Lucimycin, an antifungal peptide from the therapeutic maggot of the common green bottle fly Lucilia sericata

Article *in* Biological Chemistry · March 2014

DOI: 10.1515/hsz-2013-0263 · Source: PubMed

DOI 10.1515/hsz-2013-0263 — Biol. Chem. 2014; 395(6): 649–656

Anne-Kathrin Pöppel, Aline Koch, Karl-Heinz Kogel, Heiko Vogel, Christian Kollewe, Jochen Wiesner and Andreas Vilcinskas*

Lucimycin, an antifungal peptide from the therapeutic maggot of the common green bottle fly *Lucilia sericata*

Abstract: We report the identification, cloning, heterologous expression and functional characterization of a novel antifungal peptide named lucimycin from the common green bottle fly *Lucilia sericata*. The lucimycin cDNA was isolated from a library of genes induced during the innate immune response in *L. sericata* larvae, which are used as therapeutic maggots. The peptide comprises 77 amino acid residues with a molecular mass of 8.2 kDa and a p*I* of 6.6. It is predicted to contain a zinc-binding motif and to form a random coil, lacking β-sheets or other secondary structures. Lucimycin was active against fungi from the phyla Ascomycota, Basidiomycota and Zygomycota, in addition to the oomycete *Phytophtora parasitica*, but it was inactive against bacteria. A mutant version of lucimycin, lacking the four C-terminal amino acid residues, displayed 40-fold lower activity. The activity of lucimycin against a number of highly-destructive plant pathogens could be exploited to produce transgenic crops that are resistant against fungal diseases.

Keywords: antifungal peptides; crop protection; fungal pathogens; *Lucilia sericata*; lucimycin; wound-healing maggots.

***Corresponding author: Andreas Vilcinskas,** Fraunhofer Institute for Molecular Biology and Applied Ecology, Department of Bioresources, Winchester Strasse, D-35394 Giessen, Germany; and Institute for Phytopathology and Applied Zoology, Justus-Liebig-University of Giessen, Heinrich-Buff-Ring 26-32, D-35392 Giessen, Germany, e-mail: andreas.vilcinskas@agrar.uni-giessen.de
Anne-Kathrin Pöppel: Fraunhofer Institute for Molecular Biology and Applied Ecology, Department of Bioresources, Winchester Strasse, D-35394 Giessen, Germany; and Institute for Phytopathology and Applied Zoology, Justus-Liebig-University of Giessen, Heinrich-Buff-Ring 26-32, D-35392 Giessen, Germany
Aline Koch and Karl-Heinz Kogel: Institute for Phytopathology and Applied Zoology, Justus-Liebig-University of Giessen, Heinrich-Buff-Ring 26-32, D-35392 Giessen, Germany
Heiko Vogel: Max-Planck-Institute for Chemical Ecology, Department of Entomology, Hans-Knoell-Strasse 8, D-07745 Jena, Germany
Christian Kollewe and Jochen Wiesner: Fraunhofer Institute for Molecular Biology and Applied Ecology, Department of Bioresources, Winchester Strasse, D-35394 Giessen, Germany

Introduction

The insect immune system displays a remarkable plasticity, which allows rapid adaptation to emerging pathogens and parasites. The repertoire of antimicrobial peptides produced by insects is evolutionarily dynamic, and is characterized by rapid functional shifts and the emergence of novel peptides with distinct properties (Vilcinskas, 2010, 2013). Insects have therefore been recognized as an indispensable source of anti-infectives for the development of novel antibiotics (Ratcliffe et al., 2011) and of transgenes that can be used to engineer pathogen-resistant crops (Vilcinskas and Gross, 2005). Screening for antimicrobial effector molecules has moved beyond insect species with completed genome sequences and now includes insects that are used as preclinical research models and for therapeutic applications. For example, the larvae of the greater wax moth *Galleria mellonella* are powerful model hosts for human pathogens (Desalermos et al., 2012; Arvanitis et al., 2013; Mukherjee et al., 2013; Wang et al., 2013) and a source of novel anti-infective molecules (Vilcinskas, 2011a). Targeted screening for immunity-related effector genes in this species using diverse transcriptomic approaches (Seitz et al., 2003; Vogel et al., 2011) has led to the discovery of novel antimicrobial peptides and proteins including the antifungal peptide gallerimycin (Schuhmann et al., 2003) which has also been developed as a transgene to confer resistance against parasitic fungi in crops (Langen et al., 2006).

Knowledge-based screening focuses on promising candidate species with known and relevant properties. We therefore selected the larvae of the common green bottle fly (*Lucilia sericata*), because they are widely used for maggot therapy. This involves the treatment of wounds containing necrotic tissue, e.g., infected surgical wounds, pressure sores and ulcers, with live maggots (Church, 1996; Thomas et al., 1996; Mumcuoglu et al., 1999). The benefits of maggot therapy include the efficient removal of necrotic tissue (debridement), the acceleration of wound-healing, and wound disinfection (Nigam et al., 2006a,b).

Accordingly, the secretions/excretions of maggots have been shown to contain molecules that promote wound-healing, enzymes that digest necrotic tissues without harming healthy cells (Chambers et al., 2003), and antimicrobial compounds (Bexfield et al., 2004; Huberman et al., 2007).

