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Molecular Farming in Barley: Development of a Novel Production Platform to Produce Human Antimicrobial Peptide LL-37

*Edita Holásková, Petr Galuszka, Alžbeta Mičúchová, Marek Šebela, Mehmet Tufan Öz, and Ivo Frébort**

The peptide LL-37, a component of the human innate immune system, represents a promising drug candidate. In particular, the development of low-cost production platform technology is a critical bottleneck in its use in medicine. In the present study, a viable approach for the LL-37 production in transgenic barley is developed. First, comparative analyses of the effects of different fused peptide epitope tags applicable for accumulation and purification on LL-37 production yield are performed using transient expression in tobacco leaves. Following the selection of the most yielding fusion peptide strategies, eight different constructs for the expression of codon optimized chimeric *LL-37* genes in transgenic barley plants are created. The expression of individual constructs is driven either by an endosperm-specific promoter of the barley B1 hordein gene or by the maize ubiquitin promoter. The transgenes are stably integrated into the barley genome and inherited in the subsequent generation. All transgenic lines show normal phenotypes and are fertile. LL-37 accumulated in the barley seeds up to 0.55 mg per 1 kg of grain. The fused epitope tags are cleaved off by the use of enterokinase. Furthermore, *in planta* produced LL-37 including the fused versions is biologically active.

1. Introduction

Antimicrobial peptides (AMPs) acting as components of non-specific basal defense mechanisms are considered crucial components of the innate immunity in multicellular organisms. These small peptides display a broad spectrum biocidal activity against pathogenic bacteria or fungi. Although variable in length, composition, and secondary structure, they are generally less than 50 amino acids long and can adopt amphipathic conformation.[1] AMPs are emerging as prospective therapeutic agents especially in an era of increasing drug resistance in bacteria caused by extensive antibiotic use. Additionally, their mode of action, which generally involves electrostatic interaction between the cationic peptide and negatively charged membrane of bacteria, hinders emergence of pathogen resistance. However, large-scale production of AMPs for pharmaceutical use has been challenging.

One of the promising production techniques of AMPs is plant molecular farming, where plant cells or tissues are used for expression and production of recombinant pharmaceutical proteins or peptides. Tobacco and potato were used as model plants for production of antigens, proteins and pharmaceuticals in earlier studies.[2–4] However, recent improvements in transformation systems led to utilization of cereals, which enable high product yield while showing low levels of phenolic compounds or undesired proteolytic enzymes and require less downstream processing. Barley displays a low risk of uncontrolled gene flow and is considered biologically and environmentally safe since it is a self-pollinating plant, unlike maize. The pharmaceuticals produced in barley include human serum albumin, antithrombin III, α1-antitrypsin, lactoferrin, and lysozyme,[5,6] mammalian collagen type I α1 chain,[7,8] human growth factor Flt3 ligand,[9] barley lipoxygenase2,[10] and thaumatin from *Thaumatococcus daniellii*.[11]

In humans, host defense AMPs play a critical role in warding off invading microbial pathogens.[12] LL-37, derived from the only human member of the cathelicidin gene family encoding mammalian antimicrobial peptides, is a small peptide of 37 amino acids folded into an amphipathic α-helix. Its presence was detected in various cells and tissues such as circulating neutrophils, myeloid bone marrow cells, epithelial cells of the skin, and tissues in the gastrointestinal tract, mouth, esophagus, and lungs.[13] Recombinant production of LL-37 in *E. coli* and *Pichia pastoris* have been reported previously.[14–17] Regarding plant systems, the gene coding for cathelicidin LL-37 or its variant was integrated into genome of Chinese cabbage (*Brassica rapa* var.

E. Holásková, Dr. P. Galuszka, A. Mičúchová, Prof. M. Šebela, Dr. M. T. Öz[†] Prof. I. Frébort
Centre of the Region Haná for Biotechnological and Agricultural Research
Palacký University
Olomouc 783 71, Czech Republic
E-mail: ivo.frebort@upol.cz

[†]Present address: Department of Agronomy, University of Florida, Gainesville, FL, USA.

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chinensis),[18] tomato (*Solanum lycopersicum*),[19] and rice (*Oryza sativa* L. var. Japonica cv. Dongjinbyeo),[20] and as a result transgenic plants exhibited enhanced resistance against various plant pathogenic organisms. However, expression of LL-37 in transgenic plants for molecular farming and subsequent analysis of the heterologous product have not been reported. In our study, we report a viable approach for LL-37 production in barley endosperm and evaluation of fused signaling or stabilization tags as well as the antimicrobial activity of the product.

2. Experimental Section

2.1. Transient Expression of Chimeric *LL-37* Genes in Tobacco

To select the most desirable plant-based production strategy of LL-37, 10 different chimeric *LL-37* genes were designed combining various fusion carrier peptide domains, purification tags and/or subcellular targeting signals. The fused elements included *N*-terminal secretion signal sequence of cytokinin dehydrogenase 1 from maize (ZmCKX1sp; GenBank NM_001112121.1), *C*-terminal KDEL retention signal for endoplasmic reticulum, affinity tags for protein purification (MBP-maltose binding protein, 6xHis-polyhistidine tag) and/or the small ubiquitin like modifier (SUMO) tags (SUMOstar protein sequence[21] and the putative barley SUMO sequence predicted from the barley genome sequence data, http://webblast.ipk-gatersleben.de/barley/). Furthermore, the coding sequence for enterokinase or the Factor Xa recognition site was also included in some of the constructs to allow proteolytic cleavage of the fused protein domains. All constructs contained the Kozak consensus sequence to regulate the translation initiation. The chimeric *LL-37* DNA sequences were enlarged on both ends by appropriate nuclease cleavage sites to facilitate their subcloning into target expression vectors. The entire DNA sequences were commercially synthesized by Thermo Fisher Scientific, USA. Prepared constructs of recombinant human LL-37 (rhLL-37) are shown in **Figure 1**A and the corresponding amino acid sequences are listed in Figure S1A–J, Supporting Information.

