μM) nor phytotoxic (size of the lesion of 0.18 cm at 250 μM).

Table 3. Antimicrobial activity (MIC), hemolysis and phytotoxicity of the peptides derived from KSL-W containing a D-amino acid.

Peptide	MIC (μM)						Hemolysis[b] (%)	Tobacco lesion[c] (cm)
	Ea[a]	*Pss*[a]	*Psa*[a]	*Xap*[a]	*Xf*[a]	*Xav*[a]	375 μM	250 μM
KSL-W	6.2-12.5	6.2-12.5	1.6-3.1	0.8-1.6	3.1-6.2	6.2-12.5	20 ± 1.8	0.62 ± 0.4
BP442	12.5-25	6.2-12.5	3.1-6.2	0.8-1.6	3.1-6.2	1.6-3.1	2 ± 0.2	0.18 ± 0.1
BP443	>50	>50	25-50	12.5-25	12.5-25	12.5-25	0 ± 1.1	0.12 ± 1.1
BP444	>50	25-50	25-50	25-50	>50	>50	0 ± 0	0.50 ± 0.1
BP445	>50	12.5-25	6.2-12.5	6.2-12.5	12.5-25	6.2-12.5	1 ± 0.2	0.53 ± 0.1
BP446	>50	>50	25-50	>50	12.5-25	>50	4 ± 0.5	0.83 ± 0.1
BP447	>50	12.5-25	6.2-12.5	25-50	25-50	12.5-25	12 ± 0.9	0.90 ± 0.1
BP448	12.5-25	12.5-25	6.2-12.5	6.2-12.5	6.2-12.5	6.2-12.5	3 ± 0.7	0.68 ± 0.1
BP449	>50	12.5-25	6.2-12.5	12.5-25	12.5-25	12.5-25	2 ± 0.3	0.26 ± 0.1
BP450	12.5-25	6.2-12.5	3.1-6.2	1.6-3.1	3.1-6.2	3.1-6.2	5 ± 0.8	0.44 ± 0.1
BP451	12.5-25	6.2-12.5	3.1-6.2	1.6-3.1	3.1-6.2	3.1-6.2	4 ± 0.4	0.47 ± 0.1

[a]*Ea*, *Erwinia amylovora; Pss*, *Pseudomonas syringae* pv. *syringae*; *Psa*, *Pseudomonas syringae* pv. *actinidiae*; *Xap*, *Xanthomonas arboricola* pv. *pruni*; *Xf*, *Xanthomonas fragariae*; *Xav*, *Xanthomonas axonopodis* pv. *vesicatoria*. [b]Percent hemolysis at 375 μM plus confidence interval (α = 0.05). [c]Phytotoxicity at 250 μM determined as the lesion diameter (cm) in infiltrated tobacco leaves plus confidence interval.

Lipopeptides derived from KSL-W, in general, were not active against *E. amylovora* and the two *Pseudomonas* species (Table D in Supplementary Material and Fig 1). Only 2, 1 and 6 lipopeptides displayed MIC values of 12.5 to 25 μM against *E. amylovora*, *P. syringae* pv. *syringae* and *P. syringae* pv. *actinidiae*, respectively. In contrast, these derivatives were significantly active against the *Xanthomonas* species. MIC values <25 μM were found for 20 peptides against at least two of these species. Interestingly, lipopeptides with MIC of 3.1 to 6.2 μM were obtained, namely: **BP417** and **BP432** against *X. arboricola* pv. *pruni*; **BP411**, **BP417** and **BP432** against *X. fragariae*; **BP409**, **BP410**, **BP411** and **BP432** against *X. axonopodis* pv. *vesicatoria*. It is noteworthy to mention, that the latter peptides displayed higher activity than the parent peptide KSL-W against this bacterium.

Fig 1. MICs of the lipopeptides derived from KSL-W against the bacteria *E. amylovora* (*Ea*), *P. syringae* pv. syringae (*Pss*), *P. syringae* pv. actinidiae (*Psa*), *X. arboricola* pv. pruni (*Xap*), *X. fragariae* (*Xf*) and *X. axonopodis* pv. vesicatoria (*Xav*). The position of the acyl group is indicated with a superscript. The type of the acyl group is indicated at the bottom of each peptide code as: 1: R = C_5H_{11}; 2: R = C_3H_7 and 3: R = $C_{11}H_{23}$.

Differences on the antibacterial activity of lipopeptides were detected depending on the position and the length of the acyl chain. The analogues derivatized at the N-terminus and at the side-chain of the residues at positions 3 and 4 displayed the highest activity. For these derivatives, peptides with MIC <50 μM against 4 or 5 pathogens were identified and most of them displayed high activity against the three *Xanthomonas* species (MIC <12.5 μM). The analogues incorporating a lauroyl group were clearly the most active. In fact, 6 out of 11 sequences displayed MIC <12.5 μM against at least two

Xanthomonas species. Among them, highlighted **BP411**, **BP417** and **BP432** which exhibited MIC values of 3.1 to 6.2 μM against these bacteria. Within the hexanoyl and butanoyl derivatives, **BP410** showed the best antibacterial profile, being active against 5 pathogens with MIC <25 μM.

These lipopeptides were, in general, low toxic (Figs 2 and 3). Regarding their toxicity against erythrocytes, at 50 μM, 26 out of 33 peptides displayed a hemolysis percentage ≤13%, and for the rest of peptides this percentage ranged from 22 to 63%. At the highest concentration tested, 375 μM, the hemolysis of 23 peptides did not exceed 50% and, among them, 15 sequences exhibited only ≤16% hemolysis. Concerning their effect in tobacco leaves, these acylated derivatives were not phytotoxic at 50 μM: only 6 peptides caused a necrotic area of 0.25 to 0.44 cm. At 250 μM, they were significantly less phytotoxic than melittin, being the size of the lesion <1 cm for all peptides, ranging from 0-0.60 cm for 25 of them. Notably, among the lipopeptides with the highest antibacterial activity (**BP409**, **BP410**, **BP411**, **BP417** and **BP432**), we would highlight **BP410**, **BP411** and **BP432** because they are not toxic towards either erythrocytes (<50% at 375 μM) or tobacco leaves (size of the lesion of 0.30 to 0.53 cm at 250 μM).