Although traditional maggot therapy is highly successful, the use of live maggots is often distasteful to the patient and the maggots themselves have a limited shelf-life. Therefore, a switch to contemporary biosurgery is envisaged, which involves the application of maggot-derived substances instead of live insects. Several groups have started to isolate and characterize the beneficial molecules in maggot secretions, aiming to produce them synthetically or as recombinant proteins that can be applied as topical formulations or in novel dressings impregnated with hydrogels as carrier substrates (Vilcinskas, 2011b). One example is the defensin-like peptide lucifensin from *L. sericata*, which has potent antibacterial activity (Cerovsky et al., 2010, 2011).

We have previously used suppression subtractive hybridization (SSH) to screen systematically for immune-inducible effector molecules produced by *L. sericata* maggots (Altincicek and Vilcinskas, 2009). This powerful PCR-based method amplifies cDNAs that are differentially expressed when comparing two samples while suppressing the amplification of common cDNAs, allowing the isolation of genes that are induced by challenge with microbial elicitors of innate immune responses such as bacterial lipopolysaccharides (LPS). A key advantage of this approach is that a complete genome sequence is not required. It has therefore been applied in insects with little sequence data available, such as the rat-tailed maggots of the drone fly *Eristalis tenax*, which are the only animals capable of surviving in aquatic habitats with extreme levels of microbial contamination, such as farmyard liquid manure storage pits (Altincicek and Vilcinskas, 2007). SSH screening of *L. sericata* maggots injected with LPS to elicit a strong immune response identified several antibacterial peptides, including FG360491, which is related to the defensin-like peptide sapecin-B, and FG360498, which is related to insect diptericins. Three further open reading frames were identified in our *L. sericata* transcriptomics database, with similarities to the proline-rich antimicrobial peptides drosocin and metchnikowin from *Drosophila melanogaster*. Synthetic analogs of these peptides were found to be active against the Gram-positive bacterium *Micrococcus luteus*, but not against Gram-negative *Escherichia coli* (Altincicek and Vilcinskas, 2009).

Here we report the identification, characterization and heterologous expression of a recombinant *L. sericata* peptide with antifungal but not antibacterial activity. The peptide was thus named lucimycin, a portmanteau of lucifensin from the same species (Cerovsky et al., 2011) and the antifungal peptide gallerimycin from the greater wax moth *G. mellonella* (Langen et al., 2006).

Results

Identification of a *Lucilia sericata* transcript encoding an antifungal peptide

The analysis of our *L. sericata* transcriptomics database revealed the presence of an mRNA encoding a peptide containing a putative 18-residue signal peptide and a 77-residue mature domain (Figure 1). The mature peptide was notable due to the high content of histidine residues (22%), its predicted molecular mass was 8.2 kDa, and it had a theoretical p*I* of 6.6. In contrast to most antimicrobial peptides, the novel peptide lacked disulfide bonds (which are common in cysteine-rich peptides such as defensins) and proline-rich sequences, and was hydrophilic but not cationic (GRAVY=-1512). Secondary structure prediction revealed that the peptide is likely to form a random coil structure lacking β-sheets (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_seccons.html). Despite the lack of conspicuous sequence similarity, the high content of histidine residues in the novel peptide was reminiscent of histidine-rich mammalian saliva peptides known as histatins, which are active against the yeast *Candida albicans*.

Production of recombinant and synthetic lucimycin

In our first attempt to produce the novel peptide (provisionally named lucimycin) as a recombinant product in *E. coli*, we expressed a small synthetic gene construct encoding the peptide without tags or fusion sequences. This approach was not successful so we produced lucimycin as a fusion protein with thioredoxin and a His_6 tag, allowing it to be separated from the cell lysate by cobalt-based immobilized metal ion affinity chromatography (IMAC; Figure 2). After removing imidazole and salt from the eluent by size-exclusion chromatography, the fusion protein was cleaved with factor Xa to release the peptide, and the resulting protein fragments were separated by anion exchange HPLC (Figure 3A). The cleavage products were analyzed by SDS-PAGE, showing the presence

```
ATGGCAAAATTATTTATTATTGTTCTTTTCGCTTTAATCGCCGTTGTTTT
 M  A  K  L  F  I  I  V  L  F  A  L  I  A  V  V  L

GGCTCAACACGGCTATGGTGCCGGTGGCCATGGCCAACAAGGCTATGGTA
 A [Q  H  G  Y  G  A  G  G  H  G  Q  Q  G  Y  G ]

GCCAACATAGCAGTCATGCTCCCCAAGGTGGACATGTTGTCCGTGAACAA
[S  Q  H  S  S  H  A  P  Q  G  G  H  V  V  R  E  Q ]

GGTTTTAGTGGTCATGTTCATGAACAACAGGCTGGGCATCATCATGAAGC
[G  F  S  G  H  V  H  E  Q  Q  A  G  H  H  H  E  A ]

TGGCCATCATGAGCAAGCTGGTCATCATGAACAATCTGGTCAACAAGTTC
 [G  H  H  E  Q  A  G  H  H  E  Q  S  G  Q  Q  V ]

ATGGTCAAGGTCATGGCTATAAAAGTCATGGTTATTAAGTATATTCTAAA
[H  G  Q  G  H  G  Y  K  S  H  G  Y ] *

TAACAATTACTTAAACCAACAAAAATAAATAAAAAGAATTCGACTTAAAG
```