All DNA coding sequences were inserted with the use of T4 DNA ligase into pENTR 2B Dual Selection Vector (Thermo Fisher Scientific) through *Sal*I and *Eco*RI restriction sites creating intermediate plasmids. The genes were then subcloned into the binary vector pGWB17 downstream of the CaMV 35S promoter via Gateway LR recombination (Thermo Fisher Scientific). All of the final expression vectors were sequenced by a commercial service (SEQme, Czech Republic) and then electro-transformed into *Agrobacterium tumefaciens* strain GV3101. Tobacco plants (3 weeks old, *Nicotiana benthamiana* L.) were agroinfiltrated according to a published protocol.[22] Two most top leaves on two 4-week-old plants were punched with a syringe and 0.5 mL of *Agrobacterium* suspension ($OD_{600} = 0.4$)

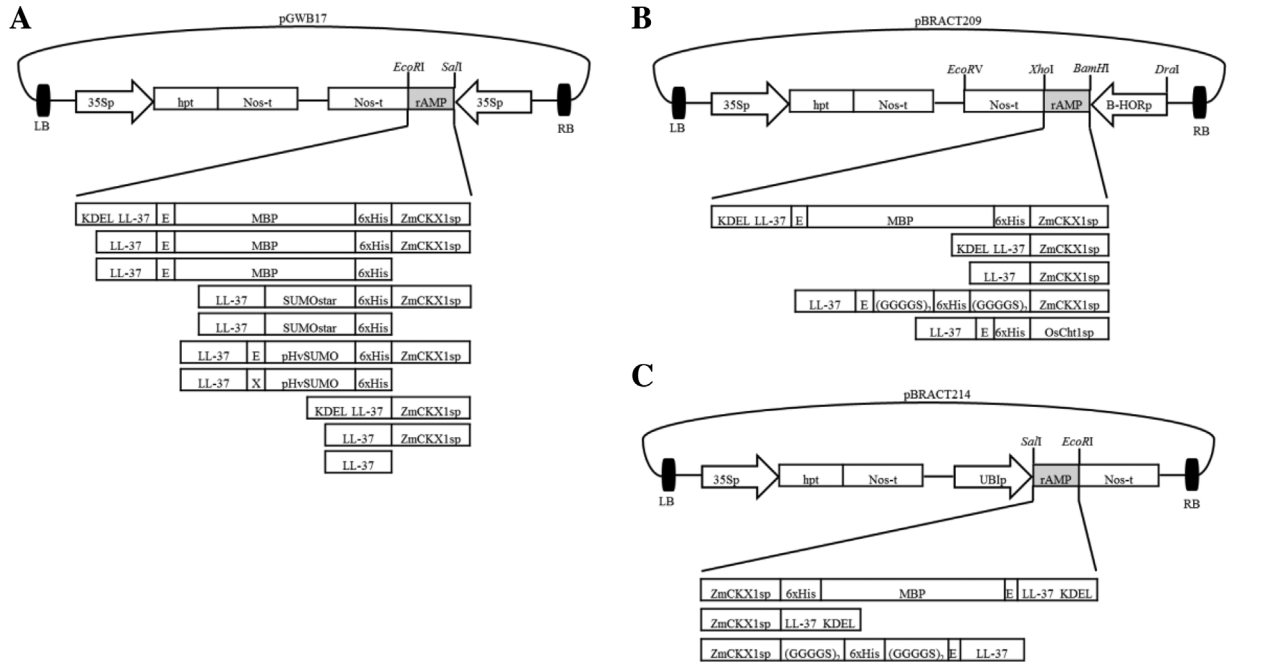

Figure 1. Scheme of the gene constructs used for the expression of rHLL-37 peptides in tobacco leaf tissue (A) and barley (B), respectively. Restriction enzyme cut sites used for cloning are indicated. 35Sp, 35S promoter from cauliflower mosaic virus; B-HORp, barley B1 hordein gene promoter; UBIp, maize ubiquitin gene promoter; hpt, hygromycin resistance selectable marker gene; Nos-t, nopaline synthase gene terminator of *Agrobacterium tumefaciens*; rAMP, recombinant antimicrobial peptide; LL-37, human cathelicidin antimicrobial peptide; ZmCKX1sp, *Zea mays* cytokinin oxidase/dehydrogenase 1 signal peptide; OsCht1sp, *Oryza sativa* chitinase 1 signal peptide; KDEL, endoplasmic retention signal; 6xHis, polyhistidine tag; MBP, maltose binding protein tag; SUMOstar, SUMO derived fusion protein tag; pHvSUMO, putative barley SUMO sequence; E, enterokinase recognition site; X, Factor Xa recognition site; $(GGGGS)_2$, flexible peptide linker; RB, right border; LB, left border.

was injected into tobacco tissue. Infiltrated area of approximately 3–5 cm^2 was collected and pooled from all leaves to analyze the expression of *LL-37* chimeric genes on both RNA and protein level.

2.2. Selection of Barley Endosperm Tissue Specific Promoter for Expression of *LL-37* Gene

Barley native genes that are specifically expressed in endosperm were identified using Genevestigator Affymetrix 22K Barley GeneChip array.[23] The strength and temporal profile of candidate promoters was analyzed by measuring expression patterns of corresponding endogenous genes on StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific) using SYBR Green reaction with gene specific oligonucleotides, which were designed with Primer Express Software 3.0.1 (Thermo Fisher Scientific) and are listed in Table S1, Supporting Information. The expression profile was analyzed in six different phenological growth stages of barley on BBCH scale,[24] that is, flag leaf sheath extending (BBCH 41), first awns visible (BBCH 49), inflorescence fully emerged (BBCH 59), late milk (BBCH 77), early dough (BBCH 83), and hard dough (BBCH 87). The efficiency of amplification was calculated from standard curves for individual genes as 98.0% for B1 hordein, 93.8% for D hordein, 103.1% for hordoindoline b, 97.8% for chymotrypsin inhibitor 2, and 96.4% for trypsin inhibitor. Reaction conditions were set up as follows: denaturation at 95 °C for 10 min, 40 cycles of amplification (95 °C for 15 s and 60 °C for 1 min) and melt curve stage (95 °C for 15 s, 60 °C for 1 min, 1 °C increment per 1 min from 60 to 95 °C and 95 °C for 15 s). Reaction was always set up for two independent biological samples and each sample was run in three technical replicates. The specificity of the amplification was proved by analysis of melting curves. Expression data were analyzed using the DataAssist software (Thermo Fisher Scientific).

2.3. Construction of Plasmids for Expression of Chimeric *LL-37* Genes in Barley

Based on the results from *Agrobacterium*-based infiltration of tobacco leaves, eight plant expression vectors containing chimeric *LL-37* genes, codon optimized for barley, were prepared and used for stable barley transformation. Expression of individual transgenes was driven either under the control of barley seed-specific B1 hordein promoter (*B1 HORp*, GenBank X87232.1) or the maize ubiquitin promoter (*UBIp*), and the nopaline synthase gene terminator (*Nos-t*). For the purpose of grain specific expression, DNA fragments corresponding to *B1 HORp* and *Nos-t* intermitted with multiple cloning sites containing *Bam*HI and *Xho*I sites were commercially synthesized (Mr. Gene, Germany). The promoter and terminator fragments were first inserted into the pENTR 1A Dual Selection Vector (Thermo Fisher Scientific) using *Dra*I and *Eco*RV restriction sites and then the sequences of chimeric *LL-37* genes were placed between them using *Bam*HI and *Xho*I sites. Finally, the individual genes were subcloned into destination vector pBRACT209 (www.bract.org, provided by John Innes Center, Norwich, UK) using Gateway® LR recombination reaction (Thermo Fisher Scientific). For constitutive expression driven by *UBIp*, the individual genes were first cloned into pENTR 2B Dual Selection Vector (Thermo Fisher Scientific), and then recombined into the binary vector pBRACT214 (www.bract.org, John Innes Center, UK) through LR Gateway® reaction downstream of *UBIp*. This resulted in constructs shown in Figure 1B. Sequences of all binary plasmids were verified by DNA sequencing (SEQme) and then inserted via electroporation into *Agrobacterium tumefaciens* strain AGL1 (obtained from Plant Breeding and Acclimatization Institute, Blonie, Poland) together with the helper plasmid pSoup.