Fig 2. **Hemolytic activity of the lipopeptides derived from KSL-W at 50 and 375 μM.** Vertical bars within each column indicate confidence interval at the mean. The position of the acyl group is indicated with a superscript. The type of the acyl group is indicated at the bottom of each peptide code as: 1: R = C_5H_{11}, 2: R = C_3H_7 and 3: R = $C_{11}H_{23}$.

Fig 3. Effect of the lipopeptides derived from KSL-W at 250 μM on the size of the lesions in infiltrated tobacco leaves. This effect was compared to melittin. Vertical bars within each column indicate confidence interval at the mean. The position of the acyl group is indicated with a

superscript. The type of the acyl group is indicated at the bottom of each peptide code as: 1: R = C_5H_{11}, 2: R = C_3H_7 and 3: R = $C_{11}H_{23}$.

Effect of peptide treatment on defense gene expression of tomato plants

The capacity of inducing the expression of genes related to plant defense responses of KSL-W and its D-diastereoisomers was evaluated (Table 4). Flagellin 15 (flg15), jasmonic acid (JA) and acybenzolar-*S*-methyl (ASM) were included in this study as positive controls due to their described capacity to enhance the plant immune system [56]. PIP-1 and Pep-13, previously described as plant defense elicitors, were also tested.

Table 4 summarizes the relative quantification for the expression of the selected genes calculated by the *ΔΔCt* method. Positive controls clearly caused the overexpression of the majority of genes. JA induced expression of all the genes except for *Harp*, *PR1*, *GluA* and *Tas14*. ASM and flg15 caused the overexpression of all the genes tested except for *Tas14* and *LOX,* respectively. PIP-1 and Pep-13 caused the overexpression of only one (*Osm2*) or two (*PPO* and *Tas14*) of the tested genes, respectively.

Concerning KSL-W and its derivatives, the parent peptide, **BP443** and **BP451** did not induce the overexpression of any of the genes. The other eight peptides caused the overexpression of from 1 to 6 genes. In particular, **BP442** and **BP446** only induced the overexpression of *BCB* and *Osm2*, respectively. **BP444** and **BP445** caused the overexpression of 2 genes; *Harp* and *LOX* (**BP444**) and *PPO* and *Tas14* (**BP445**). Peptides **BP448** and **BP450** promoted the overexpression of 3 genes. Apart from *ERT3*, **BP450** also caused the overexpression of *PR1* and *GluA*, and **BP448**, *Osm2* and *LOX*. The latter was the only peptide that induced overexpression of *LOX*. The KSL-W analogues **BP447** and **BP449** caused the overexpression of 6 and 4 genes, respectively, that were also induced by flg15.

These two peptides caused the overexpression of *Harp*, *GluA*, *PinII* and *Tas14*, and **BP447** also promoted the overexpression of *PR1* and *ERT3*.

Table 4. Expression of genes related to defense/stress response in tomato after the treatment with the reference products flagellin 15 (flg15), jasmonic acid (JA), and acybenzolar-*S*-methyl (ASM), with the plant defense elicitors PIP-1 and Pep-13, and with KSL-W and ten KSL-W derivatives. Fold induction above 2 is considered overexpression in the relative quantification by the ΔΔ*Ct* method. Significant values are indicated in bold.

	Reference products[a]			Plant defense elicitors[b]		Peptides[b]										
Genes	flg15	JA	ASM	PIP-1	Pep-13	KSL-W	**BP442**	**BP443**	**BP444**	**BP445**	**BP446**	**BP447**	**BP448**	**BP449**	**BP450**	**BP451**
Harp	**3.3**	1.9	**2.8**	0.4	0.6	0.5	1.4	1.6	**2.4**	1.9	1.7	**3.8**	1.4	**2.3**	1.3	1.0
PR1	**30.4**	2.9	**16.4**	0.1	0.1	0.1	2.7	1.5	0.6	0.6	0.8	**2.3**	0.5	1.7	**2.2**	0.5
GluA	**12.7**	0.2	**7.5**	0.1	0.1	0.7	0.6	1.7	1.4	**4.6**	1.9	**6.1**	1.7	**2.9**	**2.5**	0.4
PPO	**4.3**	**>50**	**7.8**	1.1	**3.2**	1.0	0.7	0.9	**2.1**	1.4	1.0	1.3	0.9	1.7	1.2	1.4
LOX	1.6	**9.7**	**2.4**	1.0	1.1	1.3	0.9	0.9	0.7	0.6	1.1	1.2	**3.2**	1.1	1.9	1.0
PinII	**5.2**	**>50**	**3.6**	0.2	0.8	0.3	0.4	0.7	1.2	1.3	1.3	**2.4**	1.4	**3.5**	1.2	2.0
Sub1	**12.3**	**4.0**	**4.1**	1.4	0.8	0.4	0.6	0.9	0.6	0.4	1.0	1.0	0.6	0.8	1.0	1.0
ERT3	**3.6**	**41.3**	**3.7**	0.4	0.3	0.2	0.1	1.5	1.5	1.6	1.6	**2.1**	**3.7**	1.9	**2.1**	1.0
BCB	**11.3**	**6.6**	**2.6**	0.7	1.6	1.1	**2.8**	0.8	0.9	0.6	1.1	1.1	0.4	1.3	0.9	1.4
Osm2	**11.3**	**48.4**	**2.8**	**2.0**	0.7	1.2	0.9	1.9	1.1	1.0	**2.1**	1.1	**4.0**	0.6	1.7	1.6
Tas14	**2.3**	0.9	1.1	0.9	**4.1**	1.2	1.2	1.1	1.5	**6.1**	1.4	**6.0**	0.6	**9.0**	0.7	1.4

[a] The reference products were tested at 125 µM (flg15), 2.5 mM (JA), and 300 mg/L (ASM)
[b] Plant defense elicitors and peptides were tested at 125 µM

Discussion

The great economic losses produced by plant pathogens together with the strict regulatory restrictions on the use of pesticides in several countries have urged the development of new antimicrobial agents [57,58]. Within this context, our research is focused on identifying peptides able to fight plant diseases caused by the bacteria *Erwinia amylovora*, *Xanthomonas arboricola* pv. pruni, *Xanthonomas axonopodis* pv. vesicatoria, *Xanthomonas fragariae*, *Pseudomonas syringae* pv. actinidiae and *Pseudomonas syringae* pv. syringae. Towards this aim, in this work sequences previously described as plant defense elicitors, promiscuous, multifunctional or antimicrobial were screened against the aforementioned bacteria. The best peptide was then chosen for conducting further studies.