Figure 1 The *Lucilia sericata* lucimycin mRNA and peptide sequences (GenBank accession no. KJ413251). The predicted 77-residue lucimycin amino acid mature peptide is shown in a box.

of a band representing the released peptide migrating at an apparent molecular mass of 30 kDa even though the anticipated molecular mass of the peptide was 8.2 kDa (Figure 3B). We investigated the discrepancy by LC/MS and confirmed that the molecular mass of the peptide was 8.2 kDa as expected. When we replaced the factor Xa cleavage site in the fusion protein with an enterokinase recognition sequence, digestion with this enzyme yielded a peptide representing lucimycin without the C-terminal four amino acid residues, as confirmed by LC/MS analysis (data not shown). Thus we concluded that enterokinase

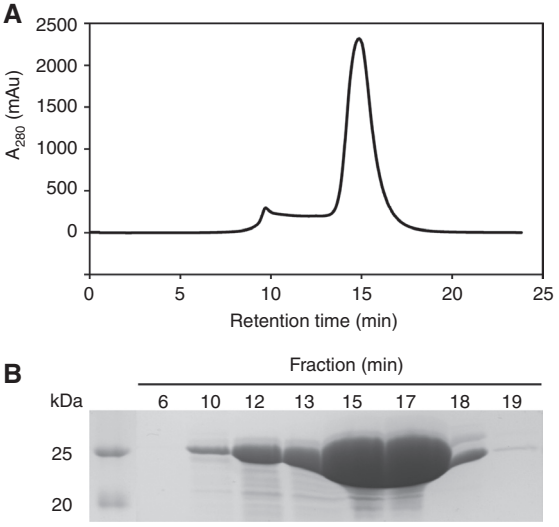

Figure 2 Purification of the lucimycin fusion protein by immobilized metal affinity chromatography.
(A) After the application of *Escherichia coli* lysate to a Co^{2+} affinity column, the His_6-tagged fusion protein was eluted in 200 mM imidazole. (B) Elution fractions were analyzed by SDS-PAGE followed by staining with Coomassie Brilliant Blue.

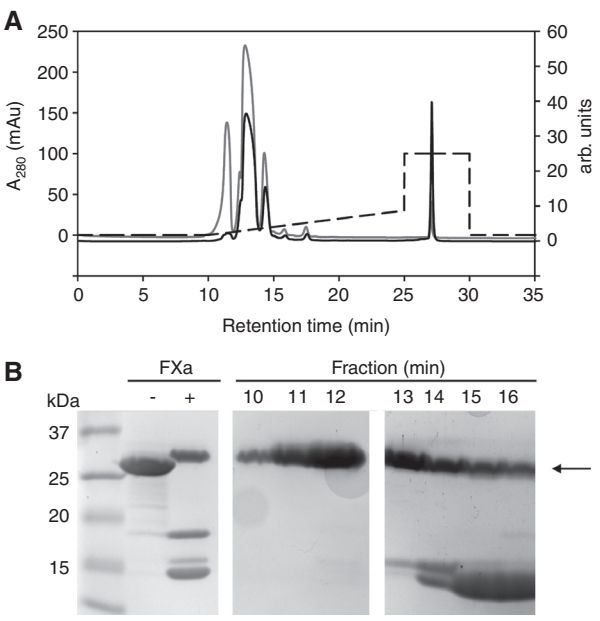

Figure 3 Purification of lucimycin by anion exchange chromatography.
(A) After cleavage of the fusion protein with Factor Xa, the sample was loaded onto a MonoQ column and the proteins were separated in a linear gradient of 0–300 mM NaCl in 20 column volumes. Grey line: absorption at 280 nm; black line: fluorescence detection.
(B) The products of the cleavage reaction with factor Xa (FXa) and elution fractions were analyzed by SDS-PAGE followed by staining with Coomassie Brilliant Blue. The protein band corresponding to lucimycin is marked with an arrow.

cleaves the peptide bond after lucimycin residue K73, in addition to the specific recognition site. As a reference, we also produced synthetic lucimycin. Solid-phase synthesis was carried out by GenScript (USA) and the integrity of the peptide was confirmed by LC/MS.

Antibacterial and antifungal activity tests

We first tested the *in vitro* antibacterial activity of the purified recombinant peptide against the Gram-negative species *E. coli* and *Pseudomonas aeroginosa*, and the Gram-positive species *Micrococcus luteus*, *Staphylococcus aureus* and *Staphylococcus epidermidis*, using both inhibition zone and microdilution assays. Lucimycin did not exhibit activity against these bacteria at concentrations up to the highest concentration tested (100 μM). Next we tested the *in vitro* antifungal activity of the synthetic and recombinant forms of lucimycin against the economically-important agricultural pathogens *Fusarium graminearum* and *Phytophthora parasitica* by counting the number of spores that germinated in the presence of 0.3–200 μM of the peptide. The IC_{50} concentration, which reduced germination by 50%, and the minimal inhibitory concentration (MIC) after 24 h, are shown in Table 1. Images showing hyphal growth at different peptide concentrations are shown in Figures 4 and 5. Recombinant lucimycin completely abolished spore germination in *F. graminearum* at a concentration of 2.5 μM and in *P. parasitica* at concentrations ≥40 μM. In both the *F. graminearum* and *P. parasitica* tests, the efficiency of the recombinant lucimycin was marginally higher than its synthetic counterpart, possibly reflecting a higher purity of the recombinant peptide. We also tested the truncated lucimycin lacking the four C-terminal amino acid residues for activity against *F. graminearum*. This derivative retained its antifungal activity, but

Table 1 MIC (minimal inhibitory concentration) and IC_{50} (half-minimal inhibitory concentration) values of synthetic and recombinant lucimycin when tested against the fungal pathogens *Fusarium graminearum* and *Phytophtora parasitica*.