2.4. Generation and Selection of Transgenic Barley Plants Expressing rhLL-37 Peptide

Stable transgenic barley lines (*Hordeum vulgare* L. cv. Golden Promise) were generated by *Agrobacterium tumefaciens* (strain AGL1) mediated transformation of immature embryos according to a previously described protocol[25] with minor modifications. These included the addition of 300 μM acetosyringone to *Agrobacterium tumefaciens* cell suspension culture immediately prior to the inoculation of donor plant material and the addition of 0.4 μM biotin to MG/L cultivation medium. The *hpt* gene (included in transformation vectors pBRACT209 and pBRACT214) encoding the hygromycin B phosphotransferase was used as the selectable marker. Plantlets were transferred into hydrated peat jiffy pellets (Rosteto, Czech Republic) and grown with the 15 °C/16 h/light (140 μM m^{-2} s^{-1}) and 12 °C/8 h/dark cycles at relative humidity 60%. After 2 weeks, plants were transferred into pots containing 1:100 w/v mixtures of perlite (Perlit, Czech Republic) and a professional substrate for plant cultivation, Gramoflor special mix (Gramoflor, Germany). Seeds obtained from selected T0 lines were used to produce T1 and T2 progeny plants, which were then grown in pots in a greenhouse. Non-transgenic lines regenerated from in vitro tissue cultures were used as control plants.

2.5. Genomic DNA Isolation, PCR Analysis, and Southern Blot Analysis

Total genomic DNA was isolated from 200–400 mg of young leaf tissue as described previously.[26] Transgene insertion was confirmed by independent PCR analysis of 50–100 ng of genomic DNA with primers (listed in Table S1, Supporting Information) specific to *hpt* gene, *B1 HORp*::*rhLL-37* gene fusion or *UBIp*::*LL-37* gene fusion. Amplification conditions were as follows: denaturation at 95 °C for 2 min, 35 cycles at 95 °C for 30 s, 55 °C for 30/102 s (for *hpt*, and promoter-*rhLL-37* detection, respectively) and 72 °C for 30 s; and final elongation at 72 °C for 5 min. Selected PCR-positive plants were further analyzed by Southern blot to define copy number of T-DNA inserts. For this purpose, 40 μg of genomic DNA was digested with *Xho*I, obtained fragments separated by agarose gel electrophoresis and transferred onto blotting nylon membrane (type B, Merck, Germany). Membranes were then exposed to *hpt* gene-specific DIG-labelled DNA hybridization probe created by PCR (for

primers see Table S1, Supporting Information) according to the manufacturer's instructions (Roche, Switzerland). The DNA fragments with the hybridized probe were visualized by the use of a DIG system for filter hybridization.

2.6. Analysis of Ploidy Level of Primary Barley Transformants

The ploidy level of primary transformants was determined by flow-cytometry using ML CyFlow flow cytometer (Partec, Münster) at an early stage of development.[27] Leaf tissue was chopped in LB01 isolation buffer containing 2% w/v polyvinylpyrrolidone to prevent the interference of phenolic compounds with DNA staining.[28] The acquired suspension of nuclei was stained with 0.01% w/v DAPI. Leaves of non-transformed barley were used as internal reference standard.

2.7. RNA Isolation, cDNA Synthesis, and Analysis of Gene Expression

Total RNA samples were isolated either from barley roots, leaves, and grains or from tobacco leaves using the RNAqueous kit and treated with Turbo DNase (Thermo Fisher Scientific), then purified with Agencourt RNAClean XP (Beckman Coulter, USA). cDNA synthesis was accomplished from 2 μg of total RNA using the M-MuLV RT reverse transcriptase (Thermo Fisher Scientific). *rhLL-37* gene expression was assessed by RT-PCR under following conditions: denaturation at 95 °C for 2 min; 32 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 90 s; final elongation at 72 °C for 10 min. Amplicons were analyzed on 3% agarose, stained with ethidium bromide and visualized using Gel Doc EZ system (Bio-Rad, USA). DNA ladders were from New England Biolabs (USA). The mRNA levels of respective actin and transcriptional elongation factor 2 genes (barley *HvACT* and *HvEF2*, tobacco *NbeACT,* and *NbeEF1*) were used as quantitative control. All corresponding primers are listed in Table S1, Supporting Information.

2.8. Purification of rhLL-37 Peptides from Plant Extracts

Frozen plant tissues were milled in MM 400 homogenizer (Retsch, Germany) with grinding jars pre-cooled with liquid nitrogen. Roots, leaves and grains of T1 generation of barley were harvested at the late milk developmental stage and senescence. Total soluble proteins were extracted with 2 mL of 0.1 M Tris-HCl pH 8.0 containing 0.3 M NaCl, and cOmplete™ EDTA-free Protease Inhibitor Cocktail (Roche) per one gram of plant tissue. The extraction buffer also contained 0.01 M imidazole, 4% v/v glycerol, and 0.3% v/v Triton X–100 for purification using the polyhistidine tag and 1 mM EDTA and 1 mM DTT in case of MBP-mediated purification. The extraction was performed for 2 h at 4 °C, then the samples were centrifuged at 12 000x*g* for 30 min at 4 °C. The pellets were re-extracted once more as described above and the supernatant fractions were combined.

Tobacco leaves were collected 3 days after infiltration and proteins were extracted with 0.02 M Tris–HCl pH 8.0 containing 0.15 M NaCl, 1 mM EDTA, 1% v/v Triton X–100, 1% w/v sodium deoxycholate, and cOmplete™ EDTA-free Protease Inhibitor Cocktail for 1 h, followed by centrifugation at 12 000x*g* for 30 min, all at 4 °C. Protein content was estimated using the Bradford assay with bovine serum albumin as standard, and the presence of rhLL-37 peptide by Western blot.