Among the 15 selected sequences, the multifunctional peptides temporin-1CEa, frenatin 2.1S and frenatin 2.2S were active against at least one of the *Xanthomonas* strains tested. Interestingly, the antimicrobial peptides KSL and KSL-W exhibited, in general, high activity against all the above pathogens, being the latter the most active with MIC values ranging from 0.8 to 12.5 μM. The antibacterial activity of KSL-W had been previously described, in particular against bacteria present in the oral cavity or in wounds [41-46]. However, its efficacy towards plant pathogenic bacteria had not yet been reported. Notably, this peptide also showed low hemolytic activity (20% at 375 μM) and low phytotoxicity in tobacco leaves (0.62 cm at 250 μM). Therefore, KSL-W constitutes an interesting lead in the search for new agents to control plant diseases of economic importance in agriculture.

Taking KSL-W as basis, analogues were prepared by reducing the length, or by incorporating a D-amino acid or an acyl chain. Results revealed that the antibacterial activity increased when increasing the peptide length, being 9 the minimum number of amino acids required for considerable activity. Analogues incorporating a D-amino acid showed a weaker antibacterial activity, but the hemolytic activity greatly decreased. Similar results have been obtained for other

antimicrobial peptides, i.e. for analogues of **BP100** and polybia-CP [52,59]. In addition, the position of the D-amino acid influenced the biological activity. In particular, the presence of a D-amino acid at the N-terminus or at positions 9 and 10 only led to a slight decrease of the antibacterial activity. In contrast, sequences bearing a D-amino acid at positions 2, 3, 5 or 6 were significantly less active. Moreover, a low phytotoxicity was observed for peptides containing a D-amino acid at the N-terminus or at positions 2 or 8. Accordingly, **BP442** with a D-amino acid at the N-terminus resulted to be the diastereoisomer with the best biological activity profile.

In the case of the lipopeptides derived from KSL-W, the antibacterial activity depended on the pathogen. Whereas, in general, they displayed high activity against the *Xanthomonas* strains, they were not active against the other bacteria tested. As previously reported, this different sensitivity was attributed to the different composition of the bacterial membrane [20,22,52]. Among this set of compounds, we found sequences with the same activity than the parent peptide KSL-W against *X. fragariae* and analogues with improved activity against *X. axonopodis* pv. *vesicatoria*.

On the other hand, in agreement with previous reports, both the position and the type of acyl chain influenced the antibacterial activity and the hemolysis of the lipopeptides [21,53-55,60,61]. In this study, the most active derivatives were those incorporating the acyl chain at the N-terminus or at positions 3 or 4. In general, the presence of a lauroyl group favoured the antibacterial activity, while no significant differences were observed between peptides bearing a butanoyl or a hexanoyl chain. Accordingly, the best peptides were **BP409**, **BP410** and **BP411**, incorporating the acyl chain at the N-terminus, **BP417** and **BP432** with a lauroyl group at position 9 and 4, respectively.

Analysis of the hemolytic activity of the lipopeptides pointed out that, in general, it increased with increasing the length of the acyl chain. A percentage of hemolysis <50% at 375 μM was obtained for all lipopeptides derivatized with a butanoyl group. In contrast, only 6 sequences containing a lauroyl chain displayed similar percentages. Thus, the incorporation of a butanoyl group rendered the least hemolytic lipopeptides, whereas a lauroyl yielded the most hemolytic ones. These results are in good agreement with previous studies reporting that an increase of the peptide

hydrophobicity is related to an increase in the cytotoxicity [21,62,63]. Moreover, the position of the acyl chain also influenced the hemolysis, being the least hemolytic the sequences acylated at the N-terminus or at positions 2 and 8, and the most hemolytic those derivatized at positions 3, 5 or 10. It is noteworthy to mention that the hemolysis was determined at concentrations up to 375 μM, which is 7 to 120 fold higher than the MIC values.

These lipopeptides had significantly less effect on tobacco leaves than melittin at concentrations up to 5 to 80 fold higher than the MIC. Comparable results were described in previous reports on short cationic lipopeptides, which revealed that a high activity against plant pathogens is not necessarily associated with a damage in plant tissues even when applied directly at the infection area [64]. Moreover, similarly to previous results on cyclic lipopeptides, no correlation was observed between phytotoxicity and hemolysis [21]. Interestingly, among the most active sequences, **BP410**, **BP411** and **BP432** were also low hemolytic and phytotoxic.

In addition, the presence of a D-amino acid in the KSL-W favored the capacity of inducing the expression of defense-related genes on tomato plants. Some of these KSL-W analogues caused the overexpression of more genes than the parent peptide and than other defense response elicitors described in the literature (PIP-1 or Pep-13). This modification resulted to be more effective when the D-amino acid was incorporated at positions 2 to 5, being peptides **BP447**, **BP448**, **BP449**, and **BP450** the ones that showed the best results.

Taking all the results of the KSL-W derivatives together (Table 5), it can be concluded that the incorporation of a D-amino acid at position 10 affords a peptide (**BP442**) with significant activity against all the bacteria tested that is neither hemolytic nor phytotoxic. It can also be observed that when a D-amino acid is present at position 5, the resulting peptide **BP447** induces the expression of 6 defense-related genes. On the other hand, it has been demonstrated that the acylation of KSL-W renders peptides selective towards *Xanthomonas* strains. In particular, those bearing a butanoyl at the N-terminus (**BP410**), or a lauroyl at the N-terminus (**BP411**) or at position

4 (**BP432**) are highly active against these bacteria and also low toxic against erythrocytes or plant tissues.