Lucimycin	*Fusarium graminearum*		*Phytophtora parasitica*	
	MIC (μM)	IC_{50} (μM)	MIC (μM)	IC_{50} (μM)
Synthetic	5.0	2.2	50	35
Recombinant	2.5	1.6	40	29

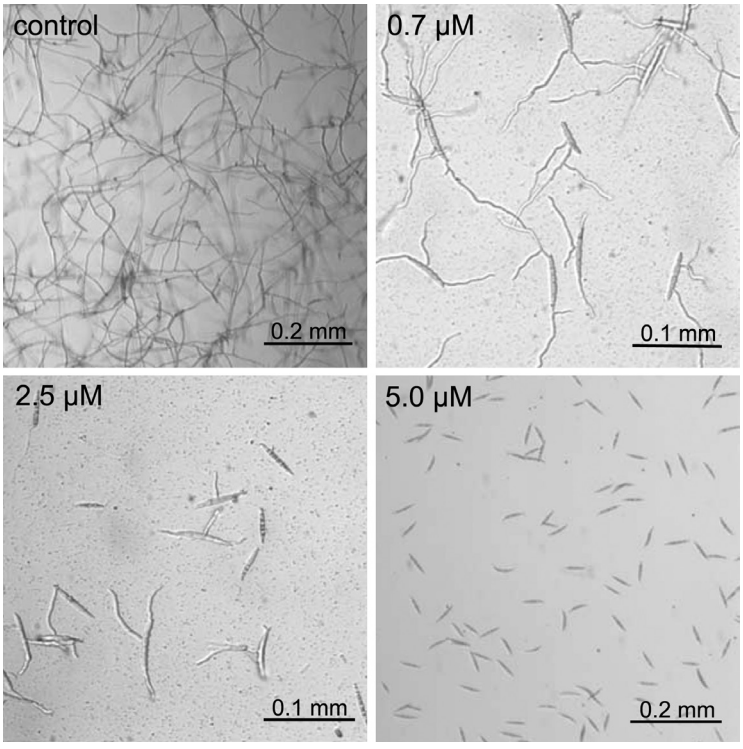

Figure 4 Inhibition of macroconidial germination in axenic *Fusarium graminearum* cultures with increasing concentrations of recombinant lucimycin.

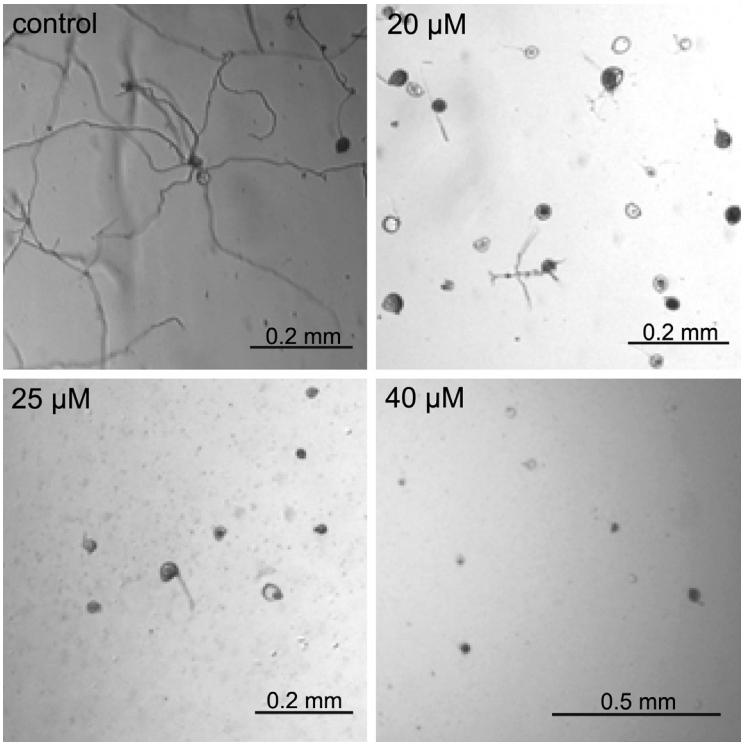

Figure 5 Inhibition of zoospore germination in axenic *Phytophtora parasitica* cultures exposed to increasing concentrations of recombinant lucimycin.

higher concentrations were required for complete inhibition (MIC=100 µM).