Histidine-tagged rhLL-37 peptides were purified using the Co^{2+}-IDA-agarose resin (Qiagen, USA) washed repeatedly with 0.1 M Tris-HCl pH 8.0, containing 0.3 M NaCl, 0.01 M imidazole, and 4% v/v glycerol, to remove non-specifically bound proteins. Retained proteins were then eluted with the same buffer containing 0.4 M imidazole. rhLL-37 peptides fused to MBP were purified on amylose resin (New England Biolabs) washed with 0.1 M Tris-HCl pH 8.0, containing 0.3 M NaCl, 1 mM EDTA, and 1 mM DL-dithiothreitol and eluted with addition of 10 mM maltose in the same buffer. In all cases, the collected protein fractions were concentrated using centrifugal filters with nominal molecular weight limit of 3 kDa (Merck Millipore, Germany) to a final concentration of 15 mg of protein/mL. For immunoblotting, 0.4 mg of purified proteins were precipitated by addition of pre-chilled acetone (−20 °C, final concentration 85%) followed by overnight incubation at 20 °C. After centrifugation at 19 500 × *g* for 60 min at 4 °C, the protein pellets were allowed to air dry, dissolved in MilliQ water and stored at −20 °C till further analysis.

2.9. Preparation of Protein-Body Enriched Fraction

To test whether the individual chimeric versions of rhLL-37 peptide accumulate in protein storage organelles, protein extracts from 200 mg of freshly collected vegetative tissues and grains in the late milk developmental phase (BBCH 77) and dry grain at harvesting stage (BBCH 99) were prepared by slightly adapting the published method.[29] The pelleted protein-body enriched fraction was resuspended in 70 μl of 8 M urea, heated for 10 min at 90 °C with continuous shaking and centrifuged at 21 000x*g* for 15 min at 4 °C. Protein samples of 0.2 mg were precipitated by acetone as above and subjected to immunoblotting analysis.

2.10. Immunoblotting

Protein samples were separated under reducing conditions on 4–12% Bis–Tris Plus precast polyacrylamide gels (Thermo Fisher Scientific) and electroblotted to a PVDF membrane (Merck, Millipore). Immunodetection of rhLL-37 protein products was carried out on iBind Western device (Thermo Fisher Scientific) following the manufacturer's protocol. Dilution of LL-37 polyclonal antiserum raised in rabbit (sc-50423; Santa Cruz Biotechnology, USA) was 1:400 and dilution of goat anti-rabbit immunoglobulin-peroxidase conjugate (sc-2004; Santa Cruz Biotechnology) was 1:1000. Protein detection was performed using enhanced chemiluminescent (ECL) substrate (Bio-Rad). To determine the accumulation level of rhLL-37 in barley tissues, different amounts of synthetic LL-37 (4445-s; Peptide Institute, Japan) were used as standards. Image acquisition and documentation was accomplished using either the Image Lab

Software (Bio-Rad), or the X-ray film (Thermo Fisher Scientific). Signal intensity was measured with Image Lab Software (Bio-Rad) using at least three different biological replicates per line. The SeeBlue Plus2 Prestained Standard (Thermo Fisher Scientific) served as a molecular size marker.

2.11. Mass Spectrometry Analysis

Matrix-assisted laser desorption/ionization with time-of-flight detection mass spectrometry (MALDI-TOF MS) was performed on a Microflex LRF20 MALDI-TOF mass spectrometer (Bruker Daltonik, Germany) equipped with a microScout ion source and a 337 nm nitrogen laser (60 Hz). Peptide samples (1 μL aliquots) were applied on a MSP AnchorChip 600/96^{TM} target plate and mixed via a standard dried droplet technique with 1 μL of ferulic acid (20 mg mL^{-1} in 7:3 v/v mixture of acetonitrile and 2.5% trifluoroacetic acid) as a matrix. Mass spectra were acquired in positive linear mode with a relative laser power adjusted at 60% compared to 20% applied for routine peptide mass fingerprinting experiments with protein digests. The acquisition method used was typical for working with large peptides and small proteins (acceleration voltage 20.0 kV, extraction voltage 18.4 kV, lens voltage 7.5 kV, delayed extraction 350 ns). External calibration was done using the Protein Calibration standard I (6 calibration points, *m/z* 5734.56-16952.55; Bruker Daltonik). Spectral data were acquired with flexControl 3.4 and processed for reading *m/z* values by flexAnalysis 3.4 software (Bruker Daltonik).

2.12. In Vitro Analysis of Antibacterial Activity of rhLL-37 Peptide

Purified rhLL-37 peptides were buffer exchanged for either 5 mM NH_4HCO_3, pH 8.0, or the enterokinase cleavage buffer (20 mM Tris-HCl pH 8.0, 50 mM NaCl, 2 mM $CaCl_2$) and concentrated using 3 kDa centrifugal filters (Merck Millipore, USA) to a maximal final concentration of 10 μg μL^{-1}. To release the fused protein tags, the proteins were mixed with enterokinase (New England Biolabs) in 1:30 v/v ratio and digested according to the manufacturer's manual. Finally, the reaction buffer was exchanged to 5 mM NH_4HCO_3, pH 8.0 as above. For cleavage efficiency analysis, the proteins were precipitated by acetone and analyzed by immunoblotting

Antimicrobial activity of barley derived rhLL-37 peptides on the proliferation of *E. coli* TOP10 cells was compared to that of synthetic LL-37 peptide (LT12016, LifeTein, USA) using the purified protein fraction from non-transgenic plants as a control. *E. coli* was grown to the rapid mid-log phase culture under aseptic conditions in LB liquid medium (density 2×10^8 colony forming units (CFU)/mL) and 0.5 μL aliquots were mixed with 10 μL of purified fractions containing between 1 and 3 μg of rHLL-37 peptide or synthetic LL-37. After 4 h of incubation at 37 °C with continuous shaking (1000 rpm), the mixture was 10^5-fold diluted with LB medium, plated over non-selective LB agar plates and incubated for 24 h at 37 °C. Antibacterial activity was evaluated by comparing the CFU values on agar plates for rhLL-37 and synthetic LL-37 to that of control. The bioassay was carried out in at least two independent experiments with three technical replicates.

2.13. Immunolabeling of rhLL-37 in Mature Barley Grains

The presence of the rhLL-37 peptide in mature grains of barley T2 lines was visualized by adapting the published procedure.[30] The grain sections were blocked in 5% w/v bovine serum albumin (BSA) in Tris-buffered saline (TBS) for 4 h, incubated with 1:40 v/v LL-37 antiserum (sc-50423; Santa Cruz Biotechnology) and then with 1:2500 v/v goat anti-rabbit alkaline phosphatase conjugate (AP307A, Merck), both in TBS containing 2.5% w/v BSA for 90 min at 37 °C followed by 90 min at 20 °C. For colorimetric detection of the rhLL-37 peptide, the nitroblue tetrazolium/5-bromo-4-chloro-3-indolyphosphate substrate (B6404, Merck) was used and the images were captured by Nikon SZM800 stereomicroscope.