Table 5. Peptides with the best biological activity profile

Biological activity properties	Peptides					
	KSL-W	**BP410**	**BP411**	**BP432**	**BP442**	**BP447**
Structure[a]		C_3H_7CO-K^1	$C_{11}H_{23}CO$-K^1	$K^4(COC_{11}H_{23})$	D-K^{10}	D-F^5
Antibacterial activity (MIC)						
Ea[b]	6.2-12.5	12.5-25	>50	>50	12.5-25	>50
Pss[b]	6.2-12.5	25-50	>50	>50	6.2-12.5	12.5-25
Psa[b]	1.6-3.1	12.5-25	>50	25-50	3.1-6.2	6.2-12.5
Xap[b]	0.8-1.6	12.5-25	>50	3.1-6.2	0.8-1.6	25-50
Xf[b]	3.1-6.2	6.2-12.5	3.1-6.2	3.1-6.2	3.1-6.2	25-50
Xav[b]	6.2-12.5	3.1-6.2	3.1-6.2	3.1-6.2	1.6-3.1	12.5-25
Hemolysis (%)[c]	20 ± 1.8	2 ± 0	32 ± 1.9	47 ± 4.3	2 ± 0.2	12 ± 0.9
Size of the lesions (cm) in infiltrated tobacco leaves[d]	0.62 ± 0.4	0.30 ± 0.1	0.52 ± 0.3	0.53 ± 0.2	0.18 ± 0.1	0.90 ± 0.1
Defense gene expression in tomato plants[e]	none	nd[f]	nd[f]	nd[f]	*BCB*	*Harp, PR1a, GluA, PinII, ERT3, Tas14*

[a] KSL-W sequence: KKVVFWVKFK-NH_2; for the other peptides is indicated the modification that was incorporated. [b] *Ea*, *Erwinia amylovora*; *Pss*, *Pseudomonas syringae* pv. syringae; *Psa*, *Pseudomonas syringae* pv. actinidiae; *Xap*, *Xanthomonas arboricola* pv. pruni; *Xf*, *Xanthomonas fragariae*; *Xav*, *Xanthomonas axonopodis* pv. vesicatoria. [c] Percent hemolysis at 375 μM plus confidence interval ($\alpha = 0.05$). [d] Diameter of the lesions (cm) measured after 48 h infiltration of lipopeptides at 250 μM. [e]Expression of genes in tomato plants after the treatment with the peptides at 125 μM. [f] nd, not determined.

Conclusions

This study has allowed the identification of KSL-W as a new lead to control plant pathogens of economic importance. This peptide is highly active against a range of bacteria being also low hemolytic and phytotoxic. It has also been demonstrated that the incorporation of a D-amino acid constitutes a suitable strategy to obtain derivatives with an improved biological activity profile or with the capacity to induce the expression of defense-related genes. Based on these promising data, we envisage that these peptides may open up an alternative approach to the challenging task of developing novel peptides with multifunctional properties like antimicrobial activity or plant defense elicitation properties.

Supplementary material

Sequences, retention times and purities by HPLC, and mass spectrometry data of the set of peptides selected from the literature, of the peptides derived from KSL-W containing a D-amino acid and of the lipopeptides derived from KSL-W.

Antimicrobial activity (MIC), hemolysis and phytotoxicity of KSL-W and lipopeptides **BP409-BP441**.