The *in vitro* antifungal activity of synthetic lucimycin was also tested against further plant pathogens, as well as fungi causing opportunistic infections in humans. Lucimycin showed no activity against *Rhizopus oryzae*, *Aspergillus fumigatus*, *Aspergillus terreus* and *Arthoderma benhamiae* at concentrations below 120 µM, but was effective against the plant pathogens *Cladosporium herbarium* and *Alternaria alternata* and the human pathogens *Lichtheimia corymbifera*, *Mucor circinelloides*, *Candida albicans* and *Trichosporon asahii* at this concentration. For all these fungal species, we determined the lucimycin concentrations that completely inhibited spore germination (MIC) and that began to inhibit mycelial growth (Table 2).

Discussion

We have discovered a novel antimicrobial peptide from the wound-healing maggot *L. sericata* showing that the spectrum of antimicrobial peptides produced by this species can be expanded beyond the antibacterial peptides that have previously been described (Altincicek and Vilcinskas, 2009; Cerovsky et al., 2011). The novel peptide, named lucimycin, has exclusively antifungal properties and displays activity against diverse fungal pathogens, including an oomycete and representatives of the phyla Ascomycota, Basidiomycota and Zygomycota.

The antifungal mechanism of lucimycin is unknown, but probably differs substantially from the antibacterial mechanisms of classical cationic peptides such as defensins and cecropins, which bind negatively-charged

Table 2 Lucimycin concentrations that began to inhibit mycelial growth and minimal inhibitory concentrations (MIC) when tested against the fungal pathogens.

Fungal species	Phylum	Inhibition of mycelial growth (µM)	MIC (µM)
Lichtheimia corymbifera	Zygomycota	1.9	15
Mucor circinelloides	Zygomycota	15	60
Rhizopus oryzae	Zygomycota	>120	>120
Candida albicans	Ascomycata	15	30
Cladosporium herbarium	Ascomycata	3.7	60
Alternaria alternata	Ascomycata	3.7	60
Aspergillus fumigatus	Ascomycata	>120	>120
Aspergillus terreus	Ascomycata	>120	>120
Arthoderma benhamiae	Ascomycata	>120	>120
Trichosporon asahii	Basidiomycota	7.5	30

phospholipids typically present in the outer leaflet of bacterial cell membranes. By adopting an amphipathic conformation, these peptides finally form pores or disrupt the membrane in a detergent-like manner (Wiesner and Vilcinskas, 2010). The slightly acidic p*I* of lucimycin suggests that it does not interact with negatively-charged membranes and this is likely to explain its lack of antibacterial activity. Charge-independent binding to glucosylceramides was demonstrated for antifungal defensins (Aerts et al., 2008), which however are structurally distinct from lucimycin. The candidacidal activity of histatin 5 involves binding to the Ssa2p protein (a member of the heat shock protein 70 family) and non-lytic internalization via polyamine influx transporters, leading to the selective leakage of ions and ATP (Kumar et al., 2011).

The primary structure of lucimycin contains a canonical zinc-binding motif (HEXXH), which is part of an eight-residue sequence with five clustered histidine residues (HHHEAGHH, residues 45–52). Therefore lucimycin is likely to bind zinc and probably other divalent metal ions. The histatins also contain one or two HEXXH motifs, in addition to one copper and nickel binding ATCUN motif (DSH), which is not found in lucimycin. Whether or not metal-binding, possibly resulting in the depletion of trace elements, contributes to the candidacidal activity of histatins is less clear, because a synthetic fragment lacking both metal-binding motifs retains its activity. Our experiments also suggest that the antifungal activity of lucimycin depends at least in part on other determinants besides metal complex formation, as we observed markedly less activity against *F. graminearum* when we tested a truncated version of lucimycin, lacking four C-terminal amino acid residues that are not part of a known metal-binding motif. This observation also suggests that the antifungal activity may not merely depend on the overall physicochemical properties of the peptide but may involve binding to a specific receptor.

We have previously shown that antifungal peptides produced by insects can be used to confer resistance against fungal pathogens in crops. For example, the expression of the *G. mellonella* antifungal peptide gallerimycin was shown to confer resistance against the fungal pathogens *Erysiphe cichoracearum* and *Sclerotinia minor* in transgenic tobacco (Langen et al., 2006). We have also demonstrated that the *D. melanogaster* antifungal peptide metchnikowin confers strong resistance against fungal pathogens in barley without harming fungal symbionts such as *Piriformospora indica* that protect barley from drought and salinity stress (Rahnamaeian et al., 2009). Most recently, we reported that the antifungal peptide thanatin from the hemipteran spined soldier bug *Podisus maculiventris* confers resistance to fungal pathogens when expressed in *Arabidopsis thaliana* (Koch et al., 2012). Of particular interest in this regard is the activity of lucimycin against an oomycete of the genus *Phytophthora*, because only a few peptides are known to exhibit activity against these plants pathogens (Ali and Reddy, 2000). *Phytophthora* is best known for its role in the Great Irish Famine of the 19th century, but even today it causes losses of US$ 3 billion per year worldwide and no effective control measures are available (Louis and Roy, 2010). We therefore aim to produce transgenic potato plants expressing lucimycin to test their resistance against late blight.