3. Results

3.1. Assessment of Plant-Based Strategies for *LL-37* Peptide Production Using Transient Expression in Tobacco Leaf Tissues

In order to choose the optimal time for sampling of agroinfiltrated plant material, tobacco leaves were collected 2, 3, 4, and 6 days after agroinfiltration with five randomly selected *rhLL-37* DNA constructs. Afterwards, levels of transgenic mRNA were relatively quantified by qRT-PCR analysis. High accumulation levels of transgene transcripts were detected 2–4 days post-inoculation but significantly decreased afterwards. Accordingly, the expression of all of the 10 designed chimeric *LL-37* genes was analyzed on the third Day after infiltration. Although RT-PCR analysis confirmed the accumulation of transgenic mRNA in all samples (Figure S2A, Supporting Information), immunoblotting analysis showed the presence of rhLL-37 peptide only for 3 out of the 10 constructs as depicted in Figure S2BC, Supporting Information. Notably, constructs lacking the secretion signal peptide ZmCKX1sp did not show any protein expression, which clearly indicated that the entry into the endoplasmic reticulum (ER) is essential for rhLL-37 peptide accumulation in plant tissue. Based on this observation, the three positive rhLL-37 peptide variants, ZmCKX1sp_LL-37, ZmCKX1sp_LL-37_KDEL, and ZmCKX1sp_6xHis_MBP_PRO_LL-37_KDEL (Figure 1B; amino acid sequences are shown in Figure S1BCF, Supporting Information) were selected for the heterologous expression in barley. Moreover, two additional variants ZmCKX1sp_$(GGGGS)_2$_6xHis_$(GGGGS)_2$_PRO_LL-37 and OsCht1sp_6xHis_PRO_LL-37 consisting of ER transit peptide ZmCKX1sp or OsCht1sp (rice chitinase 1, GenBank: D16221.1), enterokinase recognition sequence (PRO), and 6xHis tag, which was in one case flanked by a flexible linker sequence $(GGGGS)_2$ to ensure effective separation of the domains (see the amino acid sequences in Figure S1KL, Supporting Information), were designed.

3.2. Selection of a Suitable Native Promoter for Grain Endosperm Directed Peptide Delivery in Barley

Based on results obtained from Genevestigator platform, five barley endogenous genes with endosperm-preferred expression

were selected as the most promising candidates to drive tissue-specific accumulation of rhLL-37 peptide in grains. The candidate genes that included B1 hordein (GenBank X87232.1), D hordein (X84368.1), hordoindoline b (AY644004.1), chymotrypsin inhibitor 2 (X57035.1), and trypsin inhibitor (X65875.1) were subjected to real-time RT-PCR analysis to determine their temporal expression profile. As shown in Figure S3, Supporting Information, the barley B1 hordein gene promoter (*B1 HORp*) revealed the strongest expression levels of the corresponding endogenous gene in later developmental stages of wild-type barley spikes. The ability of *B1 HORp* to direct stable overexpression in barley grains was further verified with endogenous cytokinin dehydrogenase nine gene (*HvCKX9*). In homozygous transgenic plants, the relative transcript levels of this gene determined using real-time RT-PCR increased about 1000, 2600, and 4800 times in seed coat, aleurone, and embryo, respectively. However, the predominant overexpression was observed in endosperm tissue, where the level increased about 32 500 times compared to non-transgenic control plants.

3.3. Production and Characterization of Stable Barley Transgenic Lines Expressing rhLL-37 Peptide

Barley cultivar Golden Promise was selected for *Agrobacterium*-mediated transformation because it is the most susceptible cultivar and shows the best regeneration response in tissue culture.[25] PCR analysis showed from 5 to 37 independently transformed T0 barley plants for each of the DNA constructs indicated in Figure 1B. The overall transformation efficiency determined as a number of independent transgenic T0 lines obtained from the number of inoculated immature barley embryos was about 10%. The transgenes were stably integrated into the barley genome and inherited as indicated in Figure S4, Supporting Information. Most of the transgenic barley plants were diploid (92%). As chromosomal variation might negatively affect not only transgene expression, but also agronomic and quality characteristics, aneuploid plants were discarded from further analysis. According to Southern blot analysis results, most of the transformants contained multiple T-DNA inserts (see Figure S5, Supporting Information). Heterologous expression of any of *LL-37* gene variants was not phytotoxic, since no apparent negative impact on plant development and phenotype was observed as depicted in Figure S6, Supporting Information. For further propagation, only diploid T0 plants harboring either single or low copy number of T-DNA inserts, without changes in phenotype were selected.

3.4. Expression of *rhLL-37* Transgenes in Barley Lines

T1 plants of *B1 HORp* and *UBIp* transgenic barley lines showed transcription of chimeric *LL-37* genes when tested by RT-PCR (see Figure S7, Supporting Information). The *rhLL-37* gene transcripts in *B1 HORp* lines were detected only in grains, but not in roots or leaves. Opposite to that, the *UBIp* lines showed the presence of *LL-37* gene amplicons in all analyzed tissues. Hence, the functionality of the used promoters, as well as the expression of *rhLL-37* genes, was confirmed.

3.5. Accumulation of *rhLL-37* in Transgenic Barley Plants

To see whether the transgene transcripts were properly translated in barley genetic background, crude protein extracts, their purified fractions and protein bodies' enriched fractions were analyzed by immunobloting. Whereas no specific signals were detected in crude protein extracts from leaves, roots and grains of the transgenic lines, immunoblot analysis revealed the presence of rhLL-37 products of expected size in either the purified protein fractions from the grain or leaves and roots, the storage protein organelles, or both, depending on the type of production strategy and promoter used (**Figure 2**). When purifying rhLL-37 peptides containing 6xHis and MBP epitope tags, the elimination of contaminating proteins by affinity chromatography greatly enriched the content of the corresponding rhLL-37 peptide. Furthermore, when the epitope tags used for purification were cleaved off by enterokinase, the released rhLL-37 peptides well matched the expected sizes (Figure 2A). Purifications of ZmCKX1sp_6xHis_MBP_PRO_LL-37_KDEL protein on either Co^{2+}-IDA-agarose or amylose affinity column provided comparable yields. Non-tagged variants were not recovered from crude extracts in any case.