Acknowledgements

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FIGURE 1

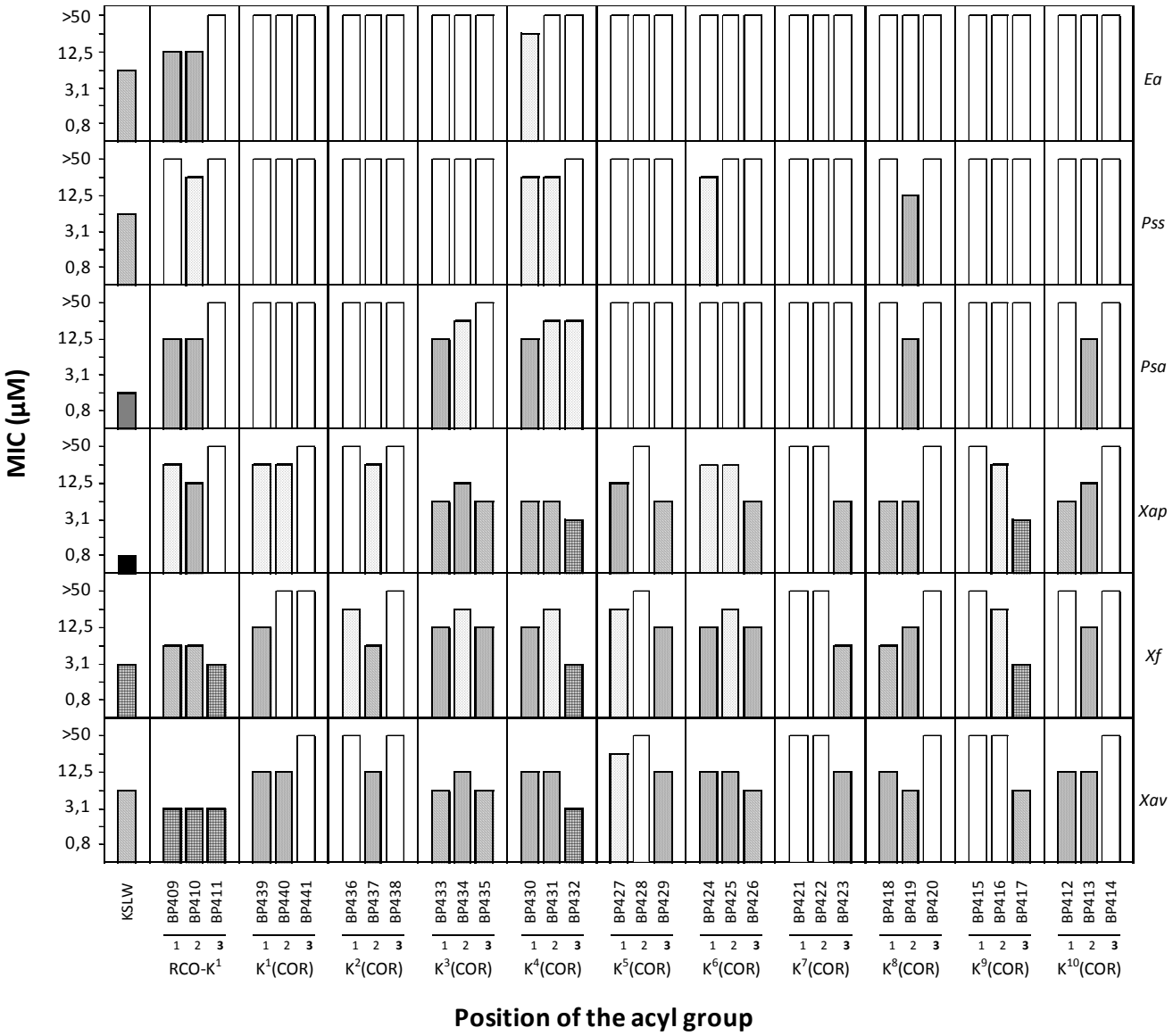

MIC (µM)
>50
12,5
3,1
0,8
Ea
Pss
Psa
Xap
Xf
Xav
KSLW
BP409 BP410 BP411
BP439 BP440 BP441
BP436 BP437 BP438
BP433 BP434 BP435
BP430 BP431 BP432
BP427 BP428 BP429
BP424 BP425 BP426
BP421 BP422 BP423
BP418 BP419 BP420
BP415 BP416 BP417
BP412 BP413 BP414
1 2 3
RCO-K1
K1(COR)
K2(COR)
K3(COR)
K4(COR)
K5(COR)
K6(COR)
K7(COR)
K8(COR)
K9(COR)
K10(COR)
Position of the acyl group

FIGURE 2

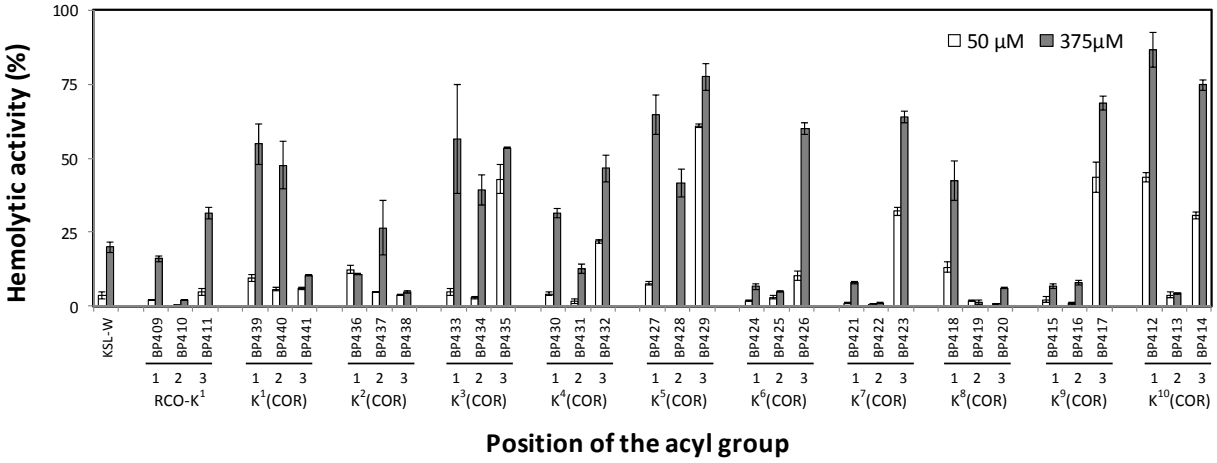
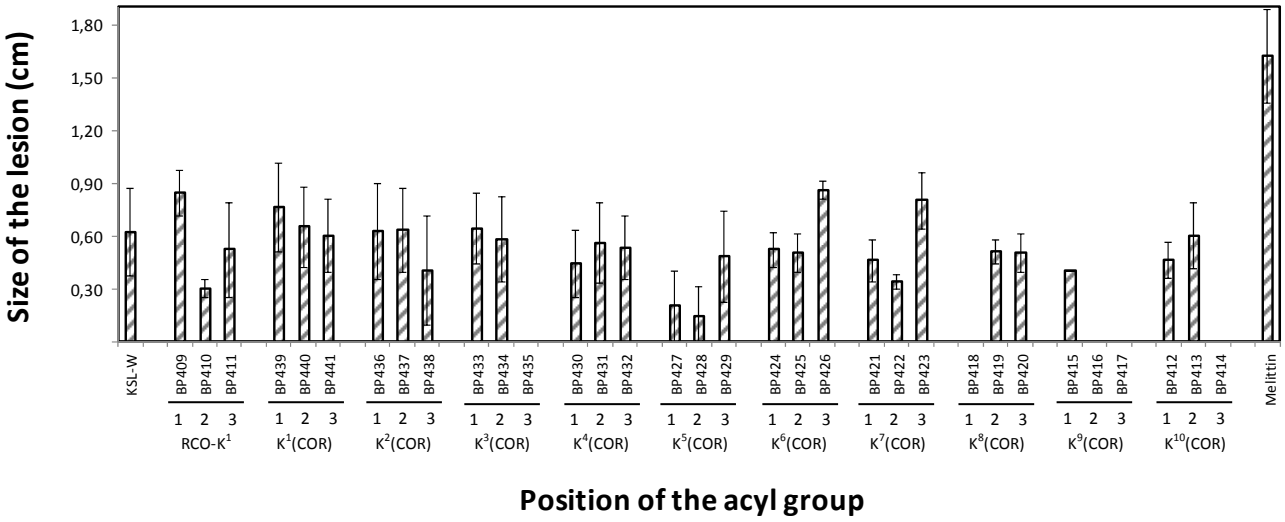