Materials and methods

Cloning and heterologous expression of lucimycin

A synthetic codon-optimized gene encoding the deduced amino acid sequence of the predicted mature lucimycin was ligated via the KpnI and EcoRI site into the *E. coli* expression plasmid pET32-(a)+ (Novagen, Darmstadt, Germany) to yield pET-FCP, encoding a translational fusion protein containing an N-terminal thioredoxin protein, followed by a His_6 tag, a factor Xa recognition site and the lucimycin peptide sequence. The expression of lucimycin with a fusion partner facilitated purification by IMAC. The factor Xa recognition site allowed the native lucimycin peptide to be cleaved from the fusion protein, leaving no residual additional amino acids. *E. coli* TOP10 cells (Invitrogen, Carlsbad, USA) were transformed with pET-FCP and the purified plasmid was introduced into *E. coli* BL21(DE) (Invitrogen, Carlsbad, USA) cells. The 37-kDa lucimycin fusion protein was expressed by cultivating a single transformant colony at 37°C in 400 ml LB containing 100 mg/l ampicillin in 1-l shake flasks at 220 rpm. Expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at an optical density (OD_{600}) of 0.5. The cells were harvested 4 h after induction by centrifugation at 10 000 *g* for 10 min at 4°C. The cell pellet was disrupted in a M110PS microfluidizer (Microfluidics, Newton, USA) in binding buffer A (100 mM NaCl, 30 mM Tris-HCl, pH 7.5). After centrifugation at 70 000 *g* for 30 min the supernatant was filtered through a 0.22-µm membrane.

Purification of the fusion protein and isolation of lucimycin

The His_6-tagged fusion protein was purified from the bacterial cell lysate by cobalt-based IMAC using an Äkta prime plus system (GE Healthcare, Buckinghamshire, UK) fitted with a column containing 20 ml TALON resin (Clontech, Mountain View, USA). The supernatant was added to the column and the column was washed with four column volumes of 15% elution buffer B (100 mM NaCl, 30 mM Tris-HCl, 200 mM imidazole, pH 7.5) before the fusion protein was eluted in 100% buffer B. Because imidazole inhibits the factor Xa cleavage reaction, it was removed by size-exclusion chromatography using a HiPrep Desalting Column 26/10

(GE Healthcare, Uppsala, Sweden). The fusion protein was eluted in 10 mM Tris-HCl, pH 8. To release the lucimycin from the fusion protein, factor Xa (Merck, Darmstadt, Germany) was added to the eluate at a ratio of 500 enzyme units per 10 mg of fusion protein in 10 ml buffer C (10 mM Tris-HCl, 2 mM $CaCl_2$, pH 8) for 16 h at 22°C.

Purification of lucimycin

The lucimycin peptide was separated from its former fusion partner and the protease by anion exchange chromatography using a Dionex HPLC system (Dionex, Idstein, Germany). The cleavage solution was loaded onto a MonoQ column 5/50 GL (GE Healthcare, Uppsala, Sweden), and washed with binding buffer D (10 mM Tris-HCl, pH 8) and the peptide was subsequently eluted with buffer E (10 mM Tris-HCl, 1 M NaCl, pH 8) by applying a 0–30% linear gradient in 20 column volumes. Because high salt concentrations inhibit the activity assays, the peptide was desalted using the HiPrep Desalting Column 26/10 (GE Healthcare) and eluted in water. The integrity of the purified peptide was confirmed by SDS-PAGE and LC/MS analysis (micrOTOF II, Bruker Daltonik, Bremen, Germany).

Solid-phase synthesis

Lucimycin was also produced by solid-phase synthesis and purified by reversed-phase chromatography by GenScript (Piscataway, USA). The integrity of the peptide was confirmed by LC/MS.

Antifungal activity tests

Fusarium graminearum strain IFA 65 (IFA Tulln obtained from Marc Lemmens Department for Agrobiotechnology Tulln, Austria) was cultivated for 7 days on synthetic nutrient agar (SNA) at 22°C. Fungal spore suspensions were prepared by washing the plate with 5 ml sterile distilled water containing 0.02% Tween-20 and scraping gently using a sterile rod. The spore solution was then passed through four layers of Miracloth (Kalbiochem, USA) to remove the mycelia. The inoculum concentration was adjusted to conidia 2×10^4/ml for the *in vitro* assay. *Phytophthora parasitica* isolate 329 (provided by Harald Keller and sourced from the *Phytophthora* collection at INRA, France) was cultured on carrot juice agar medium at 25°C for 2 weeks. Zoospores were harvested by rinsing the plates with 5 ml cold sterile distilled water. The sporangial suspension was then incubated at 4°C for 2 h and 1 h at room temperature to induce the release of zoospores. The inoculum concentration was adjusted to zoospores 2×10^4/ml. *Rhizopus oryzae* ATCC 56536, *Arthoderma benhamiae* CBS 112371, *Aspergillus fumigatus* ATCC 46645, *Alternaria alternata* FSU 9317, *Aspergillus terreus* FSU 6307, *Candida albicans* SC 5314, *Cladosporium herbarium* FSU 3771, *Lichtheimia corymbifera* FSU 9682, *Mucor circinelloides* FSU 5859 and *Trichosporon asahii* FSU 11372 were cultured on malt extract agar at room temperature for 2 weeks. Fungal spores were harvested by rinsing the plates with 7 ml sterile distilled water. The spore solution was then passed through four layers of Miracloth (Kalbiochem, USA) and the inoculum concentration was adjusted to spores 2×10^4/ml. The antifungal activity of recombinant and synthetic lucimycin was evaluated using a spore germination assay in 96-well microtiter plates (Tekan, flat bottom). We incubated 50 µl of each *F. graminearum* and *P. parasitica* spore suspension at room temperature for 24 h with 50 µl of recombinant or synthetic lucimycin at concentrations of 0.3–200 µM. The other fungal suspensions were incubated for 24 h at 25°C with synthetic peptide at concentrations of 1.8–120 µM, and with distilled water as a control. The number of germinating spores was counted and the percentage inhibition was calculated for each concentration. Germ tube morphology was also examined using an inverted stereomicroscope (Leica DM IL, Wetzlar, Germany). The experiment was carried out twice with at least three replicates per concentration.