Chimeric rhLL-37 peptides extended with ER entry signal sequence ZmCKX1sp at *N*-terminus and *C*-terminal KDEL tetrapeptide ER retention sequence were successfully recovered from protein bodies' enriched fraction (Figure 2A and 2B) and accordingly no product was extracted from protein bodies when lacking the KDEL sequence (data not shown). As shown in **Table 1**, content and stability of rhLL-37 peptides on the transition from late milk (BBCH 77) to desiccated grain (BBCH 99) strongly depended on the type of production strategy used. The seed-specific expression driven by *B1 HORp* provided much higher levels of rhLL-37 peptide in grains than the expression driven by *UBIp*. Although analysis of *UBIp* lines provided clear evidence of the presence of rhLL-37 peptide also in roots and leaves of transgenic plants (Figure 2B), the estimated amounts of products were much lower than those in late milk grains. Hence, the use of grain specific B1 hordein gene promoter seems to be much better strategy for molecular farming than ubiquitous expression. The highest levels of rhLL-37 peptide were obtained when produced as a fusion with ZmCKX1sp on *N*-terminus and *C*-terminal KDEL sequence (Table 1). Therefore, attachment of large fusion protein tags as the MBP to the amino acid sequence of the LL-37 peptide appears not to positively influence its accumulation level. ZmCKX1sp_LL-37 was the only peptide product which did not give any positive signal in immunoblot analysis, which might be due to the detection limit in crude extracts, as the product did include neither the *C*-terminal KDEL sequence nor purification tags (Table 1). Interestingly, there was no apparent correlation between the number of T-DNA gene inserts and final levels of recombinant peptide products. 6xHis tag (Co^{2+}-IDA) purified protein fractions prepared from barley lines expressing *B-HORp::ZmCKX1sp_(GGGGS)$_2$_6xHis_(GGGGS)$_2$_LL-37* analyzed by MALDI-TOF-MS showed a peptide of monoisotopic mass of 7161 Da, which was absent in the samples from control plants. The detected mass exactly corresponds to that of the rhLL-37 peptide lacking the ZmCKX1sp domain, thus indicating that the *N*-terminal signal sequence is properly cleaved off by an endogenous barley signal peptidase upon entering ER.

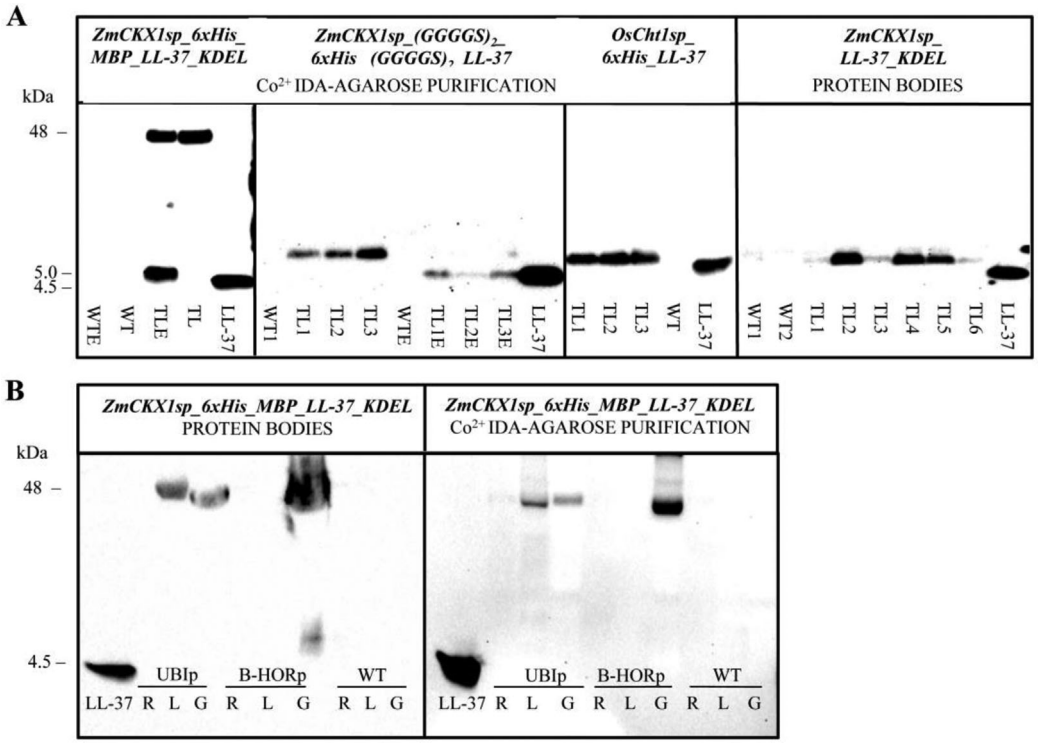

Figure 2. Immunoblot analysis of rhLL-37 peptide in several T1 transgenic lines (TLn) and control plants (WT). A) Detection of rhLL-37 in protein bodies or purified protein fractions from late milk endosperm seeds (BBCH 77) of transgenic lines carrying the indicated transgenes under the control of barley B1 hordein promoter. The sizes of individual bands corresponded either to theoretical sizes of the fusion protein products or to their digested variants. Proteins from control and transgenic lines subjected to enterokinase digestion are assigned as (WTE) and (TLnE), respectively. (B) Detection of rhLL-37 in purified protein fractions prepared from roots (R), leaves (L) and late milk endosperm grains (G) of transgenic lines expressing the indicated transgene under the control of grain specific promoter of the barley B1 hordein gene (B-HORp), or the maize ubiquitin gene promoter (UBIp). A total of 7 ng of synthetic LL-37 served as a positive control.

To obtain more detailed information about the accumulation patterns of rhLL-37 peptide, sectioned mature barley seeds were subjected to immunolabeling with the specific antibody. While the endosperm of control lines remained unstained, all transgenic barley lines showed purple staining, reflecting the presence of rhLL-37 peptide (**Figure 3**). When a construct differed only in the presence/absence of the KDEL sequence, much greater intensity in staining was achieved in the presence of the ER retention signal. Staining in the grain sections that expressed the peptides under the control of *B1 HOR* promoter

Table 1. Accumulation levels of LL-37 in grains of T1 generation of transgenic barley lines as estimated by immunoblot analysis of either the Co^{2+}-IDA purified protein extracts or the protein body enriched fraction.

	Co^{2+}-IDA-agarose purification μg of rhLL-37 per kg of grains				Isolation of protein bodies μg of rhLL-37 per kg of grains			
	Late milk endosperm (BBCH 77)		Desiccated grain (BBCH 99)		Late milk endosperm (BBCH 77)		Desiccated grain (BBCH 99)	
rhLL-37 gene	B-HORp	UBIp	B-HORp	UBIp	B-HORp	UBIp	B-HORp	UBIp
ZmCKX1sp_6xHis_MBP_LL-37_KDEL	15.8 ± 4.74	8.94 ± 2.66	0.081 ± 0.032	N.D.	107 ± 26.0	10.5 ± 4.51	8.66 ± 3.72	N.D.
ZmCKX1sp_LL-37_KDEL	N.D.	N.D.	N.D.	N.D.	548 ± 228	17.0 ± 4.37	6.02 ± 3.15	N.D.
ZmCKX1sp_(GGGGS)2_6xHis_(GGGGS)2_LL-37	330 ± 40.3	0.029 ± 0.007	0.162 ± 0.065	N.D.	N.D.	N.D.	N.D.	N.D.
OsCht1sp_6xHis_LL-37	122 ± 37.2	X	N.D.	X	N.D.	X	N.D.	X
ZmCKX1sp_LL-37	N.D.	X	N.D.	X	N.D.	X	N.D.	X