Hemolytic activity (%)
100
75
50
25
0
□ 50 µM ■ 375µM
KSL-W
BP409 BP410 BP411
BP439 BP440 BP441
BP436 BP437 BP438
BP433 BP434 BP435
BP430 BP431 BP432
BP427 BP428 BP429
BP424 BP425 BP426
BP421 BP422 BP423
BP418 BP419 BP420
BP415 BP416 BP417
BP412 BP413 BP414
1 2 3
RCO-K1
K1(COR)
K2(COR)
K3(COR)
K4(COR)
K5(COR)
K6(COR)
K7(COR)
K8(COR)
K9(COR)
K10(COR)
Position of the acyl group

FIGURE 3


Size of the lesion (cm)
1,80
1,50
1,20
0,90
0,60
0,30
KSL-W
BP409 BP410 BP411
BP439 BP440 BP441
BP436 BP437 BP438
BP433 BP434 BP435
BP430 BP431 BP432
BP427 BP428 BP429
BP424 BP425 BP426
BP421 BP422 BP423
BP418 BP419 BP420
BP415 BP416 BP417
BP412 BP413 BP414
Melittin
1 2 3
RCO-K1
K1(COR)
K2(COR)
K3(COR)
K4(COR)
K5(COR)
K6(COR)
K7(COR)
K8(COR)
K9(COR)
K10(COR)
Position of the acyl group

Supplementary Material

Antimicrobial peptide KSL-W and analogues:

Promising agents to control plant diseases

Cristina Camó, Anna Bonaterra, Esther Badosa, Aina Baró, Laura Montesinos, Emilio Montesinos,

Marta Planas* and Lidia Feliu*

Table of contents:

Table A. Sequences, retention times and purities by HPLC, and mass spectrometry data of the set of peptides selected from the literature.

Peptide	Sequence	t_R (min)[a]	Purity (%)[b]	HRMS
Plant defense elicitors				
PIP-1	YGIHTH-NH_2	5.38	>99	726.3688 $[M+H]^+$, 748.3503 $[M+Na]^+$
Pep-13	VWNQPVRGFKVYE-OH	6.31	>99	811.4266 $[M+2H]^{2+}$, 1621.8475 $[M+H]^+$
Promiscuous peptides				
Cn-AMP1	SVAGRAQGM-NH_2	5.33	>99	875.5 $[M+H]^+$, 897.4 $[M+Na]^+$
Cn-AMP2	TESYFVFSVGM-NH_2	6.97	>99	1265.5850 $[M+H]^+$, 1287.5670 $[M+Na]^+$
Cn-AMP3	YCSYTMEA-NH_2	5.78	>99	988.3481 $[M+Na]^+$
Multifunctional peptides				
p10	QKALNEINQF-NH_2	5.76	>99	1203.6445 $[M+H]^+$, 1225.6262 $[M+Na]^+$
p14	TKKTKLTEEEKNRL-NH_2	5.38	>99	1716.9983 $[M+H]^+$
Temporin-1CEa	FVDLKKIANIINSIFGK-NH_2	7.07	90	1919.1473 $[M+H]^+$
L10	WFRKQLKW-OH	6.27	>99	596.3406 $[M+2H]^{2+}$, 1191.6753 $[M+H]^+$
Frenatin 2.1S	GLVGTLLGHIGKAILG-NH_2	7.90	>99	759.4799 $[M+2H]^{2+}$, 1517.9536 $[M+H]^+$
Frenatin 2.2S	GLVGTLLGHIGKAILS-NH_2	7.17	>99	774.4836 $[M+2H]^{2+}$, 1547.9655 $[M+H]^+$
Antimicrobial peptides				
dGu-Api88	ONNRPVYIPRPRPPHPRL-NH_2[c]	5.76	92	2191.2430 $[M+H]^+$
JCpep7	KVFLGLK-OH	6.01	>99	804.5332 $[M+H]^+$
KSL	KKVVFKVKFK-NH_2	5.81	>99	625.4278 $[M+2H]^{2+}$, 1249.8494 $[M+H]^+$
KSL-W	KKVVFWVKFK-NH_2	6.19	>99	654.4198 $[M+2H]^{2+}$, 1307.8339 $[M+H]^+$

[a]HPLC retention time. [b]Percentage determined by HPLC at 220 nm after purification by column chromatography. [c]O stands for 4-hydroxyproline.

Table B. Sequences, retention times and purities by HPLC, and mass spectrometry data of the peptides derived from KSL-W containing a D-amino acid.

Peptide	Sequence	t_R (min)[a]	Purity (%)[b]	HRMS
BP442	KKVVFWVKFk-NH_2	6.07	>99	654.4211 $[M+2H]^{2+}$, 1307.8342 $[M+H]^+$
BP443	KKVVFWVKfK-NH_2	6.02	>99	654.4213 $[M+2H]^{2+}$, 1307.8338 $[M+H]^+$
BP444	KKVVFWVkFK-NH_2	5.98	>99	654.4213 $[M+2H]^{2+}$, 1307.8340 $[M+H]^+$
BP445	KKVVFWvKFK-NH_2	6.09	>99	654.4205 $[M+2H]^{2+}$, 1307.8356 $[M+H]^+$
BP446	KKVVFwVKFK-NH_2	6.26	>99	654.4195 $[M+2H]^{2+}$, 1307.8337 $[M+H]^+$
BP447	KKVVfWVKFK-NH_2	6.25	>99	654.4193 $[M+2H]^{2+}$, 1307.8332 $[M+H]^+$
BP448	KKVvFWVKFK-NH_2	6.27	>99	654.4193 $[M+2H]^{2+}$, 1307.8333 $[M+H]^+$
BP449	KKvVFWVKFK-NH_2	6.18	>99	654.4196 $[M+2H]^{2+}$, 1307.8327 $[M+H]^+$
BP450	KkVVFWVKFK-NH_2	6.11	>99	654.4201 $[M+2H]^{2+}$, 1307.8326 $[M+H]^+$
BP451	kKVVFWVKFK-NH_2	6.13	>99	654.4200 $[M+2H]^{2+}$, 1307.8334 $[M+H]^+$

[a]HPLC retention time. [b]Percentage determined by HPLC at 220 nm after purification by column chromatography.