Antibacterial activity tests

Escherichia coli D31 was cultivated for 4 h in 2 ml LB medium at 37°C. *Staphylococcus aureus* DSM 2569, *Staphylococcus epidermidis* DSM 3269 and *Pseudomonas aeruginosa* DSM 50071 were cultivated for 4 h in 2 ml Tryptic Soy Broth (TSB) at 37°C. *Micrococcus luteus* DSM 20030 was cultivated overnight in 2 ml TSB at 28°C. Bacterial suspensions were diluted to OD_{600}=0.001 (OD_{600}=0.0004 for *E. coli*) and 10 µl of bacterial suspension was added to 10 µl of recombinant peptide at concentrations of 1–100 µM in 384-well microtiter plates. Bacteria were incubated with the solvent as a negative control. The plates were incubated in an Eon Microplate Spectrophotometer (BioTek, Seattle, USA) for 16 h at 37°C (20 h at 28°C for *M. luteus*) and the optical density was measured at 20-min intervals.

Acknowledgments: This work was supported by the excellence initiative of the Hessian Ministry of Science, Higher Education and Art (HMWK) which encompasses a generous grant for the LOEWE research focus 'Insect Biotechnology' to A.V. and K.K. We thank Dr. Kerstin Voigt from the Jena Microbial Resource Collection, Leibniz-Institut für Naturstoff-Forschung und Infektionsbiologie e.V., Hans-Knöll-Institut (HKI) for the antifungal assays and Christian René Röhrich for LC/MS analysis. The authors acknowledge Dr. Richard M. Twyman for editing the manuscript.

Received October 5, 2013; accepted March 11, 2014; previously published online March 12, 2014

References

Aerts, A.M., Francois, I.E., Cammue, B.P., and Thevissen, K. (2008). The mode of antifungal action of plant, insect and human defensins. Cell. Mol. Life. Sci. *65*, 2069–2079.

Ali, G.S. and Reddy, A.S. (2000). Inhibition of fungal and bacterial plant pathogens by synthetic peptides: in vitro growth inhibition, interaction between peptides and inhibition

of disease progression. Mol. Plant. Microbe. Interact. *13*, 847–859.

Altincicek, B. and Vilcinskas, A. (2007). Analysis of the immune-inducible transcriptome from microbial stress resistant, rat-tailed maggots of the drone fly *Eristalis tenax*. BMC Genomics *8*, 326.

Altincicek, B. and Vilcinskas, A. (2009). Septic injury-inducible genes in medicinal maggots of the green blow fly *Lucilia sericata*. Insect Mol. Biol. *18*, 119–125.

Arvanitis, M., Glavis-Bloom, J., and Mylonakis, E. (2013). Invertebrate models of fungal infection. Biochim. Biophys. Acta. *1832*, 1378–1383.

Bexfield, A., Nigam, Y., Thomas, S., and Ratcliffe, N.A. (2004). Detection and partial characterisation of two antibacterial factors from the excretions/secretions of the medicinal maggot *Lucilia sericata* and their activity against methicillin-resistant *Staphylococcus aureus* (MRSA). Microbes Infect. *6*, 1297–1304.

Cerovsky, V., Zdarek, J., Fucik, V., Monincova, L., Voburka, Z., and Bem, R. (2010). Lucifensin, the long-sought antimicrobial factor of medicinal maggots of the blowfly *Lucilia sericata*. Cell. Mol. Life Sci. *67*, 455–466.

Cerovsky, V., Slaninova, J., Fucik, V., Monincova, L., Bednarova, L., Malon, P., and Stokrova, J. (2011). Lucifensin, a novel insect defensin of medicinal maggots: synthesis and structural study. Chembiochem. *12*, 1352–1361.

Chambers, L., Woodrow, S., Brown, A.P., Harris, P.D., Phillips, D., Hall, M., Church, J.C., and Pritchard, D.I. (2003). Degradation of extracellular matrix components by defined proteinases from the greenbottle larva *Lucilia sericata* used for the clinical debridement of non-healing wounds. Br. J. Dermatol. *148*, 14–23.

Church, J.C. (1996). The traditional use of maggots in wound healing, and the development of larva therapy (biosurgery) in modern medicine. J. Altern. Complement. Med. *2*, 525–527.

Desalermos, A., Fuchs, B.B., and Mylonakis, E. (2012). Selecting an invertebrate model host for the study of fungal pathogenesis. PLoS Pathog. *8*, e1002451.

Huberman, L., Gollop, N., Mumcuoglu, K.Y., Breuer, E., Bhusare, S.R., Shai, Y., and Galun, R. (2007). Antibacterial substances of low molecular weight isolated from the blowfly, *Lucilia sericata*. Med. Vet. Entomol. *21*, 127–131.