BBCH scale 77 and 99 correspond to late milk endosperm seeds and desiccated seeds, respectively. B-HORp, transgenic lines expressing *rhLL-37* under grain specific B1 hordein gene promoter; UBIp, transgenic lines expressing *rhLL-37* under maize ubiquitin gene promoter. Displayed are the mean accumulation values with standard deviations of estimated amounts of *rhLL-37* peptide of three independent lines per transformation event and at least three plants per line (with the exception of line *B-HORp::ZmCKX1sp_LL-37_KDEL*, where only 1 line was analysed). X, transgenic lines were not prepared; N.D. product not determined.

was more intense than in the case of *UBI* promoter.3.6 Analysis of antimicrobial activity of recombinant hLL-37 products

Since the antimicrobial activity of the LL-37 peptide has been documented elsewhere, only a simple test was conducted whether the rhLL-37 peptides obtained from barley grains possess a comparable biological activity against *E. coli* TOP10. Both the full-length fusion peptide products, as well as their enterokinase digested versions, were examined. Purified protein fractions were buffer exchanged to NH_4HCO_3, as carbonate containing compounds are known to increase the antimicrobial activity of LL-37 peptide.[31] As shown in **Figure 4**, additions of the synthetic LL-37 peptide to protein extracts from control plants caused concentration-dependent inhibition of the bacterial growth. For a comparative experiment, the late milk developing grains of *B1 HORp* and *UBIp* barley lines showing a high content of the recombinant peptide were selected. Whereas no inhibition of the bacterial growth was observed with untreated extracts from *ZmCKX1sp_6xHis_MBP_LL-37_KDEL* lines, about 50% inhibition was observed after enterokinase cleavage of the fused peptide tags that released the LL-37_KDEL peptide. Interestingly, a similar inhibition was observed for enterokinase treated extracts of *ZmCKX1sp_(GGGGS)$_2$_6xHis_(GGGGS)$_2$_LL-37* lines, which indicates that the antimicrobial activity of in planta produced LL-37 peptide is not lost by adding the four amino acid sequence KDEL at the *C*-terminus. Furthermore, incubation of *E. coli* with protein extracts from lines expressing *OsCht1sp_6xHis_LL-37* transgene, which peptide product is 6xHis_PRO_LL-37, resulted in 40% bacterial growth inhibition. To sum up, the rhLL-37 peptide produced in barley plants including short tag elongated versions on either *N*- or *C*- termini are biologically active.

4. Discussion

Plant-based expression systems are considered promising and competitive, owing to advantages over other biological systems. Plants possess post-translational modification machinery, display no risk of contamination with mammalian viruses and pathogens, and require comparably lower costs for large-scale production. Additionally, efficient transformation systems and simple downstream processing techniques are available for use in plant-based systems.[32,33] The main barrier to the commercial use of AMPs, including cathelicidins, is their high production costs. For example, the price of chemically synthesized LL-37 is in a line of hundreds to thousands of US dollars per mg depending on purity. A recombinant precursor of LL-37 prepared by heterologous expression in microorganisms is also available on the market. However, its price is also high and it is not biologically active.[34]

Heterologous expression of human antimicrobial peptide cathelicidin LL-37 in barley grains yielded up to 0.55 µg of recombinant peptide per gram of grain. Comparison of the amount of accumulated product in different barley lines has clearly shown that larger yields are achieved using a grain-specific than a constitutive promoter. In addition to presented

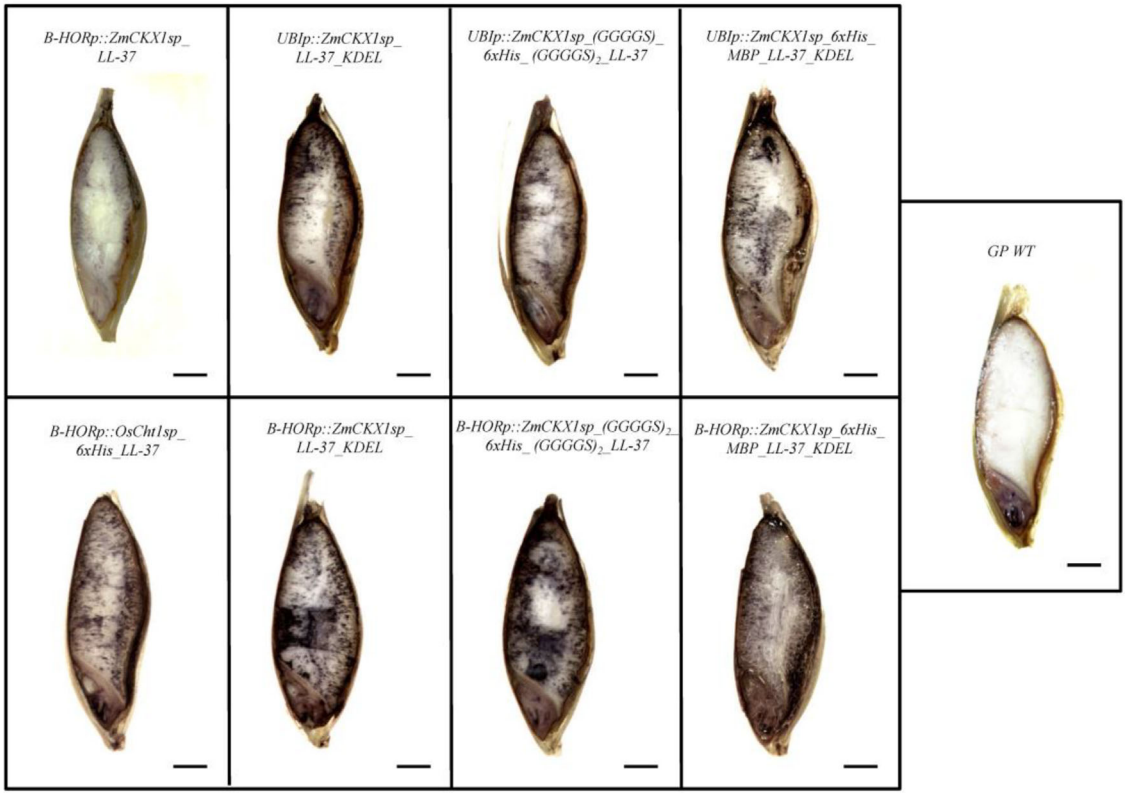

Figure 3. Immunolocalization of rHLL-37 peptide in desiccated seeds (BBCH 99) of T2 barley lines carrying the indicated transgenes. GP WT, control non-transgenic tissue culture regenerated plant. Scale bars correspond to 1 mm.