Table C. Sequences, retention times and purities by HPLC, and mass spectrometry data of the lipopeptides derived from KSL-W.

Peptide	Sequence	t_R (min)[a]	Purity (%)[b]	HRMS
BP409	$C_5H_{11}CO$-KKVVFWVKFK-NH_2	6.53	>99	469.3072 $[M+3H]^{3+}$, 703.4552 $[M+2H]^{2+}$
BP410	C_3H_7CO-KKVVFWVKFK-NH_2	6.30	>99	459.9627 $[M+3H]^{3+}$, 689.4398 $[M+2H]^{2+}$
BP411	$C_{11}H_{23}CO$-KKVVFWVKFK-NH_2	7.36	>99	497.3374 $[M+3H]^{3+}$, 745.5023 $[M+2H]^{2+}$
BP412	KKVVFWVKFK(COC_5H_{11})-NH_2	6.74	>99	483.3109 $[M+3H]^{3+}$, 724.4609 $[M+2H]^{2+}$
BP413	KKVVFWVKFK(COC_3H_7)-NH_2	6.53	>99	473.9678 $[M+3H]^{3+}$, 710.4464 $[M+2H]^{2+}$
BP414	KKVVFWVKFK($COC_{11}H_{23}$)-NH_2	7.72	>99	511.3431 $[M+3H]^{3+}$, 766.5086 $[M+2H]^{2+}$
BP415	KKVVFWVKK(COC_5H_{11})K-NH_2	6.34	>99	476.9874 $[M+3H]^{3+}$, 714.9749 $[M+2H]^{2+}$
BP416	KKVVFWVKK(COC_3H_7)K-NH_2	6.11	>99	467.6437 $[M+3H]^{3+}$, 700.9595 $[M+2H]^{2+}$
BP417	KKVVFWVKK($COC_{11}H_{23}$)K-NH_2	7.18	>99	505.0202 $[M+3H]^{3+}$, 757.0225 $[M+2H]^{2+}$
BP418	KKVVFWVK(COC_5H_{11})FK-NH_2	6.76	>99	483.3118 $[M+3H]^{3+}$, 724.4622 $[M+2H]^{2+}$
BP419	KKVVFWVK(COC_3H_7)FK-NH_2	6.52	>99	473.9685 $[M+3H]^{3+}$, 710.4469 $[M+2H]^{2+}$
BP420	KKVVFWVK($COC_{11}H_{23}$)FK-NH_2	7.66	>99	511.3422 $[M+3H]^{3+}$, 766.5082 $[M+2H]^{2+}$
BP421	KKVVFWK(COC_5H_{11})KFK-NH_2	6.44	>99	492.9859 $[M+3H]^{3+}$, 738.9734 $[M+2H]^{2+}$
BP422	KKVVFWK(COC_3H_7)KFK-NH_2	6.23	>99	483.6428 $[M+3H]^{3+}$, 724.9596 $[M+2H]^{2+}$
BP423	KKVVFWK($COC_{11}H_{23}$)KFK-NH_2	7.25	>99	521.0187 $[M+3H]^{3+}$, 781.0226 $[M+2H]^{2+}$
BP424	KKVVFK(COC_5H_{11})VKFK-NH_2	6.26	>99	463.9834 $[M+3H]^{3+}$, 695.4695 $[M+2H]^{2+}$
BP425	KKVVFK(COC_3H_7)VKFK-NH_2	6.05	>99	454.6387 $[M+3H]^{3+}$, 681.4533 $[M+2H]^{2+}$
BP426	KKVVFK($COC_{11}H_{23}$)VKFK-NH_2	7.11	>99	492.0150 $[M+3H]^{3+}$, 737.5167 $[M+2H]^{2+}$
BP427	KKVVK(COC_5H_{11})WVKFK-NH_2	6.33	>99	736.9569 $[M+2Na]^{2+}$, 1450.9245 $[M+Na]^{+}$
BP428	KKVVK(COC_3H_7)WVKFK-NH_2	6.12	>99	467.6417 $[M+3H]^{3+}$, 700.9590 $[M+2H]^{2+}$
BP429	KKVVK($COC_{11}H_{23}$)WVKFK-NH_2	7.21	>99	505.0172 $[M+3H]^{3+}$, 757.0216 $[M+2H]^{2+}$
BP430	KKVK(COC_5H_{11})FWVKFK-NH_2	6.46	>99	492.9852 $[M+3H]^{3+}$, 738.9745 $[M+2H]^{2+}$
BP431	KKVK(COC_3H_7)FWVKFK-NH_2	6.26	>99	483.6433 $[M+3H]^{3+}$, 724.9612 $[M+2H]^{2+}$
BP432	KKVK($COC_{11}H_{23}$)FWVKFK-NH_2	7.26	>99	521.0162 $[M+3H]^{3+}$, 781.0215 $[M+2H]^{2+}$
BP433	KKK(COC_5H_{11})VFWVKFK-NH_2	6.46	>99	492.9874 $[M+3H]^{3+}$, 738.9764 $[M+2H]^{2+}$
BP434	KKK(COC_3H_7)VFWVKFK-NH_2	6.26	>99	483.6433 $[M+3H]^{3+}$, 724.9601 $[M+2H]^{2+}$
BP435	KKK($COC_{11}H_{23}$)VFWVKFK-NH_2	7.27	>99	521.0198 $[M+3H]^{3+}$, 781.0247 $[M+2H]^{2+}$
BP436	KK(COC_5H_{11})VVFWVKFK-NH_2	6.85	>99	483.3102 $[M+3H]^{3+}$, 724.4619 $[M+2H]^{2+}$
BP437	KK(COC_3H_7)VVFWVKFK-NH_2	6.62	97	473.9657 $[M+3H]^{3+}$, 710.4447 $[M+2H]^{2+}$
BP438	KK($COC_{11}H_{23}$)VVFWVKFK-NH_2	7.74	93	511.3408 $[M+3H]^{3+}$, 766.5078 $[M+2H]^{2+}$
BP439	K(COC_5H_{11})KVVFWVKFK-NH_2	6.81	>99	483.3096 $[M+3H]^{3+}$, 724.4612 $[M+2H]^{2+}$
BP440	K(COC_3H_7)KVVFWVKFK-NH_2	6.58	>99	473.9675 $[M+3H]^{3+}$, 710.4471 $[M+2H]^{2+}$
BP441	K($COC_{11}H_{23}$)KVVFWVKFK-NH_2	7.73	>99	511.3411 $[M+3H]^{3+}$, 766.5080 $[M+2H]^{2+}$

[a]HPLC retention time. [b]Percentage determined by HPLC at 220 nm after purification by column chromatography.