Koch, A., Khalifa, W., Langen, G., Vilcinskas, A., Kogel, K.-H., and Imani, J. (2012). The antimicrobial peptide thanatin reduces fungal infections in *Arabidopsis*. J. Phytopathol. *160*, 606–610.

Kumar, R., Chadha, S., Saraswat, D., Bajwa, J.S., Li, R.A., Conti, H.R., and Edgerton, M. (2011). Histatin 5 uptake by *Candida albicans* utilizes polyamine transporters Dur3 and Dur31 proteins. J. Biol. Chem. *286*, 43748–43758.

Langen, G., Imani, J., Altincicek, B., Kieseritzky, G., Kogel, K.H., and Vilcinskas, A. (2006). Transgenic expression of gallerimycin, a novel antifungal insect defensin from the greater wax moth *Galleria mellonella*, confers resistance to pathogenic fungi in tobacco. Biol. Chem. *387*, 549–557.

Louis, B. and Roy, P. (2010). Engineered pathogenesis related and antimicrobial proteins weaponry against *Phytopthora infestans* in potato plant. Biotechnol. Mol. Biol. Rev. *5*, 61–66.

Mukherjee, K., Hain, T., Fischer, R., Chakraborty, T., and Vilcinskas, A. (2013). Brain infection and activation of neuronal repair mechanisms by the human pathogen Listeria monocytogenes in the lepidopteran model host *Galleria mellonella*. Virulence *4*, 324–332.

Mumcuoglu, K.Y., Ingber, A., Gilead, L., Stessman, J., Friedmann, R., Schulman, H., Bichucher, H., Ioffe-Uspensky, I., Miller, J., Galun, R., et al. (1999). Maggot therapy for the treatment of intractable wounds. Internat. J. Dermatol. *38*, 623–627.

Nigam, Y., Bexfield, A., Thomas, S., and Ratcliffe, N.A. (2006a). Maggot therapy: the science and implication for cam part I – history and bacterial resistance. Evid. Based complement. Alternat. Med. *3*, 223–227.

Nigam, Y., Bexfield, A., Thomas, S., and Ratcliffe, N.A. (2006b). Maggot therapy: the science and implication for cam part II – maggots combat infection. Evid. Based complement. Alternat. Med. *3*, 303–308.

Rahnamaeian, M., Langen, G., Imani, J., Khalifa, W., Altincicek, B., von Wettstein, D., Kogel, K.H., and Vilcinskas, A. (2009). Insect peptide metchnikowin confers on barley a selective capacity for resistance to fungal ascomycetes pathogens. J. Exp. Bot. *60*, 4105–4114.

Ratcliffe, N.A., Mello, C.B., Garcia, E.S., Butt, T.M., and Azambuja, P. (2011). Insect natural products and processes: new treatments for human disease. Insect Biochem. Mol. Biol. *41*, 747–769.

Schuhmann, B., Seitz, V., Vilcinskas, A., and Podsiadlowski, L. (2003). Cloning and expression of gallerimycin, an antifungal peptide expressed in immune response of greater wax moth larvae, *Galleria mellonella*. Arch. Insect Biochem. Physiol. *53*, 125–133.

Seitz, V., Clermont, A., Wedde, M., Hummel, M., Vilcinskas, A., Schlatterer, K., and Podsiadlowski, L. (2003). Identification of immunorelevant genes from greater wax moth (*Galleria mellonella*) by a subtractive hybridization approach. Dev. Comp. Immunol. *27*, 207–215.

Thomas, S., Jones, M., Shutler, S., and Jones, S. (1996). Using larvae in modern wound management. J. Wound Care *5*, 60–69.

Vilcinskas, A. (2010). Coevolution between pathogen-derived proteinases and proteinase inhibitors of host insects. Virulence *1*, 206–214.

Vilcinskas, A. (2011a). Anti-infective therapeutics from the lepidopteran model host *Galleria mellonella*. Curr. Pharm. Des. *17*, 1240–1245.

Vilcinskas, A. (2011b). From traditional maggot therapy to modern biosurgery. In: Insect Biotechnology, A. Vilcinskas, ed. (Dordrecht, Netherlands: Springer), pp. 67–75.

Vilcinskas, A. (2013). Evolutionary plasticity of insect immunity. J. Insect Physiol. *59*, 123–129.

Vilcinskas, A. and Gross, J. (2005). Drugs from bugs: the use of insects as a valuable source of transgenes with potential in modern plant protection strategies. J. Pest Sci. *78*, 187–191.

Vogel, H., Altincicek, B., Glockner, G., and Vilcinskas, A. (2011). A comprehensive transcriptome and immune-gene repertoire of the lepidopteran model host *Galleria mellonella*. BMC Genomics *12*, 308.

Wang, Y., Li, D.D., Jiang, Y.Y., and Mylonakis, E. (2013). Utility of insects for studying human pathogens and evaluating new antimicrobial agents. Adv. Biochem. Eng. Biotechnol. *135*, 1–25.

Wiesner, J. and Vilcinskas, A. (2010). Antimicrobial peptides: the ancient arm of the human immune system. Virulence *1*, 440–464.