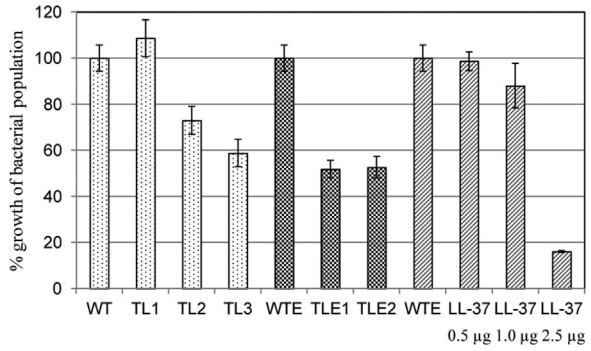

Figure 4. In vitro antibacterial activity of the synthetic LL-37 peptide and purified proteins prepared from late milk endosperm grains (BBCH 77) of T2 generation of transgenic lines. The biological activity was tested against E. coli TOP10, which have been mixed with 10 µL of Co^{2+}-IDA agarose purified fractioncontaining between 1 and 3 µg of recombinant human LL-37 and incubated for 4 h. After that, the number of viable bacterial cells was scored using the plating method. A number of bacterial colonies grown in the presence of purified protein extracts from control lines (either enterokinase digested or not) was taken as 100%. As a positive control, synthetic LL-37 applied in an aliquot of the purified control extract was used. WT, purified protein extracts from control non-transgenic tissue culture regenerated plants; TL1, purified protein extracts from lines expressing ZmCKX1sp_6xHis_MBP_LL-37_KDEL transgene; TL2, ZmCKX1sp_(GGGGS)2_6xHis_(GGGGS)2_LL-37 transgene; TL3, OsCht1sp_6xHis_LL-37 transgene. WTE, enterokinase digested purified protein extracts from control plants; TLE1, enterokinase digested purified protein extracts from lines expressing ZmCKX1sp_6xHis_MBP_LL-37_KDEL transgene; TLE2, ZmCKX1sp_(GGGGS)2_6xHis_(GGGGS)2_LL-37 transgene. Error bars represent standard error of results of two independent experiments.

work, we tested the amount of heterologously expressed protein in barley under the control of other grain-specific promoters such as oat globulin or barley trypsin inhibitor promoters; however, it was found out that used B1 hordein promoter renders the highest yield of the antimicrobial peptide. The amount of produced rhLL-37 corresponds to the expression yields of peptides obtained in other cereals. For example, peptide cecropin was produced in rice endosperm under the control of the glutenin promoter at a maximum yield of 4 µg per gram of grain.[29] Cabanos et al.[35] have achieved up to milligrams per gram of dry grain when they produced hexapeptide lactostatin under the control of the same promoter. It has to be noted, however, that the hexapeptide was incorporated into the structure of the natural storage protein A1aB1b and transcription boosted by concurrent silencing of glutenin gene. In general, higher yields of the antimicrobial peptide production in planta have been achieved only in the case of stable transformation of chloroplasts or transient expression in tobacco leaves.[36,37] Accordingly, preliminary testing of our synthesized constructs for heterologous expression in tobacco rendered yields in hundreds of micrograms per gram of fresh leaf infiltrated tissue. However, transient expression in leaf tissue is not a good system for permanent and stable production. Stable production in storage organs like grains enables time-separated processing and easier purification of a peptide product and thus is desirable and more practical than production into vegetative assimilating tissues or roots.

Analyzing of the peptide amount at different stages of grain development has shown that the largest accumulation occurs in the stage of milky endosperm where the strength of B1 hordein promoter reaches the maximum. Later in mature grains, the peptide is detected in a lower amount, indicating its instability, and degradation during grain desiccation. In order to achieve higher yields and higher peptide stability, the LL-37 gene was fused with stabilizing proteins such as SUMO or MBP. Both tags have been shown to increase protein solubility and total yield and decrease degradation in several eukaryotic expression systems.[38] While LL-37 peptide fused with the SUMO was not detected after transient expression in tobacco, MBP fusion provided signal and therefore stable barley lines were prepared. Nevertheless, total accumulation of MBP fused LL-37 was approximately one order of magnitude lower ($0.11\,\mu g\,g^{-1}$) than this of free peptide ($0.55\,\mu g\,g^{-1}$) in milky endosperm, but fused peptide was slightly more abundant in desiccated grain ($0.009\,\mu g\,g^{-1}$) than the non-fused rhLL-37 ($0.006\,\mu g\,g^{-1}$) indicating than MBP fusion protects the peptide against long-term degradation. Except the fact that the MBP tag increases stability, it can serve for affinity purification as well as the 6xHis tag, which was also introduced to purify the rhLL-37 peptide. In both cases, a purified peptide fraction was obtained, which exhibited biological activity either after cleavage of the tag in the case of MBP or even in a fusion with a smaller 6xHis tag. Higher yields of the purified peptide, however, were achieved by isolating the ER-derived protein bodies into which proteins are deposited when expressed with the secretion signal peptide and the *C*-terminal tetrapeptide KDEL ensuring retention on the ER. Previously, an addition of the KDEL signal was shown to be essential to target recombinant proteins to protein bodies[39,40] or to protein storage vacuoles.[41] Generally, targeting to storage organelles is desirable because except easier purification it offers a protective environment to store protein products at ambient temperatures for several years.[42] Effect of the ER retention sequence on the higher productivity of recombinant LL-37 has been already confirmed in transient tobacco assay where 3-fold higher accumulation was observed in the case of construct bearing the KDEL sequence.

In conclusion, we demonstrated that human cathelicidin can be produced by molecular farming in barley endosperm either as peptide sensu stricto just with the ER retention tetrapeptide or in the fusion with MBP that increases its stability in desiccated grain. The short fusion sequences do not significantly affect the biological activity of rhLL-37. Our preliminary results further indicate that fusion of LL-37 with an elastin-like protein[43] or a biosurfactant protein[44] also leads to stabilization of the short peptide in an environment of plant tissue, and could be used for low-cost purification via inverse transition cycling by changing temperature or ionic strength.[45] In order to reduce the cost of up-scale production of pure rhLL-37, the fusion partner could be fused with metal ion-catalyzed peptide bond cleavage sequence,[46] which we have successfully tested recently. The preparation of barley plants with these constructs, purification and yields of an antimicrobial peptide from grain will be the basis of further work.

Abbreviations

AMPs, antimicrobial peptides; *B1 HORp*, barley seed-specific B1 hordein promoter (GenBank X87232.1); rhLL-37, recombinant human LL-37; *UBIp*, maize ubiquitin promoter; ZmCKX1sp, *N*-terminal secretion signal sequence of cytokinin dehydrogenase 1 from maize (GenBank NM_001112121.1).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no financial or commercial conflict of interest.

Keywords

antimicrobial peptides, fusion protein strategy, genetically modified barley, heterologous expression, LL-37

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