Table D. Antimicrobial activity (MIC), hemolysis and phytotoxicity of KSL-W and lipopeptides **BP409-BP441**

Peptide	MIC (μM)						Hemolysis[b] (%)	Tobacco lesion (cm)[c]
	Ea[a]	*Pss*[a]	*Psa*[a]	*Xap*[a]	*Xf*[a]	*Xav*[a]	375 μM	250 μM
KSL-W	6.2-12.5	6.2-12.5	1.6-3.1	0.8-1.6	3.1-6.2	6.2-12.5	20 ± 1.8	0.62 ± 0.4
BP409	12.5-25	>50	12.5-25	25-50	6.2-12.5	3.1-6.2	16 ± 0.9	0.84 ± 0.1
BP410	12.5-25	25-50	12.5-25	12.5-25	6.2-12.5	3.1-6.2	2 ± 0	0.30 ± 0.1
BP411	>50	>50	>50	>50	3.1-6.2	3.1-6.2	32 ± 1.9	0.52 ± 0.3
BP412	>50	>50	>50	6.2-12.5	>50	12.5-25	87 ± 5.8	0.46 ± 0.1
BP413	>50	>50	12.5-25	12.5-25	12.5-25	12.5-25	4 ± 0.3	0.60 ± 1.9
BP414	>50	>50	>50	>50	>50	>50	75 ± 1.7	0 ± 0
BP415	>50	>50	>50	>50	>50	>50	7 ± 0.8	0.4 ± 0
BP416	>50	>50	>50	25-50	25-50	>50	8 ± 0.8	0 ± 0
BP417	>50	>50	>50	3.1-6.2	3.1-6.2	6.2-12.5	68 ± 2.3	0 ± 0
BP418	>50	>50	>50	6.2-12.5	6.2-12.5	12.5-25	42 ± 6.6	0 ± 0
BP419	>50	12.5-25	12.5-25	6.2-12.5	12.5-25	6.2-12.5	1 ± 0.7	0.51 ± 0.1
BP420	>50	>50	>50	>50	>50	>50	6 ± 0.1	0.50 ± 0.1
BP421	>50	>50	>50	>50	>50	>50	8 ± 0.5	0.46 ± 0.1
BP422	>50	>50	>50	>50	>50	>50	1 ± 0.3	0.34 ± 0
BP423	>50	>50	>50	6.2-12.5	6.2-12.5	12.5-25	64 ± 2.0	0.80 ± 0.2
BP424	>50	25-50	>50	25-50	12.5-25	12.5-25	7 ± 1.1	0.52 ± 0.1
BP425	>50	>50	>50	25-50	25-50	12.5-25		0.50 ± 0.1
BP426	>50	>50	>50	6.2-12.5	12.5-25	6.2-12.5	60 ± 1.8	0.86 ± 0.1
BP427	>50	>50	>50	12.5-25	25-50	25-50	65 ± 6.6	0.20 ± 0.2
BP428	>50	>50	>50	>50	>50	>50	42 ± 4.6	0.14 ± 0.2
BP429	>50	>50	>50	6.2-12.5	12.5-25	12.5-25	77 ± 4.6	0.48 ± 0.3
BP430	25-50	25-50	12.5-25	6.2-12.5	12.5-25	12.5-25	31 ± 1.6	0.44 ± 0.2
BP431	>50	25-50	25-50	6.2-12.5	25-50	12.5-25	13 ± 1.5	0.56 ± 0.2
BP432	>50	>50	25-50	3.1-6.2	3.1-6.2	3.1-6.2	47 ± 4.3	0.53 ± 0.2
BP433	>50	>50	12.5-25	6.2-12.5	12.5-25	6.2-12.5	56 ± 18.3	0.64 ± 0.2
BP434	>50	>50	25-50	12.5-25	25-50	12.5-25	39 ± 5.0	0.58 ± 0.2
BP435	>50	>50	>50	6.2-12.5	12.5-25	6.2-12.5	53 ± 0.2	0 ± 0
BP436	>50	>50	>50	>50	25-50	>50	11 ± 0.2	0.63 ± 0.3
BP437	>50	>50	>50	25-50	6.2-12.5	12.5-25	26 ± 9.2	0.63 ± 0.2
BP438	>50	>50	>50	>50	>50	>50	5 ± 0.3	0.40 ± 0.3
BP439	>50	>50	>50	25-50	12.5-25	12.5-25	55 ± 7.0	0.76 ± 0.2
BP440	>50	>50	>50	25-50	>50	12.5-25	48 ± 7.9	0.65 ± 0.2
BP441	>50	>50	>50	>50	>50	>50	11 ± 0.3	0.60 ± 0.2

[a]*Ea*, *Erwinia amylovora; Pss*, *Pseudomonas syringae* pv. *syringae*; *Psa*, *Pseudomonas syringae* pv. *actinidiae*; *Xap*, *Xanthomonas arboricola* pv. *pruni*; *Xf*, *Xanthomonas fragariae*; *Xav*, *Xanthomonas axonopodis* pv. *vesicatoria*. [b]Percent hemolysis at 375 μM plus confidence interval (α = 0.05). [c]Phytotoxicity at 250 μM determined as the lesion diameter (cm) in infiltrated tobacco leaves plus confidence interval.